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A screen of zebrafish mutants identifies ethanol-sensitive genetic loci

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Abstract

Background—Fetal Alcohol Spectrum Disorders (FASD) are a highly variable set of phenotypes caused by fetal alcohol exposure. Numerous factors influence FASD phenotypes, including genetics. The zebrafish is a powerful vertebrate model system with which to identify these genetic factors. Many zebrafish mutants are housed at the Zebrafish International Resource Center (ZIRC). These mutants are readily accessible and an excellent source to screen for ethanol-sensitive developmental structural mutants.

Methods—We screened mutants obtained from ZIRC for sensitivity to ethanol teratogenesis. Embryos were treated with 1% ethanol (41 mM tissue levels) from 6 hours post fertilization onward. Levels of apoptosis were evaluated at 24 hpf. At 5 days post fertilization, the craniofacial skeleton, peripheral axon projections and sensory neurons of neuromasts were examined. Fish were genotyped to determine if there were phenotype/genotype correlations.

Results—Five of twenty loci interacted with ethanol. Notable among these was that *vangl2*, involved in convergent/extension movements of the embryonic axis, interacted strongly with ethanol. Untreated *vangl2* mutants had normal craniofacial morphology while severe midfacial defects including synophthalmia and narrowing of the palatal skeleton were found in all ethanol-treated mutants and a low percentage of heterozygotes. The cell cycle gene, *plk1*, also interacted strongly with ethanol. Untreated mutants have slightly elevated levels of apoptosis and loss of ventral craniofacial elements. Exposure to ethanol results in extensive apoptosis along with loss of neural tissue and the entire craniofacial skeleton. Phenotypes of *hinf*, *mars* and *foxi1* mutants were also exacerbated by ethanol.

Conclusions—Our results provide insight into the gene/ethanol interactions that may underlie ethanol teratogenesis. They support previous findings that ethanol disrupts elongation of the embryonic axis. Importantly, these results show that the zebrafish is an efficient model with which to test for gene/ethanol interactions. Understanding these interactions will be crucial to understanding of the FASD variation.

Introduction

Fetal Alcohol Spectrum Disorders (FASD) describe the full range of adverse effects that can be caused by prenatal exposure to ethanol. Many factors contribute to the type and severity of adverse outcomes following prenatal ethanol exposure (Riley et al., 2011; Sulik, 2005). The timing and dosage of ethanol exposure are important variables regulating ethanol teratogenicity as shown by numerous studies in diverse model organisms (Bilotta et al., 2004; Cartwright and Smith, 1995; Sulik et al., 1986; Webster et al., 1980). In addition to

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these variables, it is becoming increasingly evident that genetic predisposition plays a role in FASD.

Evidence for genetic predisposition to FASD comes from both human and animal studies. In humans, the most compelling evidence comes from twin studies. Monozygotic twins have 100% concordance for FAS while dizygotic twins have 36% concordance (Streissguth and Dehaene, 1993). There is strong evidence that genes involved in ethanol metabolism associate with risk for FASD (Warren and Li, 2005). Other human studies have looked for associations with FASD using candidate genes (Etheredge et al., 2005; Mitchell et al., 2001; Romitti et al., 1999). These studies have been largely inconclusive regarding potential gene/ethanol interactions. Because the genetics underlying FASD susceptibility is likely to be very complex, animal model systems will be important to aid in identification and characterization of these genetic loci.

Early evidence for genetic involvement in FASD using animal models came from widely differing sensitivities to ethanol teratogenesis across differing wild-type strains of the same species (Cavieres and Smith, 2000; Chen et al., 2000; Su et al., 2001; Wentzel and Eriksson, 2008). More recently a handful of studies have tested directly for gene/ethanol interactions using both mouse and zebrafish. In mouse, *FancD2* has been demonstrated to interact with ethanol through its involvement in DNA damage repair (Langevin et al., 2011). FAS can cause phenotypes that closely resemble holoprosencephaly which is commonly caused by mutations in the Sonic Hedgehog (Shh) signaling pathway (Sulik, 2005). Based on this similarity, Shh pathway members have long been suspected of underlying some aspects of FAS (Ahlgren et al., 2002; Li et al., 2007; Loucks and Ahlgren, 2009). Recent analyses in mouse and zebrafish demonstrated that ethanol genetically interacts with members of the Shh pathway (Hong and Krauss, 2012; Zhang et al., 2013). Because of its amenability to genetic manipulation and external fertilization, the zebrafish is gaining in popularity for analysis of gene/ethanol interactions.

The zebrafish was first used as a model for ethanol teratogenicity as early as 1910 (Stockard, 1910). To identify loci that may be involved in ethanol teratogenesis but are not readily considered candidate genes, we recently performed a small genetic screen (McCarthy et al., 2013). In this screen, we tested five zebrafish mutant lines housed in our facility for ethanol sensitivity and discovered that ethanol interacted synergistically with *pdgfra* to produce phenotypes that would not be readily predicted from the mutant phenotype or phenotypes of ethanol-treated wild-type embryos alone. These results demonstrated that further analyses of zebrafish mutants would be a productive way to identify ethanol sensitive genes.

From numerous genetic screens a large number of zebrafish mutants have been described (<http://zfin.org>). Many of these mutants are housed at the Zebrafish International Resource Center (ZIRC), providing a valuable resource for researchers interested in gene/alcohol interactions. To screen a larger number of genetic loci for interactions with ethanol than has occurred previously, we obtained mutant lines that were available live at ZIRC. Each mutant line was screened for ethanol-induced defects to the craniofacial skeleton, peripheral axon tracts, sensory cells of the neuromast, body axis, ears and eyes. Additionally, we determined if there was ethanol-induced cell death. We found that 5 of the loci were ethanol sensitive with all the interactions disrupting the craniofacial skeleton. The strongest ethanol interaction was with *vangl2*, involved in the planar cell polarity pathway. Ethanol interacted strongly with *plk1*, and weaker with *hinfp*, both involved in the cell cycle. Ethanol interacted with *mars*, regulating protein synthesis, and the transcription factor *foxi1*. Additionally, distinct axon and cell death phenotypes are observed in *vangl2*, *plk1* and *foxi1*. Our results substantially increase the number of ethanol-sensitive loci that have been validated in an

animal model and show that distinct phenotypes are generated by different gene/ethanol interactions.

Materials and Methods

All zebrafish were obtained from ZIRC (Eugene, OR) as either adults or embryos and were raised according to IACUC approved protocols at the University of Texas at Austin. For this screen, we obtained all zebrafish mutant lines that were available live as adults or embryos. No *a priori* selection for the types of mutants that would be screen was performed, although all of the mutants are developmental structural mutants. Of these original 29 lines we were able to establish breeding stocks with genotyping protocols for 20 mutant fish lines. All of these lines were maintained as heterozygous adults. Embryos produced from incrosses of these heterozygotes were left in their chorion and exposed to 1% ethanol in embryo media at 6 hours post fertilization (hpf) and remained in ethanol until they were screened. Treated and untreated embryos all came from within the same clutch to eliminate between clutch differences. Treatment of zebrafish embryos with 1% ethanol does not cause statistically significant reduction or defects to the craniofacial skeleton, but represents a sensitizing dose of ethanol, allowing for detection of dominant enhancement of ethanol teratogenicity (McCarthy et al., 2013). We have found that tissue levels of ethanol in zebrafish embryos are much lower than media levels and 1% ethanol in EM corresponds to 41 mM in the embryonic tissue (Lovely, C.B., unpublished). A minimum of 30 embryos was used for each screening and all mutant lines were screened for all of the phenotypes discussed below.

Levels of cell death were analyzed at 24 hpf using an anti-active Caspase antibody (Promega). Primary antibody was diluted 1:200 and AlexaFluor 488 secondary antibody (Molecular Probes) was diluted 1:500. At 5 days post-fertilization (dpf) axon projections and sensory hair cells were labeled. We labeled all axons using anti-acetylated β -tubulin (Sigma-Aldrich) at 1:200 with AlexaFluor 568 secondary antibody (Molecular Probes). Because this labels all axons, limiting detection of subtle alterations to axon projections within the CNS, we concentrated the screen on peripheral axons and nerve roots (Higashijima et al., 2000). Neuromasts were labeled with DASPEI (Life Technologies) as described (Harris et al., 2003) except that the final concentration of DASPEI was 0.0008%.

At 4 dpf we also stained the craniofacial skeleton of ethanol-treated and untreated embryos with Alcian Blue and Alizarin Red to examine the morphology of the cartilages and bones, respectively (Walker and Kimmel, 2007). Embryos were scored on the basis of the presence and shape of each individual element, as compared to wild-type untreated embryos (Fig. 1). These embryos were also scored for the size and spacing of the eyes, the size of the ears and the overall size of the embryo. An ocular micrometer was used to assess these sizes.

For all clutches that deviated from ethanol-treated wildtypes and untreated mutants, a duplicate screening was performed to replicate the original finding. These replication analyses were also performed within clutches. In our screen we sought to find loci of major effect, as such, only phenotypes differing qualitatively or substantially (a decrease of at least 25% as compared to untreated mutants and treated wild-type embryos) from untreated genotypes were considered. These are the interactions most likely to be conserved to humans, as supported by our previous small screen demonstrating a synergistic interaction with *PDGFRA* and ethanol in zebrafish and human (McCarthy et al., 2013). Embryos were sorted into phenotypic classes and genotyped. Supplemental Table 1 lists all mutants, mutation type, ZIRC ID, as well as primers and restriction enzymes used for genotyping.

Results

Ethanol interacts strongly with *vangl2*

Zebrafish *vangl2* mutants have a convergent/extension defect that results in a shortening of the body axis and, at very low penetrance, synophthalmia (Marlow et al., 1998), which is associated with midline craniofacial defects. Consistent with this low penetrance, we did not observe mutants with synophthalmia or alteration to the width of the palatal skeleton in untreated clutches, although the length of skeletal elements appears slightly shortened (Fig. 2A,A',D,D'). However, all ethanol-treated mutants were synophthalmic (Fig. 2C, asterisk) and had disruptions to the palatal skeleton in which the ethmoid plate collapsed into a rod-like shape and the trabeculae failed to fuse to the more posterior neurocranium (Fig. 2C,C'). In whole mount, the pharyngeal cartilages appeared misplaced (Fig. 2D,E,F). Flat mount images show that while there is a loss of most of the ceratobranchials, the morphology of the first and second pharyngeal arch elements was fairly normal (Fig. 2F,F').

We found evidence for haploinsufficiency of *vangl2* in ethanol treated heterozygotes with one ethanol-treated heterozygote (n=42) showing a complete phenocopy of the ethanol-treated *vangl2* mutants, suggesting that there is a threshold of Vangl2 activity necessary to prevent ethanol-induced craniofacial defects.

Coincident with the craniofacial defects were alterations to peripheral axon projections. Mutation of *vangl2* causes defects to neuronal migration of hindbrain motor neurons (Jessen et al., 2002) although the peripheral projections of these neurons are grossly normal (Fig. 3A,B). In contrast, all of the cranial nerves are severely hypoplastic in ethanol-treated *vangl2* mutants (Figure 3C).

Interactions with genes involved in the cell cycle

Three genes in our screen are associated with the cell cycle: *hinfp*, *plk1* and *sass6*. Two of these, *plk1* and *hinfp*, interacted with 1% ethanol. The most profound interaction was between ethanol and *plk1*.

Polo-like kinase 1 (Plk1) is involved in regulating the mitotic cell cycle across animal species (Golsteyn et al., 1995; Jeong et al., 2010; Sunkel and Glover, 1988). Untreated mutants have severe reductions in the viscerocranium, with only the palatoquadrate and hyosymplectic remaining compared to ethanol-treated wild-type embryos (Fig. 4A,B). The neurocranium is malformed, although intact in the untreated mutants (Fig. 4A,B). Ethanol treatment resulted in a fully penetrant complete loss of the craniofacial skeleton (Fig. 4C). In wild-type embryos the developing ear, or otic vesicle, is surrounded by the cartilaginous otic capsule and this morphology is unchanged in *plk1* mutants and ethanol-treated wild-type embryos (Fig. 4A,B). The ethanol-treated mutants were also markedly smaller in size with smaller eyes and ears (Fig. 4C, the otic vesicle is outlined). Thus, there is an overall reduction in tissue in 5 dpf ethanol-treated *plk1* mutants.

As may be expected from this reduction, ethanol-treatment also resulted in severe axon defects and elevated cell death in the mutants. Isolated cell death is apparent in 24 hpf untreated *plk1* mutants as well as ethanol-treated wild-type embryos (Fig. 5A,B). The level of cell death is greatly elevated throughout the ethanol-treated *plk1* mutants (Fig. 5C). Regions undergoing massive apoptosis include the eye, pharyngeal arches (arrows) and brain (arrowheads). Consistent with massive cell death, there are substantial axon defects in 5 dpf ethanol-treated *plk1* mutants as compared to untreated mutants and ethanol-treated wild-type embryos (Fig. 5D–F). Axon projections into the pharyngeal arches are reduced in untreated *plk1* mutants (Fig. 5D,E arrows). Following ethanol treatment, nearly all axon

projections are lost in *plkl* mutants, with only cranial nerve V being clearly evident (Fig. 5F).

Histone nuclear factor p (Hinfp) is involved in cell cycle regulation (Liu et al., 2011; Xie et al., 2009). Untreated mutant embryos have reduced craniofacial skeletal elements and a smaller head and eyes (Fig. 4D,E). Ethanol-treated mutants displayed an exacerbated phenotype in 100% of cases (Fig. 4F). Meckel's cartilage, the embryonic lower jaw, was absent (Fig. 4F, arrow), while other craniofacial cartilages were further reduced in size compared to untreated mutants (Fig 4D,F).

Interactions with biosynthetic enzymes

Several mutants in important biosynthetic pathways were screened: *mars*, *pde6c* and *wdr12*. Of these, *mars* interacted with 1% ethanol.

Because of its important biosynthetic function, Methionine-tRNA synthetase (Mars) is essential for proper translation of mRNA into protein. Untreated mutants are smaller in size than either ethanol-treated or untreated wild-type embryos (Fig. 6A,B). While most of the ceratobranchials are absent, the remainder of the craniofacial skeleton is only slightly reduced in size (Fig. 6A,B). Ethanol-treatment greatly exacerbates this phenotype. In 100% of ethanol-treated mutants, all pharyngeal cartilages are substantially reduced in size (Fig. 6C). The overall size of the embryo and the eye are also greatly reduced (Fig. 6C).

Interactions with transcription factors

Our screen contained numerous transcription factors: *egr1*, *foxi1*, *lhx2b*, *mitfa*, *msgn1* and *neurog1*. We found limited interactions between 1% ethanol and these genes, with only *foxi1* mutants being sensitive to ethanol.

Foxi1 is required for proper development of skeletal elements from the second and more posterior pharyngeal arches (Solomon et al., 2003). In untreated *foxi1* mutant embryos, the ceratohyal cartilages are oriented improperly and there are defects to the dorsal hyosymplectic (Fig. 7A,A'). The number of ceratobranchials is reduced in the untreated mutants (Fig. 7A,B). In ethanol-treated mutants, the ceratohyal is greatly reduced and the hyosymplectic is absent (Fig. 7C,C'). The number of ceratobranchials as well as the size of the otic capsule, and the ear within, is reduced relative to untreated mutants (Fig. 7C).

The ethanol/*foxi1* interaction also resulted in misprojections of axons in the pharyngeal arches. Axons innervate the serially-reiterated posterior pharyngeal arches in both untreated *foxi1* mutants and ethanol treated wild-type embryos (Fig. 8A,B, arrows). While axons are present, they do not project appropriately into the arches in ethanol-treated *foxi1* mutants (Fig. 8C, arrowheads).

Discussion

We have used the zebrafish as a fast efficient model organism with which to identify developmental structural mutants that interact with ethanol. We found that 5/20, or 25%, of genes that we screened interacted with 1% ethanol. Because we have not screened all relevant FASD phenotypes, we do not claim that the remaining 75% do not interact with ethanol, but rather that the 5 loci identified here represent validated genes that interact with ethanol and that are worthy of additional study. The phenotypic outcome of the gene/ethanol interaction in all instances was unique supporting the model whereby variation in FASD is generated partly by differing gene/ethanol interactions. In our screen we used a dosage of ethanol that generates the highest penetrance of defects in ethanol-treated mutants and heterozygotes while leaving wild-type embryos intact. Additionally, we used a long

treatment of ethanol in order to uncover interactions through a wide window of development. Therefore, it is likely that, in most cases, the phenotypes of these gene/ethanol interactions in humans would be subtler than those observed in zebrafish. However, because of the conservation of gene function across vertebrates, our animal model provides invaluable information regarding the genes that may predispose to FASD. Indeed, results from our previous screen identified an interaction between *pdgfra* and ethanol in zebrafish and found support for the same interaction in humans (McCarthy et al., 2013).

While it is of great interest, it is still difficult to assess how many gene/ethanol interactions there will be during development. Our screen only contained genes important during embryonic development and, therefore, is not representative of the genome as a whole. Thus, the apparently high number of interactions is likely overestimated. Larger scale screens will be essential for our understanding of how widespread gene/ethanol interactions are, although our current results do implicate several important cellular processes in ethanol teratogenesis.

Having identified these ethanol-sensitive alleles, the next step will be a mechanistic understanding of the phenotype. Knowing the nature of the mutation will be essential for this understanding. If a mutation was hypomorphic, as with our previous analyses with *pdgfra* (McCarthy et al., 2013), ethanol could interact directly with the hypomorphic protein or directly within the signaling pathway. All of the mutations presented here are likely to be null: *plk1^{hi1856Tg}*, *hinf1^{hi286Tg}* and *mars^{hi4134Tg}* are viral insertion mutants, *foxi1^{em1}* is a small deletion and *vangl2^{m209}* is an insertion. Therefore, the ethanol interactions observed in these mutants are likely to be due to disruptions, either genetic or physical, to larger complexes that interact with each gene product. Many of these gene products are known to be part of larger protein/signaling complexes, particularly *vangl2*. Therefore, this initial screen that we present here will likely be informative to more focused screens of important developmental pathways, such as the planar cell polarity pathway.

Ethanol and convergent/extension

Of particular note is the interaction with *vangl2*. Ethanol treatment changed a weakly penetrant phenotype of synophthalmia into a fully penetrant affect. Additionally, ethanol revealed a low level of haploinsufficiency in *vangl2* heterozygotes. Previous work in zebrafish and *Xenopus* has suggested that convergent/extension movements are sensitive to ethanol-induced perturbations (Blader and Strahle, 1998; Yelin et al., 2005). Our findings show that at least one gene regulating planar cell polarity, essential for convergent extension, is sensitive to ethanol, providing a potential genetic explanation for these previous findings. Since ethanol-induced synophthalmia is fully penetrant in *vangl2* mutants, our findings also provide a useful tool for analyses to dissect how ethanol disrupts convergent/extension. Findings in zebrafish suggest that convergent extension movements are most effectively disrupted by treating embryos with ethanol at 4.3 hpf (Blader and Strahle, 1998). This is slightly earlier than our treatments, which started at 6 hpf. It will be of interest to determine if increased levels of haploinsufficiency can be obtained by earlier ethanol treatments.

The facial phenotype of ethanol-treated *vangl2* mutants are strikingly similar to that observed in another planar cell polarity pathway mutant, *wnt11* (Piotrowski et al., 1996). Furthermore, in mouse and zebrafish, *Vangl2* has been shown to physically and genetically interact with a large number of genes in the planar cell polarity pathway (Wallingford, 2012). These interactions and phenotypic similarities implicate additional planar cell polarity pathway members to test for ethanol sensitivity. Many zebrafish mutants exist for planar cell polarity components and future analyses will test the breadth of gene/ethanol

interactions within planar cell polarity genes and determine the effects on cell movements in ethanol-treated planar cell polarity pathway mutants and heterozygotes.

Ethanol and the cell cycle

In our screen, we found that ethanol interacts with two out of three genes involved in cell cycle regulation. The effects of alcohol exposure on fetal growth are well known and there is evidence that ethanol suppresses cell division which may cause retardation in growth (Pennington et al., 1983; Shibley et al., 1995). Ethanol interacted strongly with *plk1* and to a lesser extent with *hinfp*, but did not interact with *sass6*. The reason for these differences is unknown, however, it is of interest to note that both Plk1 and Hinfp interact with cyclins and cyclin-dependent kinases (Xie et al., 2009; Zou et al., 2011). In contrast, Sass6 regulates centrosome duplication (Leidel et al., 2005). In cultured cerebellar cells, ethanol has been shown to inhibit Cyclin-dependent kinase activity and decrease the expression of Cyclins (Li et al., 2001), providing a potential mechanism for the interaction that ethanol has with *hinfp* and *plk1*.

With regard to the potential cause of the striking phenotype of the ethanol-treated *plk1* mutants, it has recently been shown that Plk1 and Aurora-A have partially redundant roles in cyclin B activation and that the roles of Plk1 and Aurora-A are particularly important following DNA damage (Zou et al., 2011). Ethanol metabolites have been shown experimentally to induce DNA damage (Seitz and Stickel, 2007). Fancd2 participates in DNA repair (Garcia-Higuera et al., 2001) and, in mouse, *Aldh2;Fancd2* double mutants are particularly sensitive to maternal alcohol exposure (Langevin et al., 2011). One carbon metabolism is also important in DNA repair and ethanol-induced DNA damage is exacerbated in *Mthfr* heterozygous mice, which have reduced one carbon production and elevated homocysteine (Fowler et al., 2012). Future analyses of the interaction between *plk1* and ethanol will focus on the involvement of cyclin B activation, association with Aurora-A and DNA damage.

Ethanol and cellular biogenesis

We found that *mars*, but neither *pde6c* nor *wdr12*, interacted with ethanol. PDE6C is a phosphodiesterase essential for photoreceptor function in the eye (Huang et al., 1995), setting it apart from MARS and WDR12. Both MARS and WDR12 are involved in protein synthesis. WDR12 is essential for ribosome biogenesis and also regulates cell proliferation (Holzel et al., 2005), whereas MARS is directly involved in the elongation and editing of protein (Jakubowski, 2011). In this editing function, MARS is important in the exclusion of homocysteine (Jakubowski, 2011). As noted above, alcohol elevates levels of homocysteine (Fowler et al., 2012). Therefore, the interaction between *mars* and ethanol may be similar in nature to the interactions that ethanol has with *hinfp* and *plk1*.

Future analyses will be required to determine the etiology of the ethanol-induced phenotypes in these mutants. It will also be of great interest to determine if there are network associations that may provide predictive power for additional ethanol-sensitive mutants. The zebrafish mutation project aims to generate zebrafish mutants in all zebrafish genes and currently has mutated 32% of known genes (http://www.sanger.ac.uk/Projects/D_rerio/zmp/). This mutation project provides an excellent resource to rapidly test genes implicated in ethanol teratogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Morphology of the zebrafish craniofacial skeleton. (A) Dorsal and (B) ventral views of a 5 dpf zebrafish embryo staining with Alcian Blue/Alizarin Red. (A) The neurocranium can be divided into an anterior, crest-derived portion, and a posterior, presumably mesoderm-derived portion. The crest-derived neurocranium is the zebrafish palatal skeleton and can be further divided into the midline ethmoid plate (ep) and the bilateral trabeculae (tr). The trabeculae fuse to the parachordal cartilages (pc) of the posterior neurocranium. (B) The viscerocranium consists of cartilage elements that are reiterated along the anterior/posterior axis and are generated from pharyngeal arches. Most anteriorly, Meckel's cartilage (mc) and the palatoquadrate (pq) form the lower and upper jaws, respectively. The ventral ceratohyal (ch) and dorsal hyosymplectic (hs) are the cartilages of the second pharyngeal arch. Pharyngeal arches 3–7 generate the five pairs of ceratobranchial cartilages (cb).

Fig. 2.

Ethanol interacts strongly with *vangl2* in craniofacial development. (A–C) Wholemount and (A'–C') flat mount images of neurocrania. Relative to (A) untreated *vangl2* mutants and (B) ethanol-treated wild-type embryos, the eyes of (C) ethanol-treated *vangl2* mutants are fused (asterisk). (A'–C') The fusion of the eyes in ethanol-treated *vangl2* mutants is associated with the narrowing of the ethmoid plate (ep) and failure of the trabeculae (tr) to attach to the posterior neurocranium. (D–F) Wholemount and (D'–F') flatmount images of viscerocranial elements. (F,F') In ethanol-treated mutants, most of the ceratobranchials are lost. While

Meckel's cartilage and the ceratohyal appear mispositioned in whole mount, flatmounted cartilage elements appear shaped appropriately (n=10/10 genotyped mutants).

Fig. 3.

Ethanol-exposure causes defects to cranial nerves in 5dpf *vangl2* mutants. Cranial nerve projections are largely normal in (A) *vangl2* mutants and (B) ethanol-treated wild-type embryos. (C) Ethanol-treatment causes severe reductions in cranial nerve projections (n=6/6 genotyped mutants). Cranial nerves are numbered. pl, posterior lateral line ganglion.

Fig. 4.

Interactions with genes regulating the cell cycle. (A–F) Whole mount images focused on the viscerocranium. (A,B) Compared to ethanol-treated wild-type embryos, untreated *plk1* mutants have small eyes and loss of most viscerocranial cartilages, with only remnants of the palatoquadrate (pq) and hyosymplectic (hs) remaining). While smaller, the neurocranium is present in mutants. (C) The overall size of the entire embryo is greatly reduced and the entire craniofacial skeleton is lost in ethanol-treated *plk1* mutants (n=7/7 genotyped mutants). (D,E) Most ceratobranchials are lost in *hinfp* mutants and the remaining skeletal elements are reduced in size, relative to ethanol-treated wild-type embryos. (F) Ethanol treatment causes a further reduction in the size of the viscerocranium and loss of Meckel's cartilage (arrow, n=6/6 genotyped mutants).

Fig. 5.

Elevated cell death and axon defects in ethanol-treated *plk1* mutants. (A–C) Lateral views of 24 hpf embryos stained with anti-active Caspase antibodies. (A,B) Compared to ethanol-treated wild-type embryos there is an elevation in apoptosis in *plk1* mutants. (C) In ethanol-treated *plk1* mutants, the levels of apoptosis are dramatically increased and expand throughout the embryo (n=11/11 genotyped mutants). (D–F) Lateral views of 5 dpf embryos stained with anti-acetylated beta-tubulin antibodies. (D,E) while reduced axon projections into the pharyngeal arches are present in untreated *plk1* mutants (arrows). (F) Nearly all axon projections are absent in ethanol-treated *plk1* mutants. Additionally, only cranial ganglion V is readily identifiable in the ethanol-treated mutants (n=4/4 genotyped mutants).

Fig. 6.

Ethanol interacts with mutants in the biosynthetic Mars enzyme. (A–C) Whole mount images, focused on the viscerocranium. (A,B) Relative to ethanol-treated wild-type embryos, untreated *mars* mutants have a loss of the ceratobranchials and slight reductions to cartilages derived from the first two pharyngeal arches. (C) Derivatives of the first two pharyngeal arches are greatly reduced in ethanol-treated *mars* embryos. The neurocranium is also further reduced in ethanol-treated mutants (n=5/5 genotyped mutants). mc; Meckel's cartilage, ch; ceratohyal.

Fig. 7.

Ethanol treatment exacerbates craniofacial defects in *foxi1* mutants. (A–C) Whole mount images show that relative to (A) untreated mutants and (B) ethanol-treated wild-type embryos, (C) ethanol-treated *foxi1* mutants have reduction in the size of the otic capsule (oc), surrounding the ear. (A'–C') Flat mount images show that while (A') untreated mutants have reductions to the hyosymplectic (hs), ethanol-treated *foxi1* mutants show a reduction in the size of the ceratohyal (ch) and a loss of the hyosymplectic (n=12/12 genotyped mutants).

Fig. 8.

Axon projection defects in ethanol-treated *foxi1* mutants. (A–C) Lateral views of 5 dpf embryos stained with anti-acetylated beta tubulin antibodies. (A,B) Axon projections into the pharyngeal arches are similar in untreated *foxi1* mutants and ethanol-treated wild-type embryos. (C) While axons are present, they do not extend appropriately into the pharyngeal arches of ethanol-treated *foxi1* mutants (n=13/13 genotyped mutants).

Table 1

Phenotypes of untreated and ethanol-treated mutants.

Gene	Untreated mutant phenotypes	Mutant ethanol induced phenotypes
<i>foxi1^{em1}</i>	<ul style="list-style-type: none"> - Hyosymplectic malformations - Ceratohyl fused to ceratobranchial 1 	<ul style="list-style-type: none"> - Hyosymplectic and all ceratobranchials are absent - Mislocalized axon projections in the posterior arches - Substantially reduced ear size
<i>hinfj^{hi286tg}</i>	<ul style="list-style-type: none"> - Reduced viserocranium - Absence of some ceratobranchials - microcephaly & microphthalmia 	<ul style="list-style-type: none"> - Meckel's cartilage and all ceratobranchials absent
<i>mars^{hi4134}</i>	<ul style="list-style-type: none"> - Ceratohyl inverted or partially inverted - Ceratobranchials three through five lost 	<ul style="list-style-type: none"> - Severely reduced viserocranium - Neurocranium reduced with breaks in posterior palate - microphthalmia
<i>plk1^{hi856}</i>	<ul style="list-style-type: none"> - Complete loss of viserocranium - Neurocranium slightly reduced - Lower jaw axon projections are reduced - Microcephaly & microphthalmia - Isolated apoptosis 	<ul style="list-style-type: none"> - All cranial cartilages absent - Axon projections are nearly completely absent - Substantially reduced ear, eye and body size - Extensive apoptosis throughout the embryo
<i>vangl2^{m209}</i>	<ul style="list-style-type: none"> - Craniofacial elements all slightly shortened but present 	<ul style="list-style-type: none"> - Absence of ceratobranchials - Ethmoid plate narrowed to a rod-like structure - Disrupted axon projections - Severe synophthalmia