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Changes to histone modifications following prenatal alcohol exposure: An emerging picture

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ABSTRACT

Epigenetic mechanisms are important for facilitating gene-environment interactions in many disease etiologies, including Fetal Alcohol Spectrum Disorders (FASD). Extensive research into the role of DNA methylation and miRNAs in animal models has illuminated the complex role of these mechanisms in FASD. In contrast, histone modifications have not been as well researched, due in part to being less stable than DNA methylation and less well-characterized in disease. It is now apparent that even changes in transient marks can have profound effects if they alter developmental trajectories. In addition, many histone methylations are now known to be relatively stable and can propagate themselves. As technologies and knowledge have advanced, a small group has investigated the role of histone modifications in FASD. Here, we synthesize the data on the effects of prenatal alcohol exposure (PAE) on histone modifications. Several key points are evident. AS with most alcohol-induced outcomes, timing and dosage differences yield variable effects. Nevertheless, these studies consistently find enrichment of H3K9ac, H3K27me2,3, and H3K9me2, and increased expression of histone acetyltransferases and methyltransferases. The consistency of these alterations may implicate them as key mechanisms underlying FASD. Histone modification changes do not often correlate with gene expression changes, though some important examples exist. Encouragingly, attempts to reproduce specific histone modification changes are very often successful. We comment on possible directions for future studies, focusing on further exploration of current trends, expansion of time-point and dosage regimes, and evaluation of biomarker potential.

Different states of chromatin accessibility provide control over gene expression. These chromatin states are defined by the presence of various proteins, DNA modifications (DNA cytosine methylation), non-coding RNAs, and post-translational histone modifications. The histone code hypothesis was formed in the early 1990s as a way to view the role of histone modifications (Turner, 1993). The hypothesis postulates that each histone modification codes for specific chromatin conformations. The different modifications form a combinatorial pattern which could heritably affect gene expression (Turner, 2000). In recent years, the rigidity of the histone code hypothesis has been relaxed. Some investigators view histone modifications as more of a language: the same modifications can have different meanings in the context of nearby modifications, and are not strictly associated with any chromatin state (Oliver & Denu, 2011). At least 12 histone modifications have been reported to over 60 different amino acid residues, including methylation of lysines and arginines, phosphorylation of serine and threonine, lysine acetylation, ubiquitylation, and others (Kouzarides, 2007). Because histones and especially histone modification vary greatly between species, this review will focus on mammalian histones only. We will use the nomenclature "histone-residue-modification-number" (if applicable), where modifications are shortened from acetylation to 'ac', methylation to 'me', and phosphorylation to 'ph', for example, histone H3 lysine 4 trimethylation is H3K4me3.

1. Histone lysine acetylation

Histone lysine acetylation is a direct modification to chromatin structure. Because the acetyl group removes the negative charge from the lysine, it reduces the overall positive charge of the histone. This weakens the interactions between the histone and DNA,

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opening chromatin structure (Allfrey, Faulkner, & Mirsky, 1964). Chromatin remodeling via general acetylation of histone tails can increase RNA polymerase access to DNA by up 15-fold (Tse, Sera, Wolffe, & Hansen, 1998). However, there can also be indirect effects of acetylation on chromatin structure via reader proteins (Sanchez & Zhou, 2009). Histone lysine acetylation is highly correlated with transcriptionally active genes (Bernstein, Meissner, & Lander, 2007; Kouzarides, 2007). However, instances of acetylation correlating with repressed genes are also observed, indicating that there is complex cross-talk between histone marks and effector proteins (Duan, Heckman, & Boxer, 2005; Rada-Iglesias et al., 2007). Acetylation levels are dynamically regulated by the opposing actions of histone acyltransferases (HATs) and histone deacetylases (HDACs). HATs are multi-subunit complexes, the components of which are conserved from yeast to humans (Doyon, Selleck, Lane, Tan, & Côté, 2004). HATs use acetyl coenzyme A (acetyl-CoA) as a substrate from which to transfer the acetyl group (Kouzarides, 2007). HDACs are also a large family of conserved, multi-subunit proteins. Classes I, II, and IV are very structurally similar, each using zinc to hydrolyze the lysine-amino bond. Class III, also known as sirtuins, transfer the acetyl group onto nicotinamide adenine dinucleotide (NAD+) (Blander & Guarente, 2004). Use of NAD + links metabolism, signal transduction, and transcriptional networks. Like HATs, HDACs do not seem to have lysine-specific activities, but are expressed at varying levels in various tissues and time points during development (de Ruijter, van Gennip, Caron, Kemp & van Kuilenburg, 2003).

2. Histone methylation

Histone methylation occurs at lysine and arginine residues on histone tails. The ε-amino group of lysine residues can be mono-, di-, or tri-methylated, each having differing functional roles. Since histone methylation does not affect its charge, it carries out its effects on chromatin structure indirectly via effector proteins (Taverna, Li, Ruthenburg, Allis, & Patel, 2007; Voigt & Reinberg, 2011). Specific histone methylations are linked to nearly every chromatin state and are very consistent between mammalian cell types (Barski et al., 2007; Zhou, Goren, & Bernstein, 2011). H3K4me3 is often concomitant with transcription at transcriptional initiation sites, and is often viewed as the on/off switch of transcription (Dong et al., 2012; Santos-Rosa et al., 2002; Schneider et al., 2004). H3K4me3 can also mark poised genes together with H3K27me3 termed bivalent domains (Bernstein et al., 2006). These domains are believed to keep developmental genes poised for rapid activation while maintaining repression (Voigt, Tee, & Reinberg, 2013). In somatic cells, H3K27 methylations are linked with facultatively and constitutively repressed genes (Barski et al., 2007). H3K9 methylations are also linked with repressed transcription; H3K9me3 is deposited over large genomic regions, facilitating heterochromatin formation (Lehnertz et al., 2003; Soufi, Donahue, & Zaret, 2012). Many histone methyltransferases (KMTs) are dependent on s-adenosylmethionine (SAM), which is altered by ethanol metabolism (Barak, Beckenhauer, Tuma, & Badakhsh, 1987; Halsted et al., 2002). KMTs are quite specific, targeting not only a certain lysine residue but often catalyzing a specific methylation state. For example, KMT1A/B converts H3K9 from mono-to tri-methylation (Peters et al., 2001). However, G9a converts it to a di-methylated state (Tachibana et al., 2002). The existence of KDMs was debated for decades until the discovery of Lysine (K)-Specific Demethylase 1A (LSD1) in 2004 (Shi et al., 2004).

It is important to note that though we have increasing amounts of data on how reader proteins function, most of our data are correlative. With correlation comes the danger of implied causation, that is, the assumption that histone modifications directly cause differences in chromatin states. This assumption is inherent even in the language, when marks are described as “activating” or “repressing”, which is avoided in this review. Some believe that many histone modifications are a result of dynamic processes affecting nucleosomes, not the initiators (Henikoff & Shilatifard, 2011). While we will not delve into this topic in this review, it is an important debate to raise in any discussion of histone modifications.

3. Acquisition and propagation of histone marks

Histone marks, like most other epigenetic marks, are mutable and responsive to the environment. Indeed, many studies have linked changes in gene expression in response to various stimuli to changes in DNA methylation and histone modification (Rosenfeld, 2010). Such studies provide evidence that epigenetic processes may mediate gene-environment interactions. However, with any change in epigenetic marks, it is not easy to distinguish between correlation and causation. In any case, they often do reflect the structure of chromatin and it remains an active area of research.

For a histone modification to be passed on, it must be passed through cell division. It appears that some modifications can be inherited and are epigenetic in the classical sense, while others are not. As the DNA replication fork propagates, producing two nascent DNA strands, the parental histones are displaced and are evenly distributed to the daughter strands (Alabert & Groth, 2012; Annunziato, 2005; Margueron & Reinberg, 2010; Probst, Dunleavy, & Almouzni, 2009). Parental H3/H4 dimers tend to stay together as tetramers; H2A/H2B are loaded as dimers (Jackson, 1988; Xu et al., 2010). On average, new and parental tetramers are evenly distributed. Importantly, this means that the simplest conceptual model for histone modification propagation – copying of histone modifications within the same nucleosome – is not possible for H3 and H4, as both copies of each are either parental or new. The parental histones retain their post-translational marks (Alabert et al., 2015), and the mechanism by which the modifications are copied onto new histones remains unclear.

There are currently two models that apply to different modifications. For most histone modifications, new histones acquire modifications to become identical to the old ones. Before the next cell cycle, within 2–24 h, the modifications are written on the new histones until they become identical to the parental histones (Alabert et al., 2015). Therefore, histone modification writing is not tightly coupled to replication, and oscillates with the cell cycle.

The second model is known as the buffer model, proposed by Huang, Xu, and Zhu (2013). It applies to the repressive heterochromatin marks H3K27me and H3K9me. The buffer model protects constitutively silent genes from being activated by varying repressive mark levels after DNA replication. In general, repressive marks are found across large regions and function by recruiting effector proteins that shape the region to become inaccessible (Boyer et al., 2006). As such, exact replication of each histone modification after cell division is not necessary for gene repression. Huang et al. (2013) proposed that relatively few nucleosomes (20–30%) must bear repressive modifications to repress transcriptional activity. Therefore, the system functions as a buffer, wherein the levels of repressive marks never drop below the critical threshold and remain non-permissive to transcription. This model is supported by experimental evidence, which shows that the repressive H3K27me3 and H3K9me3 methylations are slowly written onto new histones after replication. Further, even old histones acquired more H3K27me3 and H3K9me3 to reach pre-mitosis levels after replication (Alabert et al., 2015).

4. Epigenetics and fetal alcohol spectrum disorder

Fetal Alcohol Spectrum Disorders (FASD) are a leading cause of behavioral aberration in the western world. It occurs in individuals exposed to alcohol during gestation. The effects of FASD can last a lifetime. The study of the mechanisms of FASD etiology has taken various approaches, with some success. A common approach to study molecular mechanisms has been the use of rodent models. Garro, McBeth, Lima, and Lieber (1991) found for the first time that mouse fetuses exposed to ethanol had inhibited DNA methyltransferase 1 (DNMT1) activity. Further, this inhibition was attributed to the first metabolite of ethanol, acetaldehyde (Garro et al., 1991). Since this study, ethanol has been found to impact methylation pathways in other direct and indirect ways (Fig. 1). Ethanol inhibits folate absorption in both the intestine and kidney in part by down-regulating the expression of its transporter-reduced folate carrier 1 (RFC1) (Hamid & Kaur, 2005, 2007b). Reduced folate absorption impairs pyrimidine synthesis and therefore DNA synthesis (Fig. 1). Further, acetaldehyde inhibits methionine synthase, which converts homocysteine to methionine, the precursor to SAM (Halsted et al., 2002). These actions reduce the availability of SAM, which is the primary substrate of methyltransferases and source of methyl groups.

Ethanol also induces oxidative stress as a primary effect and through its metabolism, which can alter methylation pathways. Conversion of ethanol to acetaldehyde and acetate produces reactive oxygen species (ROS) directly via CYP2E1 and via increased NADH levels (Fig. 2). Ethanol-induced mitochondrial damage produces additional ROS (Hoek, Cahill, & Pastorino, 2002). Increased ROS has numerous damaging effects on macromolecules, including DNA. Specifically, the hydroxyl radical causes mutations, including base substitution, deletion, DNA single-stranded breaks, and DNA double-stranded breaks. Single-stranded DNA can signal *de novo* DNMTs, causing hyper-methylation of these regions (Christman, Sheikhnejad, Marasco, & Sufrin, 1995). Hypo-methylation is much better characterized. With respect to methylation enzymes, under oxidative conditions, homocysteine is converted to the ROS scavenging glutathione, depleting SAM and reducing methylation (Fig. 2) (Kerksick & Willoughby, 2005). Ethanol also affects acetylation pathways. The metabolism of ethanol produces acetaldehyde followed by acetate, then acetyl-CoA. Acetyl-CoA is the substrate used by HATs as an acetyl group source (Fig. 2). Indeed, increased acetate levels lead to increased histone acetylation in experimental models (Soliman & Rosenberger, 2011). Similarly, metabolism of ethanol to acetaldehyde consumes NAD+ and produces NADH. The reduction of NAD + reduces the activity of NAD + -dependent enzymes. The sirtuin family of HDACs is NAD + dependent. As such, ethanol can inhibit sirtuin activity and increase acetylation (Fig. 2).

5. Histone modification in FASD models

The first studies on histone modification and alcohol were focused on the effects of ethanol directly on liver cells. They provided several key insights into the mechanism by which histone modifications respond to ethanol. These studies found that H3K9ac (and not other lysine acetylations) was increased in a time- and dose-dependent manner (Kim & Shukla, 2005; Park, Miller, & Shukla, 2003). In 2007, Pal-Bhadra et al. examined H3K9me2 and H3K4me2 in cultured hepatocytes. There were site-specific histone modification changes correlated with gene expression changes in response to ethanol (Pal-Bhadra et al., 2007). Reductions in H3K9me2 and increases in H3K4me2 occurred in the up-regulated genes. In the down-regulated genes, H3K9me2 increased, with minimal changes in H3K4me2 (Pal-Bhadra et al., 2007). This study showed that changes in histone modification could be correlated with changes in gene expression, implying that they do have functional relevance.

At the time of this review, at least 14 publications have examined histone modifications in FASD models (Table 1). While these studies have a great deal in common, each has taken a different

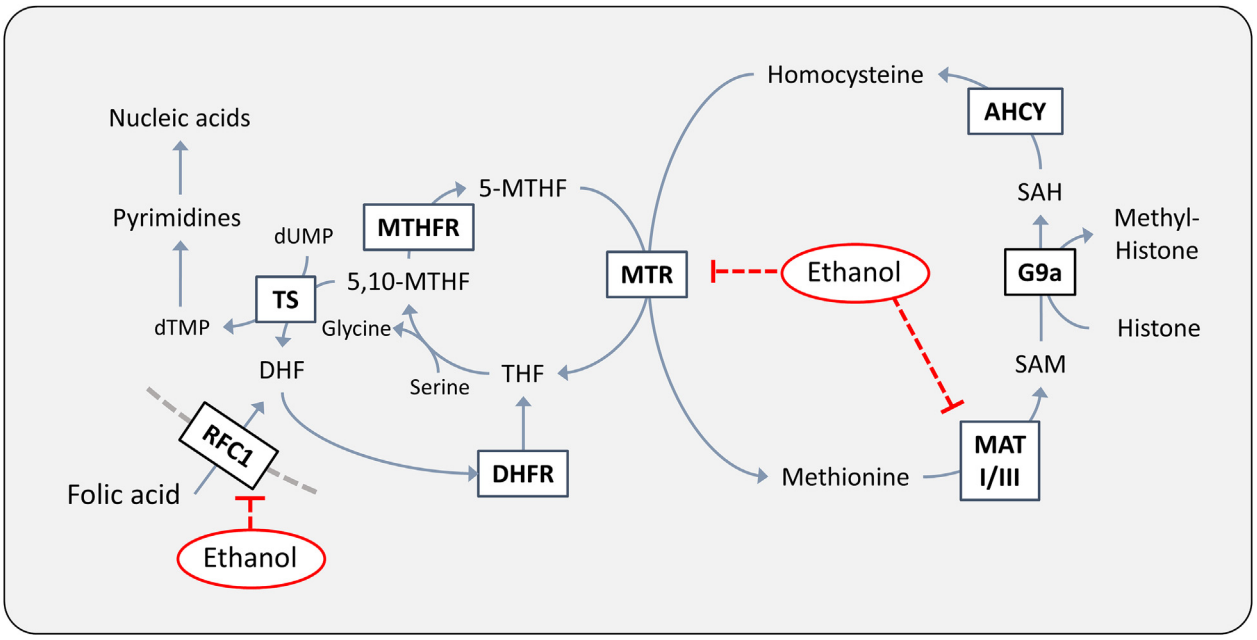

Fig. 1. Inhibitory actions of ethanol on one-carbon metabolism. Dotted lines indicate indirect inhibitory actions. Ethanol indirectly inhibits the folate cycle (left) by its first metabolite acetaldehyde (Fig. 2), inhibiting MTR (Kenyon, Nicolaou, & Gibbons, 1998) and by blocking folate uptake via RFC1 by down-regulating its expression (Hamid & Kaur, 2007a). Ethanol-induced oxidative stress (Fig. 2) irreversibly inactivates MAT I and MAT III in the liver (Seitz & Stickel, 2007). AHCY: adenosylhomocysteinase; DHF: dihydrofolate; DHFR: dihydrofolate reductase; dTMP: deoxythymidine monophosphate; dUMP: deoxyuridine monophosphate; G9a (also known as EHMT2): euchromatic histone-lysine *N*-methyltransferase 2; MAT: methionine adenosyl transferase; 5-MTHF: 5-methyltetrahydrofolate; 5,10-MTHF: 5,10-methylenetetrahydrofolate; MTHFR: methylenetetrahydrofolate reductase; MTR: methionine synthase; RFC1: reduced folate carrier 1; SAH: S-adenosylhomocysteine; SAM: S-adenosylmethionine; THF: tetrahydrofolate; TS: thymidylate synthase.

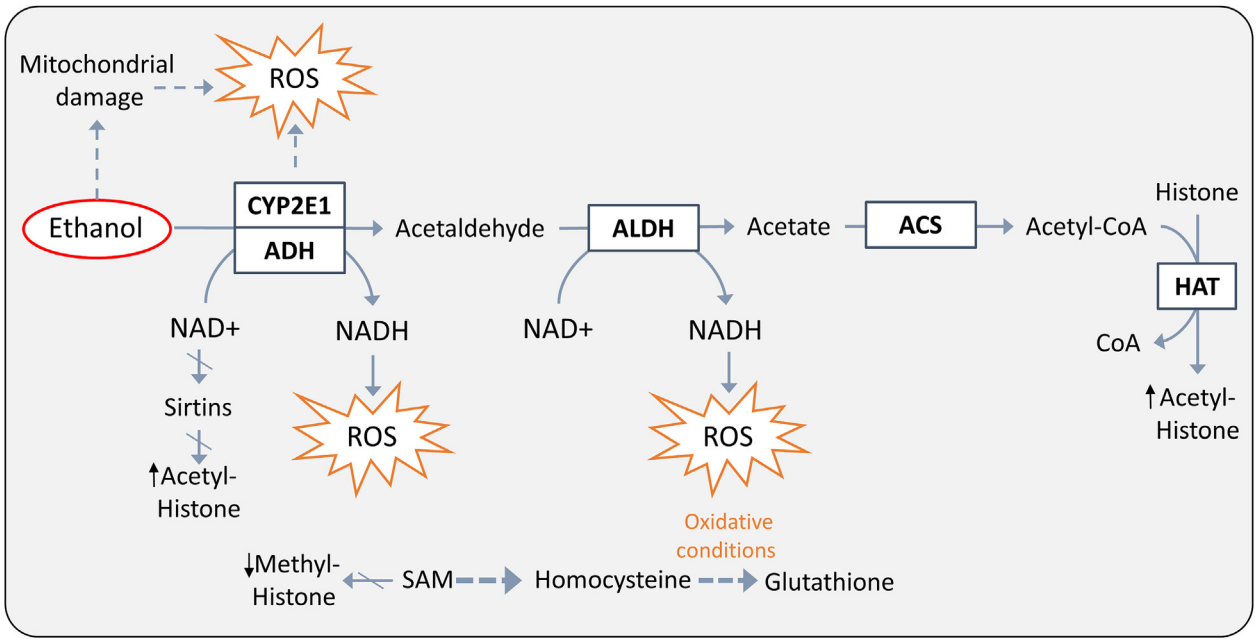

Fig. 2. Effects of the ethanol metabolism on epigenetic modifications. Slashed lines indicate reduction/impairment of that step. Dashed lines indicate simplified mechanism. Ethanol is metabolized to acetaldehyde, then acetate, both of which produce NADH, which leads to increased ROS production. Ethanol also induces mitochondrial damage, which leads to further ROS production (Hoek et al., 2002). Oxidative conditions drive production of glutathione, deleting SAM and therefore reducing methyl donors for histone (and other) methylation. Increased acetyl-CoA because of ethanol metabolism drives histone (and other) acetylation. Depletion of NAD + by ethanol metabolism reduces the activity of NAD + -dependent sirtins, which prevents histone de-acetylation. Metabolism of ethanol to acetaldehyde by CYP2E1 is simplified. Not shown is intermediate production of a geminal diol and water, which converts to acetaldehyde and an oxygen radical (shown as ROS above). Conversion of homocysteine to glutathione is greatly simplified. Conversion of SAM to homocysteine is simplified (Fig. 1). ACS: acetyl-CoA synthetase; ADH: alcohol dehydrogenases; ALDH: aldehyde dehydrogenase; CYP2E1: cytochrome P450 2E1; HAT: histone acetyltransferase; ROS: reactive oxygen species; SAM: S-adenosylmethionine.

perspective and focus. They all examined rodents – 11 studies examined mice and three studies examined rats. The models used across these studies are diverse. Choice of a model of prenatal ethanol exposure (PAE) is critical, as timing and dosage are the most important factors in predicting the outcomes (Kleiber, Diehl, et al., 2014; Mantha, Kleiber, & Singh, 2013; Miller, 2006; Patten, Fontaine, & Christie, 2014; Wozniak et al., 2004). The first consideration is ethanol exposure time point – early gestation, late gestation, or postnatal. Related to the ethanol exposure time point is the duration of exposure, which determines which developmental processes will be affected. The dosage of ethanol can also be modulated. Usually, the dosage is either binge exposure, which maximizes effects, or low continuous exposure, which more closely mimics blood alcohol levels (BACs) in humans. The vehicle of exposure (injection, gavage, preference drinking) is also variable. Finally, the endpoint will determine what types of effects are studied: acute vs. long-term. The 14 studies of histone medication in FASD are equally distributed in terms of gestational or post-natal exposure, and also binge or low continuous dosage (Table 1). There are specific marks that are studied more than others. The most studied is H3K9ac, followed by H3K4me3, H3K27me3, and H3K9me2. The majority of studies examine global changes for each modification, as opposed to gene-specific or whole-genome approaches. In the following sections, we will describe these findings that are centered on two systems: heart and brain.

6. Studies of histone modification changes in FASD

6.1. During heart development

Congenital heart disease is a potential outcome of prenatal ethanol exposure. It is present in 67% of individuals with fetal alcohol syndrome (FAS) (Burd et al., 2007). Using the C2C12 mouse cardiac progenitor cell line, Jie Tian's group examined the effects of ethanol on H3K9ac in culture. They found an increase in global H3K9ac that was dose-dependent, with the higher dose causing a greater increase in the H3K9ac (Zhong et al., 2010). There was also increased H3K9ac in regulatory regions of *Gata4, Mef2c,* and *Tbx5,* with increased expression of *Gata4* and *Mef2c* (Wang et al., 2012; Zhong et al., 2010). To explore the mechanistic basis of this increase in acetylation, the HAT inhibitor curcumin was also co-administered with ethanol. 25-μM curcumin completely ablated global, and heart gene-specific H3K9ac increases, as well as increased *Gata4* and *Mef2c* expression. These data suggest that ethanol may act through HATs to increase H3K9ac, affecting gene expression and altering fetal cellular development.

More recently, Tian's group used two *in vivo* models. Specifically, intragastric injection of ethanol in C57BL/6J (Pan et al., 2014) and Kun Ming mice (Peng et al., 2014) from gestational day (GD) 7.5 to GD15.5. They collected fetal hearts from GD11.5, through post-natal day (P) 7. Global H3K9ac was increased in both control and ethanol-exposed animals up to GD19.5 when it fell sharply, but ethanol-exposed mice had a significant increase over the controls. HAT activity was increased at GD17.5, while HDAC activity was unchanged. ChIP-qPCR of the *Gata4, α-MHC,* and *cTnT* promoters also showed ethanol-responsive H3K9ac changes correlating with expression increases (Peng et al., 2014). The effect of HAT inhibitor anacardic acid was also explored. Anacardic acid co-administration with ethanol reversed ethanol-induced H3K9ac global and gene-specific increases as well as gene expression changes. In another paper published in 2014, Tian's group explored global and cardiac gene-specific changes in H3K14ac (Zhang et al., 2014). Global H3K14ac and HAT activity were increased with ethanol treatment and were ablated with curcumin. The cardiac-specific genes *Dhand* and *Ehand* followed this pattern in both promoter H3K14ac and expression. These results in fetal heart provide a clear and

Table 1
Summary of studies examining effects of developmental ethanol exposure on histone modifications.

Study	Model	Exposure regime	Endpoint/tissue	Genes investigated	Mark(s)	Effects of ethanol
Chater-Diehl et al., 2016	C57BL6J mice	P4&P7, two 2.5 g/kg ethanol injections, 2 h apart	P70/hippocampus	All promoters	H3K4me3; H3K27me3	• General increase in H3K4me3, decrease in H3K27me3 • Enrichment of oxidative stress genes
Veazey et al., 2015	Primary mouse fetal cerebral cortical neuroepithelial stem cells	80, 120, 160, 240 mg/dL ethanol in culture for 3 days	Day 3 and Day 7 (4 days of ethanol recovery)/cell culture	22 promoters of growth factor signaling genes directing neuronal patterning	H3K4me3; H3K27me3; H3K9ac; H3K9me2	• Dose dependent, but in general modest H3K4me3 changes, more pronounced H3K27me3 and H3K9ac changes, and profound H3K9me2 changes
	C57Bl/6J mice	GD7, two 2.9 g/kg ethanol injections, 4 h apart	GD17/fetal cortex	(above)	(above)	• Changes in marks correlate with severity of fetal malformation • Overall pattern of change similar to cell culture results, in general a decrease in H3K27me3, increase in H3K9ac and H3K9me2
Subbanna et al., 2015	C57Bl/6J mice	P7, two 2.5 g/kg ethanol injections, 2 h apart	P7 (8 h after first injection)/ hippocampus and neocortex	*Cnr1 (Cb1r)* exon 1	H4K8ac; H3K9me2	• Increased H4K8ac and decreased H3K9me2 in both brain regions
Subbanna et al., 2014	C57Bl/6J mice	P7, two 1.0 g/kg ethanol injections, 2 h apart	P7 (4–24 h after injection)/ hippocampus and neocortex	*G9a* exon 1, global	H4K14ac; H3K9me2; H3K27me2	• Increased H4K14ac but not H4K8ac or H3K9me2 at *G9a* exon 1 in both brain regions • Increased global H3K9me2 and H3K27me2 at all time points in both brain regions, localized to degenerating neurons
Goldowitz et al., 2014	BXD (C57Bl/6J × DBA/2J inbred line)	P7, two 5 g/kg ethanol injections, 2 h apart	P7 (9 h after second injection)/cerebral cortex	Global	γH2AX; H3K14ac	• Increase in global γH2AX, no change in global H3K14ac
Pan et al., 2014	Kun Ming mice	GD7.5 to GD15.5 gavage exposure of dam to 10 μL/g/d, 56% ethanol	GD11.5, GD14.5, G17.5, GD18.5, P1 and P7/embryonic heart	Global	H3K9ac	• Global increase in H3K9ac in early embryonic days
Peng et al., 2014	Kun Ming mice	GD7.5 to GD15.5 gavage exposure of dam to 10 μL/g/d, 56% ethanol	GD14.5, GD16.5, P0.5, P7/embryonic heart	Global, *Gata4, cTnT, α-MHC*	H3K9ac	• Global H3K9ac increase on each day, increased H3K9ac in *Gata4* promoter on each day • Both changes attenuated by anacardic acid
Zhang et al., 2014	C57Bl/6 mice	(56%, 6 g/kg/d) via intragastric injection	GD14.5/embryonic heart	Global, *Dhand* and *Ehand*	H3K14ac	• Global increase in H3K14ac, increases at *Dhand* and *Ehand* promoters
Bekdash et al., 2013	Sprague-Dawley rats	GD7 to GD21 6.7% v/v ethanol liquid diet	P60-P65/arcuate area of hypothalamus (POMC neurons)	Global	H3K4me2,3; H3K9me2; H3K9ac; H3S10ph	• Reduced H3K4me2,3, increased H3K9me2 both normalized by choline co-administration • Reduced H3K9ac and H3S10ph not normalized by choline.
Veazey et al., 2013	Primary neurosphere cultures from G12.5 C57Bl/6J mice	60, 120, or 320 mg/dL ethanol in culture for 5 days	Day 5 of treatment/ cell culture	59 regions (stem cell-related gene promoters, *Hox* gene promoters, bivalent genes and transposons)	H3K4me3; H3K27me3	• All 8 stem cell-related genes and 10 *Hox* genes showed changes (usually reductions) in at least one mark in 320 mg/dL group • Changes in specific bivalent and transposable elements, reduced H3K27me3 across transposable elements
Subbanna et al., 2013	C57Bl/6J mice	P7, 1.0 or 2.5 g/kg ethanol injection, 2 h apart	P7/8, 4–24 h after first injection/ hippocampus and neocortex	Global	H3K9me2; H3K27me2	• Reduced global H3K9me2 in both regions, no change in H3K27me2 • Reduced total H3, correcting this there was increased H3K9me2/H3 and H3K27me2/H3 in both tissues. • Increased H3K27me2 neuron staining associated with neurodegeneration, reduced H3K9me2 neuron staining
Govorko et al., 2012	Sprague-Dawley rats			Global		

(*continued on next page*)

Table 1 (*continued*)

Study	Model	Exposure regime	Endpoint/tissue	Genes investigated	Mark(s)	Effects of ethanol
		GD7 to GD21 6.7% v/v ethanol liquid diet	P60-P80/arcuate area of hypothalamus (POMC neurons)		H3K4me2,3; H3K9me2; H3K9ac;	• Reduced global H3K4me2,3 and H3K9ac and increased H3K9me2 in POMC neurons
Wang et al., 2012	Mouse cardiac progenitor cell line (C2C12)	200 mM for 24 h	After 1 day of treatment	Global, *Gata4*, *Mef2c* and *Tbx5*	H3K9ac	• Increased H3K9ac
Guo et al., 2011	Long-Evans rats	P2-P12 inhalation for 4 h/day	P2, P4, P6, P8, P10, P12, within 1 h of final treatment/ cerebellum	Global	Acetyl-H3, Acetyl-H4	• Reduced AcH3 and AcH4 from P2-P10, no change in total H3 or H4
Zhong et al., 2010	Mouse cardiac progenitor cell line (C2C12)	50 or 200 mM ethanol for 24 h	After 1 day of treatment	Global	H3K9ac	• Increased H3K9ac, dose-dependent

Studies are sorted by data published. GD = gestational day, P = postnatal day, see text for histone modification nomenclature description.

consistent data set. They show that exposure of fetal heart cells to ethanol in culture and *in vivo* results in increased H3K9ac, and that these changes also occur at genes relevant to heart development. Increased acetylation correlates with increased heart gene expression and increased HAT activity.

6.2. During neonatal brain development

In mice and rats, the first postnatal week is neurodevelopmentally equivalent to the third trimester in humans (Dobbing & Sands, 1979). This time period coincides with critical neurodevelopmental processes, including synaptogenesis and the brain growth spurt (Dobbing & Sands, 1979; Rice & Barone, 2000). Binge exposure to ethanol during this period causes widespread apoptosis (Ikonomidou et al., 2000; Olney et al., 2002) and substantial changes to behavior, gene expression, and DNA methylation in the brain (Kleiber, Laufer, Stringer, & Singh, 2014; Kleiber, Mantha, Stringer, & Singh, 2013; Mantha et al., 2013; Otero, Thomas, Saski, Xia, & Kelly, 2012). The first study of histone modifications in the brain in FASD used inhalation exposure of rat pups to ethanol from P2–P12 (Guo et al., 2011). Acetylated histone H3 (AcH3) and H4 (AcH4) (as determined by an antibody against fully lysine-acetylated histone tail) were reduced in cerebellum. These results are at odds with the consistent finding of increased H3K9ac in the heart. However, the differing tissue, antibody, and exposure time point may account for this discordance.

Goldowitz et al. (2014) investigated the relationship between histone modifications and DNA damage in ethanol-induced apoptotic neurodegeneration. A recombinant mouse line was injected with ethanol on P7, and the effects in the cerebral cortex and hippocampus were evaluated 7 h later (Goldowitz et al., 2014). The histone variant γH2AX was globally increased in the cerebral cortex. γH2AX is a phosphorylated H2A variant that accumulates at DNA double-strand breaks (Kuo & Yang, 2008; Rogakou, Pilch, Orr, Ivanova, & Bonner, 1998). γH2AX is required for further apoptotic cascades, making it a good early indicator of apoptosis (Fernandez-Capetillo, Allis, & Nussenzweig, 2004; Rogakou et al., 1998). These results suggest that ethanol induces DNA double-strand breaks, which then leads to increased γH2AX. This may be a direct or indirect effect.

Another group investigated whether the HMT G9a was involved in the apoptotic effects of ethanol exposure on P7. G9a (also known as euchromatic histone-lysine n-methyltransferase EHMT2) and G9a-like protein (GLP) catalyze the H3K9me2 and H3K27me2 closed-chromatin modifications (Shinkai & Tachibana, 2011; Tachibana et al., 2002). These HMTs are also involved in early synaptic remodeling (Schaefer et al., 2009). In two studies, C57BL/6J mice were exposed to ethanol using a P7 dual-injection model. G9a mRNA, protein expression, and protein activity were increased after ethanol exposure in both hippocampus and neocortex (Subbanna et al., 2013). The *G9a* gene itself also showed increased H3K14ac in exon 1 (Subbanna et al., 2014). G9a up-regulation was associated with cTau (degenerating) neurons (Subbanna et al., 2013). H3K9me2/H3 and H3K27me2/H3 ratios were increased in both regions, while total H3 was reduced (Subbanna et al., 2013, 2014). Interestingly, treatment with the G9a inhibitor Bix prior to ethanol exposure prevented increases in H3K9me2 and H3K27me2, as well as apoptotic neurodegeneration (Subbanna et al., 2013, 2014). Bix pre-treatment prevented deficits in long-term potentiation, memory, and social recognition in adult mice (Subbanna & Basavarajappa, 2014). The group also examined CBR1, an inhibitory G protein-coupled receptor which controls neurotransmitter release (Mátyás, Watanabe, Mackie, Katona, & Freund, 2007). H4K8ac was increased at *Cbr1* exon 1, and H3K9me2 was decreased in both the hippocampus and neocortex (Subbanna, Nagre, Umapathy, Pace, & Basavarajappa, 2015).

These studies together suggest several specific mechanisms by which P7 ethanol exposure induces apoptosis and neurodegeneration. Alterations in histone-modifying proteins such as CREB and G9a appear to be a component of ethanol-induced apoptosis. It is clear that increased CBR1 and G9a expression/activity occur soon after ethanol exposure, and are responsible for triggering downstream effects resulting in neurodegeneration. Alterations in gene-specific and global histone modifications are also associated with neurodegeneration, and might be a key component of it.

6.3. During gestational brain development

The effects of ethanol on early gestational brain development may occur via different mechanisms from neonatal exposure in rodents. Very different processes occur in early versus late brain development. As such, exposure at this time leads to differing, but related behavioral phenotypes (Mantha et al., 2013). Exposure of the brain to ethanol during early gestation interferes with molecular regulation of stem cell differentiation and patterning (Camarillo & Miranda, 2008; Vemuri & Chetty, 2005). Rajesh Miranda and Michael Golding's group have published two papers on the effects of gestational ethanol exposure on histone modification changes at specific genes in the brain. Early changes in chromatin structure at development-related genes in stem cells are critical for maintaining cell identity. Histone modifications – especially the co-occurrence of H3K4me3 and H3K27me3 in bivalent domains – are a key part of developmental regulation (Bernstein et al., 2006). Given previous studies on the epigenetic effects of ethanol, and its role in disrupting neuronal stem cell fate, Miranda and Golding's group investigated this relationship. Fetal cerebral cortical neuroepithelial stem cells were isolated from GD12.5 C57BL/6J mice and cultured as neurospheres in ethanol for 5 days. Eight gene promoters with roles in regulating neural stem cells and ten *Hox* gene promoters showed reduced levels of H3K4me3 or H3K27me3 using chromatin immunoprecipitation (ChIP) real-time PCR (ChIP-qPCR) (Veazey, Carnahan, Muller, Miranda, & Golding, 2013). Ten bivalent gene promoters were studied, with only *Vdr* showing any changes – a reduction of H3K4me3. Only two genes, *Dlx2* and *Pax6,* showed expression changes (Veazey et al., 2013). The authors next used ChIP-qPCR of repetitive transposable element sequences – up to 45% of the human genome – to examine global H3K4me3 and H3K27me3 changes (Slotkin & Martienssen, 2007). There was significant reduction of H3K27me3 for all transposon types, and there was a trend toward reduction in H3K4me3, though it was non-significant (Veazey et al., 2013). Only seven of 30 epigenetic modifying enzymes that were investigated showed differential expression, none of which regulates H3K27me3.

In a follow-up study, the authors examined histone modification signatures that changed following recovery from ethanol, and compared these changes to an *in vivo* mouse model. In general, after 3 days of ethanol exposure there were modest H3K4me3 changes, more pronounced H3K27me3 H3K9ac changes, and large-scale H3K9me2 changes at all ethanol concentrations (Veazey, Parnell, Miranda, & Golding, 2015). There was also a lack of correlation between marks (i.e., loss of closed-chromatin marks did not co-occur with gain of open-chromatin marks) and dose-dependent

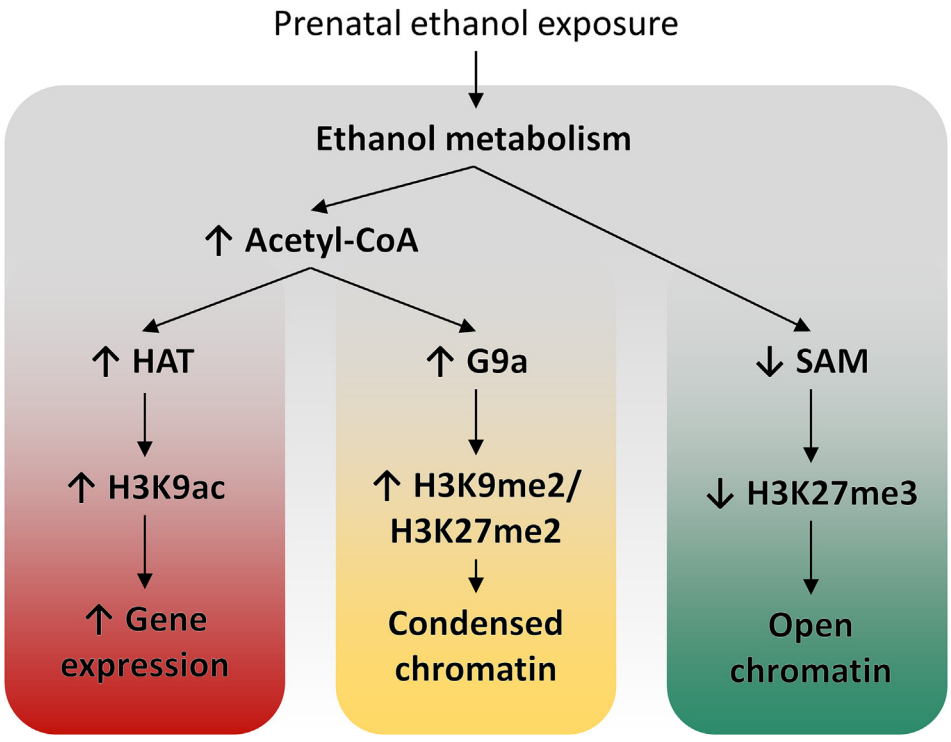

Fig. 3. Histone modification signatures consistently observed across FASD studies. A simplified view of three histone modification changes observed across studies and their possible mechanisms of genesis. In red, increased H3K9ac occurs consistently in short-term FASD studies, often attributed to increased HAT activity as a result of metabolism of ethanol to acetyl-CoA (Pan et al., 2014; Peng et al., 2014; Veazey et al., 2015; Wang et al., 2012; Zhong et al., 2010). In yellow, increased H3K9me2 and H3K27me2 observed in several studies (Subbanna et al., 2013, 2014; Veazey et al., 2013, 2015). These increases may be introduced by increased G9a activity and perpetuated through cell division through the buffer model. In green, decreased H3K7me3 is observed across some FASD studies (Chater-Diehl et al., 2016; Veazey et al., 2013, 2015). Causes and effects of these changes are less studied, but may be introduced by reduced SAM, and result in more open chromatin. Colored boxes represent differing temporal and/or spatial localization of implicated processes. G9a (also known as EHMT2): euchromatic histone-lysine *N*-methyltransferase 2; HAT: histone acetyltransferase; SAM: S-adenosylmethionine. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

relationships (Veazey et al., 2015). Allowing the neurospheres a 4-day recovery period elicited differing effects on open- and closed-chromatin marks; H3K27me3 and H3K9me2 became greatly enriched after recovery (Veazey et al., 2015). There were no changes in H3K9 nor H3K27 HMT activity. Expression of the H3K9 methylases *G9a* and *Setdb1* were suppressed after ethanol treatment and enhanced after recovery, consistent with the H3K9me3 data. However, the H3K9 demethylases *Kdm1a* and *Kdm4c* were suppressed after treatment as well, inconsistent with the H3K9me3 data. Therefore, altered expression/activity of epigenetic modifying enzymes may play some role in ethanol-induced histone modification changes, but do not account for all observed changes. To compare to *in vivo* effects, pregnant C57BL/6J mice were injected with 2.9 g/kg ethanol twice on GD7, and pups were assessed at GD17. In general, the histone modification profile of malformed ethanol-exposed pups correlated with the cell culture data – there was a reduction of H3K27me3 at more than half of the candidates, an enrichment of H3K9ac at some, and a dramatic increase in H3K9me2 at most (Veazey et al., 2015). The collective results of these studies suggest that histone modifications are each affected by ethanol exposure in cerebral stem cells in distinct genomic and temporal patterns. Further, changes in H3K9me2 persist and are even enhanced after recovery. The robust closed-chromatin mark changes are interesting, as H3K9 and H3K27 methylations are stable and heritable through development, likely replicating through the buffer model, acting as true epigenetic marks of repressed chromatin (Fig. 3) (Huang et al., 2013). They are thus very strong candidates for the transmission of a lasting ethanol-induced signature (Veazey et al., 2015).

6.4. In adult brain

The effects of ethanol in the brain can last a lifetime, affecting neuronal number and physiology, DNA methylation, and behavior (Gil-Mohapel, Boehme, Kainer, & Christie, 2010; Haycock, 2009; Kleiber, Diehl, et al., 2014). As such, many FASD researchers have endeavored to characterize the profile of the adult brain epigenome following prenatal ethanol exposure. In two studies, Dipak Sarkar's group investigated the effects of prenatal ethanol exposure on proopiomelanocortin (POMC) neurons in rats. The *Pomc* gene codes for proopiomelanocortin, which is cleaved into two classes of peptides controlling energy homeostasis, stress response, immune system function, and brain reward pathways (Cone, 2005; Luger, Scholzen, Brzoska, & Böhm, 2003; Mountjoy, 2010). The POMC system is affected by fetal ethanol exposure in humans and animal models, and the *Pomc* gene is down-regulated by PAE (Hellemans, Sliwowska, Verma, & Weinberg, 2010; Kelly et al., 2009).

In two studies, Sakar's group investigated whether epigenetic changes are involved in altered *Pomc* expression. Pregnant Sprague-Dawley rats were exposed to ethanol from GD7–21, and cells from the arcuate nucleus (ARC) of the hypothalamus were collected from the offspring in early adulthood, between P60–80. The ARC contains the majority of POMC neurons. Using immunohistochemistry, global H3K9me2, H3K4me2, H3K4me, and H3K9ac levels were assessed in ARC. There were reduced numbers of H3K4me2-and H3K4me3-positive POMC cells, increased H3K9me2-positive POMC cells, and reduced H3K9ac-positive POMC cells (Bekdash, Zhang, & Sarkar, 2013; Govorko, Bekdash, Zhang, & Sarkar, 2012). Gestational choline supplementation normalized the methylation changes (Bekdash et al., 2013). In agreement with these data, both studies found decreased mRNA of *Set7/9,* which encodes a H3K4 methylase, and increased *G9a* and *Setdb1,* which encode H3K9me2 methylase and H3K9me1 and H3K9me2 methylases, respectively. Each of these was normalized by choline supplementation (Bekdash et al., 2013; Govorko et al., 2012). In addition, they found numerous DNA methylation changes at the *Pomc* promoter that were inherited by subsequent generations.

In a recent study, our group also explored the long-term effects of early ethanol exposure on histone modification in the brain. We wanted to perform the most comprehensive examination of histone modification in FASD to date. We used C57BL/6J mice injected with ethanol on P4 and P7 and collected hippocampus at P70. Using ChIP coupled to a promoter microarray (ChIP-chip), we investigated H3K4me3 and H3K27me3 enrichment at all promoters in the genome in response to PAE. There were hundreds of changes for each mark, long after ethanol exposure. We found about four times more H3K4me3 changes than H3K27me3 changes (Chater-Diehl, Laufer, Castellani, Alberry, & Singh, 2016). H3K4me3 changes were slightly skewed toward increases in methylation, whereas H3K27me3 changes were much more likely to be decreases in methylation (Chater-Diehl et al., 2016). There was little co-occurrence of H3K4me3 and H3K27me3 changes in gene promoters, and very little correlation with gene expression changes for either mark. However, there was a significant enrichment of oxidative stress pathway genes in both the gene expression and histone methylation data. These findings show that while individual genes may not show correlation between changes, gene expression, and epigenetic marks, the pathways and processes affected can be related. This relationship may indicate an underlying mechanism manifesting as changes in expression and epigenetic modification of oxidative stress genes. Together, these three long-term studies show that despite their often short-lived reputation, histone modification changes persist long after early ethanol exposure. Further, these changes may be maintained by alteration in expression of HMTs. The functional relevance of these changes has yet to be assessed. However, this is an issue for most histone modification studies in general.

7. Patterns in modification changes

Finding trends in tissues or time points is not possible, given the limited number of histone studies. Given the heterogeneity introduced by ethanol timing and dosage differences, more repeated studies would be beneficial to determine repeatability and thus reflect reliability of these results. Though the models and time points used for each study are different, many of the same marks are investigated in each. There was increased H3K9ac in six out of eight studies that have examined it. This is not surprising, as the role of increased histone acetylation in alcohol-induced liver disease has been established (Kim & Shukla, 2005; Park et al., 2003). Ethanol increases acetate levels because of ethanol metabolism and inhibits HDACs. Interestingly, the two studies that found decreased H3K9ac were by the same laboratory – Dipak Sarkar's group – using the gestational liquid diet rat model. The fact that H3K9ac was altered so long after exposure suggests a maintained alteration to enzymatic content/function/expression. Indeed, the authors found *Set7/9*, *G9a*, and *Setdb1* expression differences.

In terms of methylations, all four studies examining H3K27me3 have found a reduction, both globally and at specific genes, though some genes showed enrichment (Veazey et al., 2015 is counted as two studies, *ex vivo* and *in vivo*) (Chater-Diehl et al., 2016; Veazey et al., 2013, 2015). There have been two studies of H3K27me2 specifically, in neocortex and hippocampus, showing global increases in response to ethanol (Subbanna et al., 2013, 2014). All three studies examining H3K4me3 found subtle changes, with candidate genes showing near equal distribution of enrichment and depletion of the mark (Chater-Diehl et al., 2016; Veazey et al., 2013, 2015). H3K9me2 was increased in five of six studies (Bekdash et al., 2013; Govorko et al., 2012; Subbanna et al., 2014,

2015; Veazey et al., 2013, 2015). In Veazey et al. (2015), the H3K9me2 changes were the most consistent and dramatic. These data suggest that a global alteration of closed-chromatin marks occurs after PAE and potentially persists over a long period. Levels of these marks are believed to self-perpetuate through the buffer model, which may explain why they are maintained after PAE (Huang et al., 2013). Together with increased H3K9ac, these changes in repressive marks are the most consistent and may point to key mechanisms in FASD etiology (Fig. 3).

8. Possible mechanisms of histone modification changes

The work in fetal heart has implicated increased HAT activity as a possible mechanism for the consistent H3K9ac increases in that model (Pan et al., 2014; Wang et al., 2012). Studies in other tissues have found similar data on HDACs being unaffected (Perkins, Lehmann, Lawrence, & Kelly, 2013), while other have implicated them (Yao, Nguyen, & Nyomba, 2014). Studies have also implicated a reduction in HAT activity in response to PAE (Guo et al., 2011). HAT and HDACs are at the convergence of various cell signaling cascades, and are the targets of post-translational modifications allowing them to respond to environmental cues and direct a gene expression response (Turner, 2000). HATs may be up-regulated in response to PAE by one of more of these mechanisms, and likely by increased acetyl-CoA because of ethanol breakdown, which is a HAT substrate.

There has also been exploration of the possible mechanisms of histone methylation changes. The best evidence for these changes is in G9a (EHMT2). G9a catalyzes the closed-chromatin H3K9me2 and H3K27me2 modifications and is essential for early development and synaptic remodeling (Shinkai & Tachibana, 2011; Tachibana et al., 2002). Basavarajappa's and Sakar's groups have found associations between increased G9a mRNA and protein levels in response to early ethanol exposure (Govorko et al., 2012; Subbanna et al., 2013). Further, G9a up-regulation was associated with neuronal degeneration (Subbanna et al., 2013). Taken with the consistent enrichment of H3K9me2, these data suggest G9a alteration may be a key component of FASD etiology. It may be that ethanol-induced increases in HAT activity are responsible for increased *G9a* expression and H3K9me2 (Fig. 3).

Data for other methylations and HMTs is somewhat conflicting. It seems that mechanisms other than altered HMT expression/activity are at play. One possible explanation is increased oxidative stress and reduced methyl donors (Fig. 2). We would expect that because ethanol leads to a shortage of all methyl groups, there would be a reduction in all methylations. Though reductions in histone methylation are observed, there are many increases in methylation as well. This predicted effect seems to be very consistent in the effects of PAE on DNA methylation, where increases in methylation are very common (Kaminen-Ahola et al., 2010; Liu, Balaraman, Wang, Nephew, & Zhou, 2009; Zhou, Chen, & Love, 2011). It may be that specific ethanol timing and dosage regimes in specific cells predominately affect oxidative stress/one-carbon metabolism leading to reduced histone methylation, while others predominantly affect HMT expression/activity leading to increased histone methylation (Fig. 3). It is not surprising that these possibilities are not yet resolved, given the diversity of possible timing and dosage regimes, cell types, animal models, and the small number of studies conducted.

9. Correlation with gene expression

In several studies, gene expression and histone modification changes co-occur at specific genes of interest; this is not the case when examining multiple genes. Chater-Diehl et al. (2016) and Veazy et al. (2015) both examined H3K4me3 and H3K27me3 on a wider scale in conjunction with gene expression. Neither study found large numbers of instances of genes bearing histone methylation changes that also exhibited altered expression. There are at least three possible explanations for this observation. First, the changes in expression may have occurred already and the mark remains. Second, it is possible that alteration of one mark is not sufficient to alter the chromatin state of the cell. Technologies are still lacking to assay multiple histone marks in the same region, let alone the same nucleosome. Related to this, a hypothesis gaining acceptance in recent years is that most histone marks are not causative of gene expression changes, but rather are a residual indicator of nucleosome remolding events (Henikoff & Shilatifard, 2011). A third possibility is that though the altered marks are present at a promoter, they only affect an expression change under a specific environmental condition, for example, during inflammation. Indeed, this interaction effect between PAE and inflammation on gene expression has been recently described by Kobor and Weinberg's group (Lussier et al., 2015). Future work should also investigate the contribution of histone marks to this phenomenon. Finally, promoters are the only region to be investigated in all the current studies. Enhancers are also marked by histone modifications and may be far more dynamic and environmentally responsive; they should be investigated further in epigenetic studies of disease (Ong & Corces, 2011). It is likely that the low correlation of gene expression and histone modification changes after PAE is some combination of these four factors.

10. Histone modifications as potential biomarkers

The potential of histone modifications as biomarkers is almost always cited when differences are found in a disease. The actual utility of histone modifications as biomarkers has only been seriously considered recently. As such, we do not have a gauge on how effective histone modification biomarkers will be for FASD, as they are not in wide use for any condition. Biomarkers can serve several roles. An ideal biomarker should be part of the causal pathway of the disease. It should also be well understood, and not related to any unknown factors that are also related to the exposure. If this is the case, it can reduce the validity of the relation between biomarker and disease (Mayeux, 2004). There are two main types of biomarkers: biomarkers of exposure which are used to make predictions, and biomarkers of disease which are used for diagnosis (Mayeux, 2004). Biomarkers of disease in FASD are very challenging, because we know so little about their etiology. One can imagine a mark that correlates with the severity of neurodevelopmental challenges, but unless a mechanism is understood, its validity would be suspect. In theory, histone modifications should have good utility as biomarkers of fetal alcohol exposure. Specific marks at specific genes may indicate how much fetal alcohol exposure has occurred. Importantly, this quantity can be inferred from patient reports and gauged against the molecular changes. However, these types of markers would be independent of the behavioral outcome, which is known to vary greatly even with the same exposure.

So far, a reliable signature of early ethanol exposure is elusive. Veazey et al. (2015) suggest H3K9me2 as a biomarker based on their data. The other H3K9me2 data summarized here support that notion. H3K9 methylations do have many of the hallmarks of a good biomarker, as they propagate their own inheritance directly and are not dependent on sequence (Audergon et al., 2015; Huang et al., 2013). More research is needed regarding the cause and consequence of this mark before a biomarker could be considered. For now, DNA methylation is more amenable to serve as a good biomarker in FASD, because we understand more about its

functional role, and there are more highly developed detection methods.

11. Limitations of current studies

While there is breadth in the current studies of histone modification in FASD, there are limitations to the current body of research. All of the current studies are in rodent models or mouse-derived cell cultures. Almost all studies are in mice, specifically C57BL/6J mice, with only three in other strains and three in rats. Model homogeneity allows easy comparison across studies. However, the genetic background used can cause major differences in findings on genetic studies of disease (Doetschman, 2009; Yoshiki & Moriwaki, 2006). The studies thus far have had limited attempts at reproduction of the data of other groups. The directions of each group have been very independent, which is not surprising given the number of potential modifications and models to study. There have been some attempts at reproduction of data within research groups, which have been very successful (Bekdash et al., 2013; Pan et al., 2014; Veazey et al., 2015). All the studies to date have been limited by technology. The study of histone modification today is based on antibodies, which can be non-specific and generate variable results. Further, they are not very effective when investigating multiple histone modifications simultaneously. New technologies are being developed with the goal of overcoming these limitations. An example is histone modification interacting domains (HMIDs) assays. This approach uses endogenous histone modification binding domains instead of antibodies to assess chromatin in a ChIP-like assay (Kungulovski et al., 2014). HMIDs have the advantage of being highly reproducible between experiments as well as being easy to produce. Very recently, a single-nucleosome imaging approach was developed that can completely assay combinatorially modified nucleosomes (Shema et al., 2016). These technologies hold the promise to expand histone studies and address many of the unanswerable questions about their role and regulation.

12. Future directions

The studies of histone modification in FASD to date have explored diverse exposure paradigms, time points, and modification states. They illustrate the complexity and variability of FASD etiology. Though the studies are varied in terms of specific findings, they do point to several trends. First, increased H3K9me2 and H3K9ac, along with reduced H3K27me3, produce the most consistent effects, with the most potential to affect cellular dynamics and explain FASD etiology (Fig. 3). Altered G9a and HAT expression/activity are also consistently coincident with enrichment of these modifications. Future work should be focused on the relationship between these marks and modifying enzymes using differing time points, exposure models, and cell types to determine their role and scope in FASD. Future studies should also attempt to repeat findings, given the success experienced by specific groups. The promise of new technologies on the horizon should allow more reproducible studies. The difficulties encountered with poor correlations when comparing modifications to each other or to expression data may be better understood by the improved scope of these new technologies. Exploration of these avenues is critical to the understanding of how histone modifications contribute to the etiology of FASD.

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