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# The Genetics of Fetal Alcohol Spectrum Disorders (FASD)

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## Abstract

**Background—**The term Fetal Alcohol Spectrum Disorders (FASD) defines the full range of ethanol-induced birth defects. Numerous variables influence the phenotypic outcomes of embryonic ethanol exposure. Among these variables, genetics appears to play an important role yet our understanding of the genetic predisposition to FASD is still in its infancy.

**Methods—**We review the current literature that relates to the genetics of FASD susceptibility and gene-ethanol interactions. Where possible, we comment on potential mechanisms of reported gene-ethanol interactions.

**Results—**Early indications of genetic sensitivity to FASD came from human and animal studies using twins or inbred strains, respectively. These analyses prompted searches for susceptibility loci involved in ethanol metabolism and analyses of candidate loci, based on phenotypes observed in FASD. More recently, genetic screens in animal models have provided additional insight into the genetics of FASD

**Conclusions—**Understanding FASD requires that we understand the many factors influencing phenotypic outcome following embryonic ethanol exposure. We are gaining ground on understanding some of the genetics behind FASD, yet much work remains to be done. Coordinated analyses using human patients and animal models are likely to be highly fruitful in uncovering the genetics behind FASD.

## Introduction

Alcohol exposure is the most common cause of environmentally induced human birth defects. Despite this, it was not appreciated until 1968 that a suite of birth defects may be associated with prenatal ethanol exposure (Lemoine et al., 1968) and 1973 when Fetal Alcohol Syndrome (FAS) was clinically recognized (Jones and Smith, 1973). We now know that ethanol can cause a wide range of birth defects, that are collectively referred to as Fetal Alcohol Spectrum Disorders (Riley et al., 2011).

The authors declare no conflicts.

While ethanol exposure causes FASD, not all exposures result in recognizable FASD. For instance only 4.3% of children with heavy exposure to ethanol will develop full blown FAS (Abel, 1995), suggesting additional susceptibility factors are involved. As early as 1988, an indication of a role of genetics in susceptibility to FAS was identified due to families with a child with FAS being dramatically more likely to have a second child with FAS (Abel, 1988). While genetics is merely one possible reason for this association, twin studies provided compelling evidence for genetics. In a study of monozygotic and dizygotic twins, monozygotic twins were 100% concordant for diagnosis while dizygotic twins were only 64% concordant (Streissguth and Dehaene, 1993). This study strongly suggests that genetic loci regulating susceptibility to, or resistance against, FASD must be present in the human population.

Similar to humans, the initial evidence for genetic basis for FASD in animal models came from studies of closely related individuals. Inbreeding has generated strains of organisms with high levels of homozygosity within the strain. Either through selective breeding or genetic drift in isolated populations, numerous substrains of rodents, chicks and fish have arisen in the scientific community. Amongst these strains and substrains of any particular species there are differences in their inherent susceptibility to the teratogenic effects of developmental ethanol exposure. Interestingly, the susceptibility to the effects of ethanol often depend on the dependent variable tested and the time of exposure. As we examine these strains, their respective genetic differences and careful attention to the relevant embryology and developmental neurobiology will assist in identifying either specific mechanisms or genetic pathways involved in ethanol's teratogenesis at specific developmental stages and on particular systems.

One of the first studies demonstrating strain differences was by Chernoff (1980), who maintained three strains of mice on a liquid diet containing ethanol prior to and throughout pregnancy and then examined the incidence and severity of fetal abnormalities. The CBA/J strain exhibited the most abnormalities, followed by C3H/lg mice and then the C57BL/6J strain. Not surprisingly, the incidence of defects was directly related to maternal blood alcohol concentration (BAC) and inversely related to liver ADH activity. It is unknown if the pregnant dams consumed the same amount of diet, and thus alcohol, but assuming they did, this study indicates a significant maternal factor, rather than fetal factors.

Since this characterization, numerous other studies since have compared the relative teratogenicity of ethanol in various strains of mice and have kept the amount of alcohol consistent. In a comparison of C57BL/10 vs. DBA/1 mice following early gestational ethanol exposure, both strains exhibited lower thresholds to audiogenic seizures, but this effect was much greater in the C57BL/10 mice, possibly due to an altered serotonergic system (Yanai, 1983). The C57BL/10 mice were also hypoactive, whereas the DBAs were not, and while both strains demonstrated reduced predatory behavior, this effect was much greater in the DBA strain. The various C57 substrains are consistently more susceptible to the effect of developmental ethanol exposure. In a whole embryo culture model of neurulation-stage ethanol exposure, both the C57BL/6N and DBA/2 strain exhibited significant malformations of the forebrain, optic system and hindbrain, although there was a differential sensitivity outside of the central nervous system (Ogawa et al., 2005). However,

a later study by this group with a narrower exposure window found delays of the forebrain, optic vesicle, midbrain hindbrain, and other derivatives of the caudal neural tube in the C57BL/6N strain, but only forebrain and optic vesicle deficiencies in the DBA/2 strain, while the 129S6/SvEvTac strain was not affected at all (Chen et al., 2011). These studies demonstrated interesting rostrocaudal and exposure period differential susceptibility that could be leveraged to focus on more specific mechanisms and genetic differences.

The C57BL/6J mice, one of the original C57 strains, were the first to be shown capable of recapitulating the craniofacial aspects of FAS and are more susceptible to ethanol teratogenesis during neurulation than many other strains, including, DBA/2, short-sleep, long-sleep, A/J, A/lbg, and 129S6 mice (Boehm et al., 1997; Downing et al., 2009; Downing et al., 2012; Sulik et al., 1981). In fact, the C57BL/6J mice are probably the most susceptible of the C57 sub-strains as demonstrated in a recent study comparing the C57BL/6J and C57BL/6N sub-strains during gastrulation-stage exposure, where the 6J mice exhibited ocular defects (rostrally-derived structures) at nearly twice the rate of their 6N counterparts (Dou et al., 2013). Finally, a recent experiment involving embryo transfers between C57BL/6J and DBA/2 mice confirmed that, with the possible exception of the maternal alcohol metabolizing enzymes, it is the genotype of the embryo and not the maternal genotype that is a more critical genetic determinant of an individual's susceptibility to ethanol's teratogenesis (Gilliam, 2014).

Rats have also been useful in assessing genetic contributions to FASD. In a third trimester equivalent model, the MR strain of rat demonstrated significant cerebellar deficiencies similar to the well-characterized Sprague-Dawley rat, whereas the M520 was less affected (Goodlett et al., 1989). However, the M520s exhibited body and brain growth restrictions by adulthood, perhaps demonstrating abnormalities of the pituitary gland, a structure partially derived from more rostral midline structures. While the anatomical defects are unknown, alcohol exposure throughout gestation increased seizure susceptibility in a strain of rat specifically bred to be more prone to convulsive epilepsies, while the WAG/Rij strain did not demonstrate this effect (Russo et al., 2008). Many FASD studies have been performed in the Sprague-Dawley rat, and two substrains of this strain have been developed that differ in their susceptibility to a neurulation-stage ethanol exposure (Wentzel and Eriksson, 2008). While these substrains metabolize ethanol at different rates, the more susceptible U strain still exhibited a greater incidence of ethanol-induced birth defects in an embryo culture system. Follow-up comparisons of these closely related sub-strains could reveal important ethanol susceptibility genes.

Strain-dependent susceptibility to the effects of developmental ethanol exposure are not restricted to rodents. A study by Debelak and Smith (2000) examined 11 strains of chick embryos following ethanol exposure during early neurulation and found that the strains could be classified into very sensitive, moderately sensitive or insensitive to ethanol-induced apoptosis of cranial neural crest cells, which give rise to facial structures. Another study in gastrulation-stage embryos found that the craniofacial effects of these various strains can be separated into those who exhibit midfacial flattening, overall facial flattening, or even facial expansion (Su et al., 2001). Interestingly, in this latter study it appears that more factors than apoptosis alone contribute to facial morphology as some strains that had excessive ethanol-

induced apoptosis did not have any facial dysmorphology and degree of facial dysmorphology in susceptible strains did not correlate with the amount of apoptosis. It is possible that the gastrulation-stage exposure results in a down-regulation of Shh, which is necessary for normal face and brain development, or that both mechanisms act together. Subsequent transcriptomic comparisons of two chick strains, one susceptible [W98S] and one resistant [W98D], during late neurulation revealed numerous strain differences, mostly in genes involved in ribosome biosynthesis, among many others, demonstrating the potential of this approach in elucidating underlying genetic susceptibilities (Garic et al., 2014).

Due to external fertilization, ease in genetic and transgenic procedures and their similarity during early development to mammals, zebrafish are an excellent model in which to study FASD susceptibility genes. As with other species, fish also have multiple strains that differ in their sensitivity to developmental ethanol exposure. The first study examined three strains of zebrafish (EK, AB and TU) for survival and craniofacial malformations following ethanol exposure (Loucks and Carvan, 2004). The EK strain exhibited the most cell death, and both the EK and AB strains had more craniofacial malformations than the TU strain. Interestingly, different craniofacial skeletal elements were affected by ethanol exposure in each strain, a finding that could assist in uncovering both genetic factors and mechanisms. The lower amount of craniofacial dysmorphology in the TU strain may be due to increased embryo lethality in the TU strain (i.e., ethanol may be killing the most-affected embryos). However, another study demonstrated that a shorter exposure to alcohol resulted in significantly different brain neurochemistry in the AB strain, while the TU strain was resistant to the effects of ethanol (Mahabir et al., 2014). Further demonstration of the EK strain's sensitivity has also been shown in a study of ethanol during eye morphogenesis (Arenzana et al., 2006).

Defining differences among various strains in terms of their sensitivity to developmental ethanol exposure is most useful if we actually know the genetic differences among these strains. Luckily, we are at a point where we can begin to explore these genetic differences using technologies such as whole transcriptome sequencing (RNA-seq) and other high-throughput sequencing techniques. However, it must be cautioned that as we go forward with these studies that we are aware of potential strain differences in developmental trajectories, particularly in early development. For example, the C57BL/6N substrain begins gastrulation approximately 6 hours sooner than does the closely related C57BL/6J substrain (Dou et al., 2013). In rapidly developing species such as mice or fish, a 6 hour difference is substantial in terms of early embryonic events. Likewise, researchers should pay careful attention to the relevant embryology/developmental biology and ensure that we are studying the appropriate areas/cell types from which an affected structure is derived. If we design our studies carefully, we can make great progress in understanding specific genes that modify susceptibility to prenatal ethanol exposure.

## FASD Genetics

Despite the magnitude of the problem, our understanding of the genetics underlying FASD is still very much in its infancy. Here, we provide a background and evidence of gene-ethanol interactions causing FASD in humans and animal models (See Table 1 for a current list of

genes shown to modulate the teratogenicity of ethanol). As with the classic definition of gene-gene interactions, a gene-ethanol interaction merely relates to a resulting phenotype and should not be taken to mean a direct physical binding of a gene product and ethanol. Indeed, for some of these interactions, the endogenous gene product is nonexistent and therefore physical interactions are impossible. Reciprocally, the physical binding of ethanol to a gene product would not necessarily have to lead to a genetic interaction, although physical binding is one possible mechanism for a genetic interaction. For the vast majority of the gene-ethanol interactions discussed here, we do not know the precise molecular mechanisms that lead to ethanol-induced phenotypes. However, where possible we comment on potential causes of these gene-ethanol interactions.

## Genes involved in ethanol metabolism

Ethanol is cleared from the body first by its conversion to the highly reactive and toxic acetaldehyde, which is, in turn, converted to acetate. The predominant enzyme involved in alcohol conversion to acetaldehyde is Alcohol Dehydrogenase (ADH), with CYP2E1 and Catalase playing smaller roles. Acetaldehyde is subsequently converted to acetate via Aldehyde Dehydrogenase (ALDH). Studies in mouse have shown that inhibition of Adh activity but not Aldh increases the teratogenicity of ethanol (Ukita et al., 1993; Webster et al., 1983), suggesting that the clearance of alcohol itself is critical to avoid teratogenesis. On the other hand, direct administration of acetaldehyde is teratogenic in both mouse and zebrafish (Reimers et al., 2004; Webster et al., 1983), suggesting there may be some role for this metabolite in FASD. However, across several strains of chickens levels of acetaldehyde had to be beyond physiological levels to cause embryo malformations (Hartl and Shibley, 2002). Ethanol-exposure alters the activity of Adh and Aldh enzymes in rat (Boleda et al., 1992; Messiha and Varma, 1983), further complicating which metabolic enzymes are most relevant to FASD. Thus, there is mixed evidence for the actual teratogenic agent associated with ethanol consumption. It is likely that, depending upon context, either ethanol or acetaldehyde can be teratogenic and that until ethanol is metabolized to acetate the developing embryo is susceptible to harm.

In the human population, the rate of metabolism of these teratogenic substances may vary due to allelic differences in alcohol metabolizing enzymes. A more complete discussion of these alleles and their rates of metabolism can be found elsewhere (Warren and Li, 2005). While alleles of *ADH* and *ALDH* effect the overall risk of alcoholism (Crabb et al., 2004), to date the focus of study in human FASD has been on the major ethanol metabolic enzyme, ADH1.

ADH1 forms as a complex of proteins synthesized from three loci: *ADH1A*, *ADH1B* and *ADH1C*. Three alleles of both *ADH1C* and *ADH1B* have been described each with differing affinities for alcohol (Crabb et al., 2004). Of these, only the three alleles of *ADH1B* have been tested for the rate of clearance of ethanol in humans. In a Japanese population, individuals carrying *ADH1B*2* or *ADH1B*3* alleles cleared ethanol at a significantly higher rate than individuals homozygous for *ADH1B*1* (Neumark et al., 2004), consistent with their *in vitro* kinetics (Crabb et al., 2004). However, in other studies examining African American individuals there were no significant differences in clearance based on *ADH1B*

genotype (Marshall et al., 2014; McCarthy et al., 2010; Taylor et al., 2008). These apparent differences could be due to study population or sample size differences, although it also likely that the genotype at a single locus will not be completely predictive of overall ethanol clearance. Regardless, enzymes affecting the rate at which alcohol and/or acetaldehyde are cleared remain likely candidate loci regulating FASD susceptibility.

In humans, there is evidence that *ADH* alleles predicted to rapidly metabolize ethanol protect against FASD. The *ADH1B*2* allele is significantly underrepresented in mothers and their non-FAS-affected children (Viljoen et al., 2001). Maternal genotypes with at least one *ADH1B*3* allele also correlate with a lower incidence of FASD (Das et al., 2004; Jacobson et al., 2006; McCarver et al., 1997). These studies differ in whether offspring genotype associated with outcome. Similarly, the slow metabolizing *ADH1C* variant, *ADH1C*2*, associates with increased risk of ethanol-associated oral clefting (Boyles et al., 2010). In contrast, a different study suggests that a maternal *ADH1B*1/ADH1B*3* genotype is the most susceptible to having offspring with FASD (Stoler et al., 2002). While there are associations of differing *ADH* alleles with overall alcohol consumption, which could complicate some of these findings (Jacobson et al., 2006; Stoler et al., 2002; Warren and Li, 2005), at least 3 of these studies showed no differences in reported overall drinking across genotypes (Boyles et al., 2010; Das et al., 2004; McCarver et al., 1997). Collectively, this provides support for an involvement of *ADH* alleles in FASD, with alleles predicted to metabolize ethanol more quickly being underrepresented in FASD. The precise nature of this correlation remains unknown. Unfortunately, there are no reports on genetic interactions with *ADH* causing FASD in animal models, which could help resolve these results.

The animal data available on ethanol metabolizing enzymes have focused on *Aldh* and highlight the potential for complex interactions of maternal and zygotic genotypes in the genesis of FASD. Fanconi anemia is a disease in human caused by mutation in any one of a number of *FANC* genes, whose products form an assembly that repairs DNA damage (Duxin and Walter, 2015). In mouse, *Fancd2* interacts with *Aldh2* in the susceptibility of embryos to FASD (Langevin et al., 2011). $Aldh2^{-/-}$;$Fancd2^{-/-}$ and $Aldh2^{+/-}$;$Fancd2^{-/-}$ pups born to $Aldh2^{+/-}$ dams had a significantly elevated occurrence of eye defects and exencephaly (Langevin et al., 2011). Other zygotic genotypes were not susceptible to these ethanol-induced defects (Langevin et al., 2011). It is unclear if this complex interaction is involved in human FASD. However, it is interesting to note that individuals with Fanconi Anemia are predisposed to cancer due to DNA damage, predominantly due to reactive aldehydes, (Duxin and Walter, 2015) and rat models suggest that embryonic alcohol exposure may speed the acquisition of tumors (Polanco et al., 2010; Zhang et al., 2016). We know virtually nothing about the long-term health issues faced by individuals with FASD (Moore and Riley, 2015) and these findings may provide insight into these life long issues.

## An alternative (or additional) potential mechanism for ADH-ethanol interactions

In 1991, two authors separately published a hypothetical cause of FASD in which alcohol teratogenicity involves ADH but is a secondary effect due to altered cell signaling. ADH is also responsible for the conversion of retinol (vitamin A) into retinaldehyde, an essential step in the generation of retinoic acid. Because retinoic acid is a critical morphogen in the

development of many structures sensitive to ethanol teratogenesis (Cunningham and Duester, 2015), Duester and Pullarkat independently proposed that competition between ethanol and retinol for ADH could reduce retinoic acid levels, resulting in birth defects (Duester, 1991; Pullarkat, 1991). Several reports in a rat model, even prior to the publication of these hypotheses, demonstrated that maternal ethanol consumption alters the levels of some, but not all, RA pathway constituents in fetal tissues (Grummer et al., 1993; Grummer and Zachman, 1990, 1995). Most in support of the retinoic acid hypothesis is the finding that maternal ethanol consumption results in elevated levels of retinol but reduced levels of retinoic acid in fetal heart (DeJonge and Zachman, 1995) and that ethanol appears to alter the activity of *RAR* transgenic expression in cultured mouse embryos (Deltour et al., 1996). Increasing the levels of retinoic acid partially restores development in avian, amphibian and fish models of FASD (Aksamija et al., 2009; Marrs et al., 2010; Muralidharan et al., 2015; Satiroglu-Tufan and Tufan, 2004; Twal and Zile, 1997) and, reciprocally, ethanol can reduce the teratogenicity of excess retinoic acid signaling in frog (Yelin et al., 2005). Collectively, results across different model systems support the hypothesis that ethanol can disrupt retinoic acid signaling.

The effect of ethanol on retinoic acid signaling may be highly dependent upon cell type. In contrast to the prediction of the retinoic acid hypothesis, ethanol greatly elevates the levels of retinoic acid in the hippocampus of fetal mice (Kane et al., 2010). Ethanol-induced microphthalmia in zebrafish is not rescued by elevating retinoic acid signaling (Kashyap et al., 2011; Zhang et al., 2015). Chen and colleagues found that while ethanol did inhibit the generation of retinoic acid from liver extracts, it failed to do so from zygotic extracts (Chen et al., 1996). Further, as noted below in “Candidate Genes: Facial Clefting in Humans”, in a human data set a receptor for retinoic acid failed to associate with ethanol and craniofacial defects (Etheredge et al., 2005). Thus, despite decades of study and the molecular evidence for the retinoic acid hypothesis it remains unknown if mutation of any pathway members associates with susceptibility to FASD.

## The Genetics of Ethanol-Induced Facial Clefting in Humans

Drinking during pregnancy is a risk factor for isolated cleft lip with or without cleft palate (Munger et al., 1996). There has been extensive study into the genetics regulating orofacial development (Bush and Jiang, 2012) and several human studies have sought to determine if some of these candidate genes associated with ethanol and facial clefts. These studies have focused on members of the Transforming growth factor (TGF) pathway, the Bmp target, *MSX1*, and the Retinoic Acid receptor, *RAR-α*. In a study of 316 Danish children born with either cleft lip and/or palate, neither of the candidate genes *TGF-β3* or *MSX1* associated with ethanol usage and facial clefting (Mitchell et al., 2001). Similarly, a log-linear approach to more sensitively identify gene-environment interactions in a group of 222 children from Denmark with non-syndromic cleft lip/palate, failed to find association with *TGF-β3* and *MSX1* (Etheredge et al., 2005). This study also found no evidence for gene-ethanol interactions with *RAR-α* or *TGF-α* (Etheredge et al., 2005). Analyses of 214 cleft lip and/or palate cases in the Iowa birth defects registry found a significant association of ethanol consumption and *MSX1*, but not *TGF-α* or *TGF-β3* in cleft lip and/or palate (Romitti et al.,

1999). Thus, these candidate approaches in humans have had limited success in identifying loci mediating susceptibility to FASD.

There are even a more limited number of genome-wide association studies (GWAS) that have been performed to identify gene-ethanol interaction. In a GWAS analysis of 550 case-parent trios of non-syndromic cleft palate cases for gene-environment interactions, SNPs in *MLLT3* and *SMC2* were significantly associated with ethanol usage and facial clefting (Beaty et al., 2011). MLLT3 contains a nuclear targeting sequence and translocation fusions between it and MLL result in leukemia (Strissel et al., 2000). In mouse, regions of the CNS, limb buds, facial prominences and skeletal primordia express *Mllt3* and *Mllt3* mutants have vertebral fusions consistent with disrupted Hox signaling (Collins et al., 2002), the facial skeleton was not specifically analyzed in these mice. SMC2 is part of the Structural Maintenance of Chromosomes gene family and functions in the Condensin complex (Hirano, 2002). This complex maintains chromosome condensation during mitosis and it critical for proper chromosome segregation (Hirano, 2002). SMC2 also appears to have non-mitosis functions, as *in vitro* depletion of *SMC2* disrupts nuclear shape (George et al., 2014). To our knowledge, *in vivo* phenotypes of Smc2 loss-of-function in vertebrates are unknown, however RNAi-mediated knockdown of *Smc2* results in increased death of embryonic stem cells (Fazzio and Panning, 2010). Continued genome-wide analyses in humans with follow-up analyses in animal models should help provide insight into how these genes and others are involved in FASD.

## The Sonic Hedgehog (Shh) Pathway

Shh is a morphogen that, like retinoic acid, is critical for the development of many structures affected in FASD. A more extensive review of Shh signaling can be found elsewhere (Ingham and Placzek, 2006), but briefly Shh is a secreted ligand that binds the receptor Patched (Ptch). In the absence of ligand, Ptch represses the activity of the 7 pass transmembrane protein Smoothened (Smo). In the presence of ligand, Smo is derepressed and processes Gli transcription factors, notably Gli1 and Gli2, into activator forms to turn on gene expression. Other cell surface proteins, Cdon and Boc, act as Shh coreceptors and assist in activating the Shh pathway.

The Shh pathway was initially implicated in FAS due to phenotype. The facial and neural phenotypes associated with FAS in humans and animal models resemble those of holoprosencephaly (Sulik, 2014) and embryonic ethanol exposure is a risk factor for holoprosencephaly in humans (Cohen and Shiota, 2002). Most mutations known to cause holoprosencephaly disrupt the Shh pathway (Solomon et al., 2010). Further, a chicken model of FASD resulted in craniofacial defects and neural crest apoptosis similar to that observed when Shh signaling was blocked by antibodies (Ahlgren and Bronner-Fraser, 1999; Cartwright and Smith, 1995). In this system, ectopic application of Shh rescued these defects (Ahlgren et al., 2002). Collectively, these findings lead to the hypothesis that one teratogenic mechanism of ethanol was the inhibition of Shh signaling.

Despite these early indications of a role of Shh in ethanol teratogenesis, it wasn't until a decade later that there was direct evidence for a genetic interaction between ethanol and the Shh pathway. Under normal conditions, loss of *Cdon* in a 129S6 genetic background only

results in mild defects similar to microform holoprosencephaly. This genotype is highly sensitive to ethanol exposure and displays profound neural and facial phenotypes covering the range of holoprosencephaly as well as palatal defects following embryonic ethanol exposure (Hong and Krauss, 2012). Wild-type and heterozygous littermates were largely normal in these analyses (Hong and Krauss, 2012). In a set of follow up experiments, the same group found that elevating Shh signaling by loss of a single allele of *Ptch1* was able to restore proper development in ethanol-treated *Cdon* mutant embryos (Hong and Krauss, 2013). In humans, haploinsufficiency for *CDON* causes holoprosencephaly (Bae et al., 2011), however, ethanol only appears to exacerbate the homozygous mutant phenotype in this mouse strain (Hong and Krauss, 2012). The reason for this discrepancy is unclear, but it may relate to the use of the 129S6 genetic background because mouse *Cdon* mutants on a C57BL/6N background have more severe holoprosencephaly (Hong and Krauss, 2012).

In addition to *CDON*, haploinsufficiency of either *GLI2* (Roessler et al., 2003) or *SHH* (Roessler et al., 1996) causes holoprosencephaly in humans. Under control conditions, mouse *Shh* and *Gli2* heterozygotes develop normally (Chiang et al., 1996; Mo et al., 1997). However, heterozygosity for *Shh* significantly enhanced the facial and neural defects caused by ethanol and this enhancement was even more profound in *Gli2* heterozygotes (Kietzman et al., 2014). Similar results are found in a zebrafish FASD model in which partial knockdown of *shha* via morpholinos sensitizes embryos to ethanol-induced neural differentiation defects (Zhang et al., 2013). Interestingly, ethanol does not appear to cause haploinsufficiency, at least for facial phenotypes, in zebrafish *smo* mutants (McCarthy et al., 2013), but does appear to enhance facial defects in a hypomorphic *shha* mutant line (our unpublished results). Collectively, these results demonstrate that the Shh pathway does genetically interact with ethanol, but not all members of the pathway interact equally. The reason for this “inequality” is not clear, but has important implications in determining genetic risk for FASD in human populations.

There are several possibilities for why ethanol genetically interacts with the Shh pathway. As mentioned above, ethanol may compete with retinol for ADH (RADH), inhibiting retinoic acid production. Retinoic acid is initially critical for inducing Shh expression in the notochord and overlying neural plate and continues to be an important morphogen and signaling molecule throughout development. The developing forelimb also requires RA for initial induction of forelimb outgrowth, and reductions of RA, or inhibition of the RA receptor results in postaxial ectrodactyly (loss of digits) similar to prenatal ethanol exposure (Johnson et al., 2007). Exogenous RA administration can rescue at least some of these effects. Further proof of this as a causative mechanism for some of the more severe brain (e.g. holoprosencephaly), craniofacial and digital effects of ethanol exposure is that the timing of the ethanol exposure is just slightly prior to the onset of *Shh* expression. In contrast, direct inhibition of Shh signaling via cyclopamine, a potent Smo antagonist, induces holoprosencephaly only at slightly later stages of development, when *Shh* is actively expressed (Heyne et al., 2015; Lipinski et al., 2014). This mechanism would also explain why ethanol exposure reduces *Shh* expression during gastrulation, but apparently not during neurulation. It is still unclear if ethanol is preventing the induction of *Shh* expression through inhibition of RA production, or through another mechanism.

A second possibility for an ethanol-induced disruption of the Shh pathway is based on the finding that Shh proteins must undergo post-translational modification in order to be properly packaged, secreted and to diffuse to its target cells (Dennis et al., 2012; Porter et al., 1996a; Porter et al., 1996b). One of these key modifications is the addition of cholesterol to the N-terminal end of Shh and the lack of this lipid modification results in altered Shh signal propagation. It was observed nearly 20 years ago that disruption of cholesterol homeostasis either through chemical inhibition of cholesterol biosynthesis during gastrulation or mutation of key cholesterol synthesis genes (i.e. DHRC7, which is mutated in Smith-Lemli-Opitz syndrome) results in dysmorphologies similar to those observed in ethanol exposure during gastrulation and these effects are likely exerted through the Shh pathway (Dehart et al., 1997; Lanoue et al., 1997; Porter et al., 1996b). This hypothesis is supported by the finding in zebrafish that ethanol can block the modification of Shh by cholesterol, resulting in decreased Shh pathway activation, and supplementation with cholesterol can rescue ethanol's effects (Li et al., 2007). This same group has also demonstrated that ethanol exposure can inhibit Caveolin-1/Shh complexes (Mao et al., 2009). Caveolin-1 is both dependent on, and modulates cholesterol homeostasis, and decreasing caveolin-1/Shh complexes results in decreased Shh secretion and subsequent signaling (Frank et al., 2006; Hailstones et al., 1998). Together, these data suggest that ethanol exposure can alter Shh signaling through a mechanism involving cholesterol, although more research is needed to fully understand this phenomenon.

Finally, the possibilities surrounding ethanol-induced cell death and disruptions in Shh signaling must also be considered. Ethanol has often been demonstrated to directly induce apoptosis at many periods of development (see below) and ethanol exposure during gastrulation has been shown to induce cell death in the general region of the embryo that will give rise to the face and brain (Dunty et al., 2001). However, at these early stages, these spatiotemporal data are difficult to interpret and it is not clear if these dying cells are causative or merely ancillary. For these reasons, it is unclear if ethanol is killing the cells expressing Shh or the cells receiving the Shh signal. Adding to the confusion is the fact that Shh itself is a potent cell survival factor and decreasing Shh expression, secretion or transduction may cause massive apoptosis secondary to the ethanol exposure (Delloye-Bourgeois et al., 2014; Thibert et al., 2003). Some of our recent unpublished data has demonstrated that mice lacking either one or both copies of the pro-apoptotic gene *Bax* show an inverse gene dose-dependent resistance to the detrimental effects of a gastrulation-stage ethanol exposure. Additional data from our lab and others showing that reducing oxidative stress (e.g. through administration of NAC) can at least partially ameliorate the effects of ethanol during early gestation also point to a mechanism involving cell death (Chen et al., 2013; Chen et al., 2015; Parnell et al., 2010; Wentzel and Eriksson, 2008; Wentzel et al., 2006). These data showing a clear relation between apoptotic cell death and resiliency to ethanol-induced dysmorphology demonstrate that cell death may interact with Shh signaling to mediate ethanol's teratogenesis. Regardless of the relationship, the idea of cell death must be considered alongside studies designed to investigate genes involved in modulating the effects of prenatal alcohol exposure.

### Nitric oxide synthase 1 (Nos1)

*In vitro* studies have shown that cerebellar granule cells are highly sensitive to ethanol-induced cell death (Pantazis et al., 1993). While nitric oxide can be pro-apoptotic (Brune et al., 1998), it has been shown to protect neurons from apoptosis (Ciani et al., 2002), making it a candidate to protect against ethanol-induced neuronal cell death. Indeed, elevating nitric oxide levels protects against ethanol-induced cerebellar granule cell death (Pantazis et al., 1998). This protective effect suggested that reduced nitric oxide could sensitize embryos to ethanol teratogenesis. Indeed, the ethanol-induced reduction in cortical, cerebellar and hippocampal size and neuronal cell loss was significantly more severe in *Nos1* mutants, compared to wild-type embryos (Bonthius et al., 2002; Karacay et al., 2015). These cerebellar neuronal losses are associated with poorer performance on balance beam and rotorod tests, demonstrating an associated behavioral deficit (Bonthius et al., 2015). Thus, the genesis of neural and behavioral deficits in FASD may depend, at least partly, on nitric oxide signaling.

How ethanol interacts with the nitric oxide signaling pathway remains to be elucidated and is likely to be complex. In cultured cerebellar neurons, NF-kB signaling acts downstream of nitric oxide signaling, with activation of NF-kB being required for the protective effects of nitric oxide signaling (Bonthius et al., 2009). In a rat model of FASD, ethanol reduces the levels of DNA-bound NF-kB in the cerebellum (Acquaah-Mensah et al., 2002), suggesting a possible mechanism for the *Nos1*-ethanol interaction. However, in whole embryo mouse and Xenopus models of FASD, ethanol elevates the level of NF-kB (Peng et al., 2005; Zheng et al., 2014). Additionally, Nerve growth factor and Fibroblast growth factor 2, but not Brain-derived neurotrophic factor, require nitric oxide signaling for their neuroprotective effects against ethanol (Bonthius et al., 2003). Thus, as is likely to be the case for most gene-ethanol interactions, the *Nos1*-ethanol interaction is likely to be context dependent.

### Platelet-derived growth factor receptor alpha (Pdgfra)/PI3K/mTOR

We recently combined a candidate gene and genetic screening approach to identify mutations that enhanced the teratogenicity of ethanol (McCarthy et al., 2013). We tested five mutants with disrupted facial development for ethanol sensitivity and discovered that *pdgfra* interacted strongly with ethanol. Notably, while under normal conditions *pdgfra* heterozygotes develop without defect, a normally subteratogenic dose of ethanol causes profound facial defects in two-thirds of heterozygous embryos (McCarthy et al., 2013). The strength of this interaction prompted us to examine a human dataset generated by the Collaborative Initiative on FASD, where we found strong support for gene-ethanol interactions with both PDGF receptors, *PDGFRA* and *PDGFRB*. Using zebrafish, we found that the *pdgfra*-ethanol interaction was synergistic in nature as ethanol greatly elevated the levels of apoptosis in mutants and heterozygotes relative to untreated mutants or ethanol-treated wild-type embryos. These data provide compelling support in both a model organism and human that ethanol interacts genetically with the Pdgf signaling pathway.

Insight into how ethanol may interact with the Pdgf pathway came from *in vitro* work with ethanol and *in vivo* analyses in mouse and frog. In mouse, the major effector of Pdgfra signaling during facial development is PI3K (Klinghoffer et al., 2002) and PI3K functions

downstream of Pdgfra to promote survival of mesoderm in frog (Van Stry et al., 2005). PI3K can activate AKT, which in turn can activate mTOR, an important pathway in cell survival (Dibble and Cantley, 2015). *In vitro* studies have demonstrated that ethanol can inhibit the PI3K/AKT/mTOR pathway at multiple levels (Hong-Brown et al., 2010; Xu et al., 2003). We found that activation of this pathway rescued mutants from the effects of ethanol and that signaling at or downstream of mTOR was disrupted in ethanol-treated *pdgfra* mutants (McCarthy et al., 2013). This finding suggests that other growth factor pathways, such as Insulin, that use the PI3K/AKT/mTOR pathway are likely to be ethanol-sensitive loci. While this prediction has yet to be tested, elevating Insulin signaling protects against FASD in a Drosophila model (McClure et al., 2011) and ethanol reduces Insulin-PI3K signaling in cerebella of rat pups (Xu et al., 2003). Because PI3K/AKT/mTOR is a major pathway linking nutrient sensing and growth factor signaling, it is possible that multifactorial gene-environment interactions through this pathway mediate FASD susceptibility.

### Others

In a follow-up genetic screen, we determined if ethanol interacted with mutant lines of zebrafish available from the Zebrafish International Resource Center (Swartz et al., 2014). We screened 20 mutant lines and found that a minority of them, 5, interacted with ethanol to produce exacerbated facial and/or neural defects. Ethanol exacerbated the phenotypes of *hinfp, foxi1, mars* and *plk1* mutants. The fifth mutant, *vangl2*, is a member of the Wnt Planar Cell Polarity (Wnt/PCP) pathway and interacted strongly with ethanol, causing cyclopia in all mutants and revealing haploinsufficiency. The cyclopic phenotype observed in the ethanol-*vangl2* interaction closely parallels phenotypes observed in zebrafish embryos treated with high doses of ethanol (Blader and Strahle, 1998). The Wnt/PCP pathway is critical for regulating convergent/extension movements in the early embryo that elongate the body axis, and separate the eye fields in zebrafish. There are several reports of ethanol disrupting convergent/extension movements (Sarmah et al., 2013; Yelin et al., 2005). Thus, it is likely that combined genetic and environmental inhibition of Wnt/PCP signaling is responsible for the *vangl2*-ethanol interaction, yet the mechanism by which ethanol may inhibit Wnt/PCP signaling remains to be determined.

## Concluding remarks

How extensive are gene-ethanol interactions? Or an oft-asked question: isn't ethanol just making a sick embryo sicker? Due to the small size of ethanol and its pleiotropic effects it would seem possible that ethanol would interact with most genes. The collective body of work presented here though clearly demonstrates that not all genotypes are equally sensitive to ethanol. From the genetic screens that we have performed, where we have also reported on genes that did not appear to interact with ethanol, it would seem that only a minority of genes interacts with ethanol, at least for phenotypes we have examined. Six of the 25 loci that we have tested interacted with ethanol and only two of these showed haploinsufficiency. By their nature our screens have been skewed towards genes involved in early embryogenesis and, therefore, may be overrepresented in loci that predispose to FASD. Due to the high prevalence of FASD, homozygous null mutation at a locus is likely to only explain a tiny fraction of human FASD cases, therefore, haploinsufficiency may be a better

model of reduced gene function, caused by coding or enhancer variants in humans. Much more extensive shelf screens could give more accurate insight into the commonality of gene-ethanol interactions, but based on our screens, it is likely that a low percentage of genes will interact strongly with ethanol. However, given the size of the human genome, currently estimated at 20,000 genes, even such a low percentage would result in many hundreds of ethanol sensitive loci. Clearly, much more work is needed to identify and characterize the effects of these genes involved in FASD susceptibility.

Given that many genes are likely to interact with ethanol, it might follow that there is no single functional type of gene that were sensitive to ethanol. Using the genes that have we have discussed that interact with ethanol, we performed gene function analysis in DAVID (https://david.ncifcrf.gov/home.jsp). This analysis examines co-occurrence between functional categories, such as gene ontology (GO) terms and a set of genes. Because *Shh*, *Gli2* and *Cdon* are all part of the Hedgehog pathway, we only used *Shh* in the analyses to avoid skewing the dataset towards functions regulated by this pathway. This analysis uncovered 8 clusters of functional terms enriched in these ethanol-sensitive genes (See Supplemental File 1 for the raw data output). Fig. 1 lists these clusters in descending order of enrichment; clusters are named according to the general properties of the functional terms within the cluster (See Supplemental File 1 for a complete list). This analysis would suggest that there is not a defining functional characteristic of loci that genetically interact with ethanol. Continued identification of gene-ethanol interactions, in particular using unbiased approaches, will be critical to fully understand the many genetic functions that ethanol inhibits. However, it is of interest that these clusters relate to functional processes have been implicated in FASD (Dennery, 2007; Sant'Anna and Tosello, 2006; Shibley and Pennington, 1997).

Why should we care about the genetics of FASD? Perhaps the most compelling reason is the fact that FASD is so widely variable in phenotypic outcome. We cannot fully appreciate how to deal with FASD medically unless we understand all of the factors that affect this phenotypic variability. As we understand how genetics leads to phenotypic outcomes, we will certainly be able to improve our diagnosis and ability to determine individual risk. Equally important, though, is that identifying a genetic interaction with ethanol gives insight into pathways, both genetic and signaling, that are causative of FASD and may provide interventions or preventatives against FASD.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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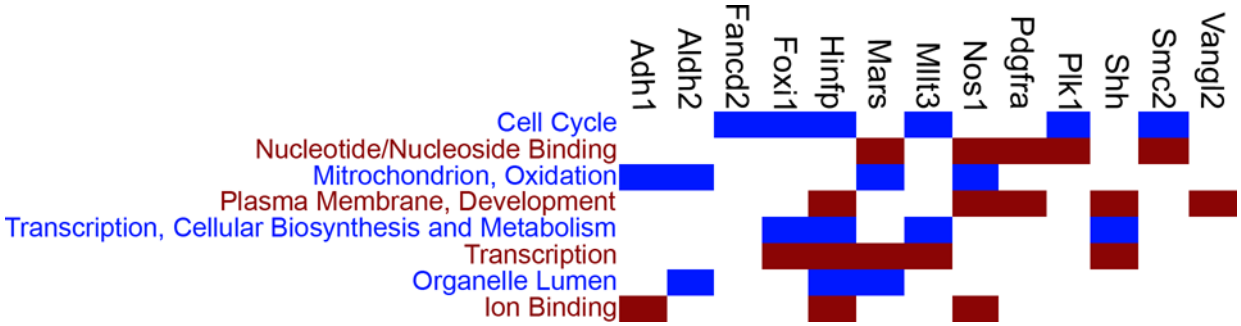


**Fig. 1.**

## Table 1

List of Gene-ethanol interactions.

| Gene(s) | Species | Phenotype | Reference(s) |
|---|---|---|---|
| *ADH1B* | Human | FAS | McCarver et al., 1997<br>Stoler et al., 2002<br>Das et al., 2004<br>Jacobson et al., 2006 |
| *ADH1C* | Human | Oral clefting | Boyles et al., 2010 |
| *MLLT3*<br>*SMC2* | Human | Oral clefting | Beaty et al., 2011 |
| *Aldh2;Fancd2* | Mouse | Eye defects<br>Exencephaly | Langevin et al., 2011 |
| *Cdon*<br>*Gli2*<br>*Shh* | Mouse<br>Zebrafish * | Holoprosencephaly,<br>Facial defects | Hong and Krauss, 2012<br>Kietzman et al., 2014<br>Zhang et al., 2013 |
| *Nos1* | Mouse | Neural and behavioral | Bonthius et al., 2002<br>Karacay et al., 2015<br>Bonthius et al., 2015 |
| *PDGFRA* | Human<br>Zebrafish | Facial | McCarthy et al., 2013 |
| *hinfp*<br>*foxi1*<br>*mars*<br>*plk1*<br>*vangl2* | Zebrafish | Facial<br>Some neural | Swartz et al., 2014 |

* denotes an interaction using a morpholino.