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CHARACTERIZATION OF ETHANOL-INDUCED EFFECTS ON ZEBRAFISH RETINAL DEVELOPMENT: MECHANISTIC PERSPECTIVE AND THERAPEUTIC STRATEGIES

A Dissertation

Submitted to the Faculty

of

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Pooja Muralidharan

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This is dedicated to Jay and our Parents

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LIST OF ABBREVIATIONS

CMZ	Ciliary marginal zone
Dpf	Days post fertilization
FA	Folic acid
FAS	Fetal alcohol syndrome
FASD	Fetal alcohol spectrum disorder
GSK3β	Glycogen synthetase kinase 3 β
Hpf	Hours post fertilization
INL	Inner nuclear layer
MGC	Müller glial cell
ONL	Outer nuclear layer
RA	Retinoic acid
RGC	Retinal ganglion cells

ABSTRACT

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Fetal alcohol spectrum disorder (FASD) is a result of prenatal alcohol exposure, producing a wide range of defects including craniofacial, sensory, motor and cognitive deficits. Many ocular abnormalities are frequently associated with FASD including microphthalmia, optic nerve hypoplasia, and cataracts. FASD is highly prevalent in low socioeconomic populations, where it is also accompanied by higher rates of malnutrition and alcoholism. Using zebrafish as a model to study FASD retinal defects has been extremely insightful in understanding the ethanol-induced retinal defects at the cellular level. Zebrafish embryos treated with ethanol from mid-blastula transition through somitogenesis (2-24 hours post fertilization; hpf) showed defects similar to human ocular deficits including microphthalmia, optic nerve hypoplasia, and photoreceptor differentiation defects. Ethanol exposure altered critical transcription factor expression involved in retinal cell differentiation. Retinoic acid (RA) and folic acid (FA) nutrient co-supplementation rescued optic nerve and photoreceptor differentiation defects. Ethanol exposure during retinal morphogenesis stages (16-24 hpf), produced retinal defects like those seen with ethanol exposure between 2-24 hpf. Significantly, during ethanol-sensitive time window (16-24 hpf), RA co-supplementation moderately rescued these defects, whereas, FA co-supplementation showed significant rescue of optic nerve and photoreceptor differentiation. RA, but not FA, supplementation after ethanol exposure could restore ethanol-induced optic nerve and photoreceptor differentiation defects. Ethanol exposure did not affect timing of retinal cell differentiation induction, but later increased retinal cell death and proliferation. Ethanol-treated embryos showed increased retinal proliferation in the outer

nuclear layer (ONL), inner nuclear layer (INL), and ciliary marginal zone (CMZ) at 48 hpf and 72 hpf. In order to identify the genesis of ethanol-induced persistent retinal defects, ethanol effects on retinal stem cell populations in the CMZ and the Müller glial cells (MGCs) were examined. Ethanol treated retinas had an expanded CMZ indicated by histology and Alcama, a retinal stem cell marker, immunolabeling, but reduced expression of *rx1* and the cell cycle exit marker, *cdkn1c*. Ethanol treated retinas also showed reduced MGCs. At 72 hpf, ONL of ethanol exposed fish showed fewer photoreceptors expressing terminal differentiation markers. Importantly, these poorly differentiated photoreceptors co-expressed the basic helix-loop-helix (bHLH) proneural differentiation factor, *neurod*, indicating that ethanol exposure produced immature and undifferentiated photoreceptors. Reduced differentiation along with increased progenitor marker expression and proliferation suggest cell cycle exit failure due to ethanol exposure. These results suggested that ethanol exposure disrupted stem cell differentiation progression. Wnt, Notch and proneural gene expression regulate retinal stem cell proliferation and transition into progenitor cells. Ethanol exposure disrupted Wnt activity in the CMZ as well as Notch activity and *neurod* gene expression in the retina. RA and FA co-supplementation were able to rescue Wnt activity in the CMZ and rescue downstream Notch activity. To test whether the rescue of these Wnt-active cells could restore the retinal cell differentiation pathways, ethanol treated embryos were treated with Wnt agonist. This treatment could restore Wnt-active cells in the CMZ, Notch-active cells in the retina, proliferation, and photoreceptor terminal differentiation. We conclude that ethanol exposure produced persistent defects in the stem cell Wnt signaling, a critical pathway in retinal cell differentiation. Further analysis of underlying molecular mechanisms will provide insight into the embryonic origins of ethanol-induced retinal defects and potential therapeutic targets to cure this disorder.

CHAPTER 1. INTRODUCTION

1.1 Objectives

The purpose of this study is to characterize the ethanol effects on retinal development, to dissect the molecular and cellular mechanisms underlying ethanol-induced retinal defects, and to identify potential treatments that can rescue ethanol-induced defects. Prenatal ethanol exposure is known to be one of the most frequent preventable birth disorders. Studies in human patients show frequent association of retinal defects in FAS patients. Yet, mechanisms underlying ethanol-induced retinal defects remain unidentified. We postulate that zebrafish could be used as a model system to explore at the cellular level the defects induced by ethanol during the process of retinal morphogenesis. Understanding the genesis of these defects can lead to identification of preventive measures and therapeutic targets.

The central hypothesis of the thesis is that ethanol exposure during early development leads to persistent retinal defects and that nutritional compound supplementation with RA and FA can rescue retinal defects.

To test this hypothesis, we defined the following objectives:

1. Characterize the effects of ethanol on retinal development, specifically retinal cell differentiation;
2. Identify specific ethanol sensitive time windows during early development that can lead to retinal developmental defects;
3. Determine if nutritional supplements, such as RA and FA can rescue ethanol-induced retinal defects;
4. Characterize the ethanol effects on retinal stem cell populations and signaling pathways to elucidate underlying mechanism that lead retinal cell differentiation defects;
5. Dissect the effect of RA and FA supplementation on retinal stem cell populations.

The outcome of this study may provide critical insights on molecular mechanisms underlying ethanol-induced retinal defects and provide potential therapeutic and preventive targets for ocular defects in FASD patients.

1.2 Organization

This thesis is organized into five chapters, where CHAPTER-1 introduces the objectives and organization of this dissertation. CHAPTER-2 provides a literature review on the fetal alcohol spectrum disorder and retinal developmental events. CHAPTER-3 describes the materials and methods used in this research. Following this chapter, findings in this study are presented, each of which are individually introduced and discussed. CHAPTER-4 summarizes the characterization of ethanol-induced effects on retinal development and retinal cell differentiation. CHAPTER-5 summarizes the effect of nutrient co-supplementation with ethanol on retinal development at the cellular level. CHAPTER- 6 summarizes the results related to characterization of ethanol effects on retinal stem cell populations and specific differentiation pathways. CHAPTER- 7 summarizes the future directs and the impact of this study in the field of FASD.

CHAPTER 2. LITERATURE REVIEW

2.1 Fetal alcohol spectrum disorder

Prenatal alcohol exposure can cause fetal alcohol spectrum disorder (FASD), which is the most frequent preventable birth defect syndrome. FASD is an umbrella term, which includes a severe fetal alcohol syndrome (FAS), alcohol-related neurodevelopmental disorder (ARND), and alcohol related birth defects (ARBD). FASD effects range from physical developmental abnormalities to learning, cognitive, and behavioral deficits. The prevalence of FAS alone is estimated to be 1.5 in 1000 live babies born in the US. FASD prevalence is estimated to be more than ten times higher than that of FAS, reaching 2-5% of the population (May et al, 2009). This is especially exaggerated in low socio-economic populations where FASD prevalence has been estimated to be as high as 8.9% (May et al, 2009).

Since FAS was defined in 1973, a significant amount of research has been done to understand alcohol's teratogenic effect during development. However, it is still not clear how alcohol consumption produces a wide spectrum of physical and mental disabilities. This may be due to differences in dosages, timing of alcohol exposure (stage-dependent and/or duration-dependent) or differences in comorbid environmental and genetic factors. Model organisms such as zebrafish allow control over variables such as dose, duration, and timing of alcohol exposure while approaching these questions. Zebrafish also offers the convenience of genetic tool availability, and retinal developmental events are well-characterized and conserved between vertebrates.

2.2 Vertebrate retinal structure

The retina, a light sensing organ in vertebrates, has a typical laminated structure, which is conserved among vertebrates. Retina consists of six major neuronal cell types and Müller glial cells (MGCs) arranged in six distinct layers (Fig 2.1). The photosensory cells are rods and cones. Zebrafish have one type of rod and four types of cones: red, green, blue and UV cones. The photoreceptors lie in the outer nuclear layer (ONL), the outermost layer, and are supported by the retinal pigmented epithelium (RPE). Photoreceptor cells synapse in the outer plexiform layer onto the interneurons, bipolar cells, the cell bodies of which are located in the inner nuclear layer (INL). The bipolar cells then synapse onto the retinal ganglion cell (RGC) in the inner plexiform layer. The RGCs lie in the retinal ganglion cell layer bundle the axons to form the optic nerve that projects them to the retinorecipient parts of the brain, particularly in zebrafish, the optic tectum, for further processing. There are other interneurons such as horizontal cells and amacrine cells in the INL to modulate the signals transmitted at the outer and inner plexiform layers respectively. MGCs are the major glial cell type of the retina, which not only maintain ionic homeostasis but may also represent a stem cell population within the retina (Muralidharan et al, 2013; Powell et al, 2016).

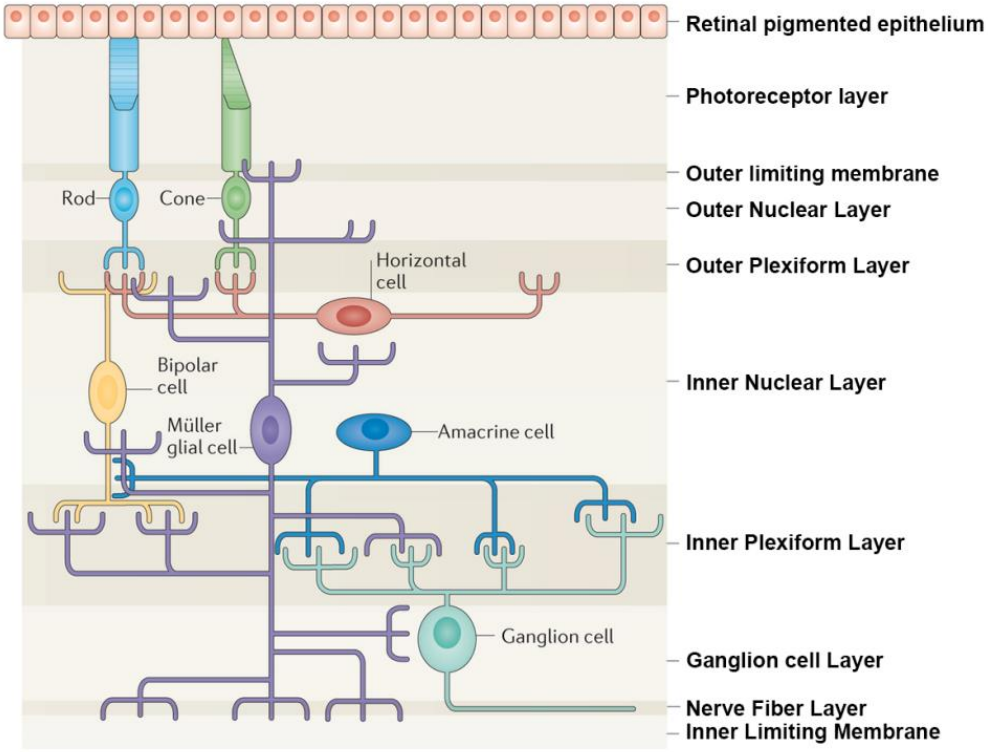

Figure. 2.1 A schematic representation of the major retinal cell types in the retina.
The retina is divided into three laminar layers: ONL, INL, and ganglion cell layer (GCL). Six different neuronal cell types and one glial cell type are distributed among these layers: rod and cone photoreceptor cell bodies are located in the ONL; the cell bodies of bipolar, horizontal and amacrine interneurons, along with the cell bodies of Müller glia, are located in the INL; and the cell bodies of ganglion cells are located in the GCL. Ganglion cell axons run just beneath the GCL and comprise a nerve fiber layer (NFL). Synapses between photoreceptors and interneurons form in the outer plexiform layer (OPL), and synapses between interneurons and ganglion cells form in the inner plexiform layer (IPL). Müller glial processes span all retinal layers and contribute to the formation of the inner limiting membrane (ILM) and outer limiting membrane (OLM). The RPE consists of pigmented cells that absorb light, make contact with and metabolically support the photoreceptors (used with permission (Goldman, 2014)).

2.3 Vertebrate retinal development

Retinal structure and developmental mechanisms are highly conserved among vertebrates. Retinal development is well-characterized in most animal models (Lupo et al, 2005; Ohkubo et al, 2002). The retina is an extremely sensitive indicator of prenatal abnormalities induced by teratogen exposure, and assessment of ocular defects is relatively easy, clinically accessible, and well defined. As the retina arises from the central nervous system (CNS), studying retinal defects provides insights in neural developmental defects, in part by isolating it from other developmental events. Due to similarities in retinal structure and CNS including various gene expression patterns and signaling pathways, ethanol effects on retinal development is also insightful in understanding CNS defects. The major retinal developmental pathways are conserved among vertebrates, and zebrafish provides an excellent model to study developmental events in mechanistic detail. Rapid development, easy visualization, accessibility, and availability of genetic tools are key assets of zebrafish as a model system for the study of retinal development and disease.

During CNS development, expression of eye field transcription factors (including *rx1*, *pax6* and *six3*) in the anterior neural plate specifies the eye field. The eye field is bisected by sonic hedgehog (Shh) signaling from the midline and following evagination of the optic primordium leads to the formation of optic vesicle. Various signaling and cell specification events are initiated by inductive signals from the optic stalk. Interactions of the optic vesicle with the non-neural ectoderm and surrounding cranial neural crest cell derived periocular mesenchyme induce lens placode and cornea formation. Reciprocal signaling leads to neural retinal invagination to form optic cup. A series of signaling events then lead to retinal morphogenesis. This leads to specification of RPE cells on the proximal optic cup and the distal portion leading to formation of retinal neuroblasts. Bmp (dorsal), retinoic acid (RA), Shh, and Fgf (ventral) signaling subdivide and pattern the retina during retinal morphogenesis (Lupo et al, 2005). This is followed by retinal neurogenesis. Differentiated retinal neurons are generated at specific times in a sequential order. Shh signaling from the midline and optic stalk regulates initiation of retinal neurogenesis (Esteve & Bovolenta, 2006; Ohkubo et al, 2002). First, a ventral patch of the retina, adjacent to the optic stalk, undergoes differentiation. Specific signaling events propagate a neurogenic wave of

differentiation around to the nasal, dorsal, and temporal retina. During this time, specific cell types differentiate sequentially, migrate to specific retinal layers, and terminally differentiate. This process is regulated by specific external and internal cues including Wnt, Notch, RA, Fgf, and Shh. In an overlapping manner, the retinal neuroblasts sequentially differentiate into RGCs, horizontal cells and cones, amacrine cells, rods, bipolar cells, and finally, MGCs (Young, 1985). Once differentiated, these cells express specific terminal differentiation markers and typically do not re-enter the cell cycle, with the exception of MGCs.

2.4 Retinal stem cell populations

During retinogenesis, all retinal cell types are produced by the multipotent progenitors including MGCs. Retinal neurogenesis continues throughout the life of the fish. Specific cell populations have been identified as the multipotent retinal stem cells residing in the ciliary marginal zone (CMZ). Cells from the CMZ can give rise to most retinal cell types including MGCs. MGCs, in turn, produce fate-restricted rod progenitor cells which then terminally differentiate in the ONL to form rod photoreceptors (Stenkamp, 2011). In response to an injury, zebrafish MGCs can reenter cell cycle give rise to produce neurogenic cluster of multipotent progenitors that can differentiate into almost all retinal cell types (Nagashima et al, 2013).

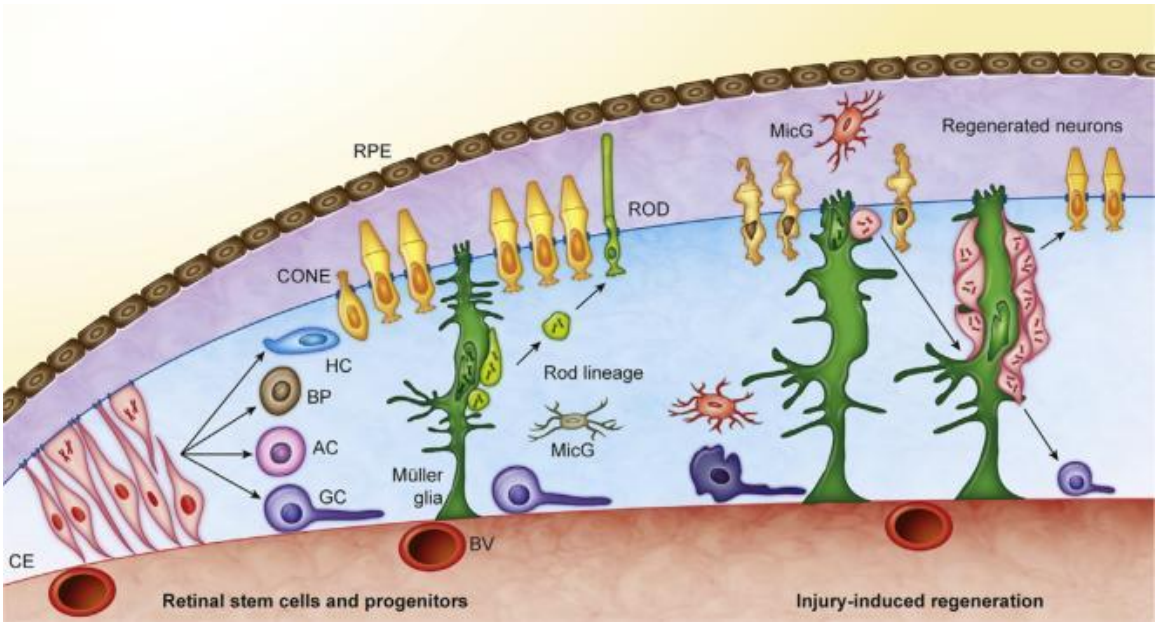

Figure 2.2 Neurogenesis in the adult teleost fish retina during growth and regeneration. CMZ with multipotent retinal stem cells and committed retinal progenitors (magenta) at the junction between the neural retina and the ciliary epithelium (CE). New retinal neurons are generated sequentially: first retinal ganglion cells (GC), then interneurons (amacrine cells, AC; bipolar cells, BP; horizontal cells, HC), then cone photoreceptors. The apical surface of the neural retina faces the RPE at the back of the eye; the basal surface of the neural retina is vascularized (blood vessels, BV). Radial processes of Müller glial cells (green) span the apical-basal extent of the retina and lateral processes enwrap the neurons. Müller glia are somatic retinal stem cells supporting the rod lineage: they divide infrequently, with an asymmetric, self-renewing division, to give rise to proliferating, committed progenitors that migrate to the apical surface to generate rod photoreceptors within the differentiated retina. When retinal neurons are destroyed, microglia (MicG) are activated and remove cellular debris. The nuclei of Müller glia translocate to the apical surface, divide asymmetrically to give rise to proliferating, multipotent retinal progenitors that accumulate around the radial glial fiber and migrate to the appropriate retinal laminae to regenerate neurons (e.g., cone photoreceptors or retinal ganglion cells) (used with permission (Lenkowski & Raymond, 2014)).

2.4.1 Ciliary marginal zone

The CMZ is a group of cells present in peripheral retina that contain the multipotent retinal stem cell and progenitor cell populations. In fish and amphibian, a significant part of the retina is added from the CMZ after the initial differentiation of the retina (after 48 hpf)

(Hitchcock & Raymond, 1992; Raymond & Hitchcock, 1997). The CMZ is a stem cell niche and several intrinsic and surrounding factors affect its establishment and maintenance (Raymond et al, 2006). Unlike zebrafish, the CMZ is not clearly defined in human retinas. However, studies in mammalian systems and primates hint the presence of self-renewing and proliferative cells.

Based on cell cycle and proneural gene marker expression pattern, CMZ can be roughly divided into three major zones. Peripheral CMZ with low expression of cell cycle activators, middle CMZ with high expression of *cyclind1* and high proliferation, and central CMZ with high cell cycle inhibitor ($p57^{kip2}$), and proneural gene expression (Ohnuma et al, 2002; Raymond et al, 2006). Evidence from zebrafish, *Xenopus*, and chick retinal models show that the maintenance and regulation of differentiation multipotent retinal stem cells in the CMZ is tightly regulated by Wnt, Sox2 and Notch signaling cycle (Agathocleous & Harris, 2009; Agathocleous et al, 2009; Denayer et al, 2008; Kubo & Nakagawa, 2009; Kubo et al, 2003; Kubo et al, 2005; Meyers et al, 2012; Nadauld et al, 2006; Stephens et al, 2010; Van Raay et al, 2005; Yamaguchi et al, 2005). In the peripheral CMZ, Wnt signaling can inhibit neural retinal differentiation and maintain a proliferative, undifferentiated state through Notch by blocking proneural activity. (Agathocleous et al, 2009; Kubo et al, 2003; Yamaguchi et al, 2005). Canonical Wnt signaling activation initiates a cascade resulting in GSK3β inhibition and β-catenin translocation into to nucleus leading to binding with TCF/LEF transcription factors and transcriptional activation of target genes. Notch activation causes nuclear translocation of notch-intracellular domain that, on binding with other co-factors, leads to bHLH transcriptional repressor expression, Hairy-enhancer of split (*hes*) genes. *hes* genes expression can actively repress neurogenesis. Notch and Sox2 signaling coordinate to determine proneural gene expression levels, which eventually induce the precursors and progenitors of neural cells differentiate. As stem cell differentiation progresses into middle and central CMZ with reduced Notch activity, these mitotic precursor cells express other differentiation transcription factors, which vary with their cell type, and finally, exit the cell cycle and undergo terminal differentiation. Following activation of proneural genes, other signaling pathways such as Shh, RA, Fgf, and Bmp regulate proliferation, differentiation and patterning (Hyatt et al, 1996a; Hyatt et

al, 1996b; Masai et al, 2000; Prabhudesai et al, 2005; Valdivia et al, 2016). For example, rod photoreceptor precursors and progenitors, express differentiation factors such as, *neurod*, *crx*, and *rx1*, migrate to the ONL, express rod-specific transcription factors, and finally terminally differentiate into rods (Stenkamp, 2011). Later born glial cells require Notch activation, which promotes MGC differentiation (Bernardos et al, 2005).

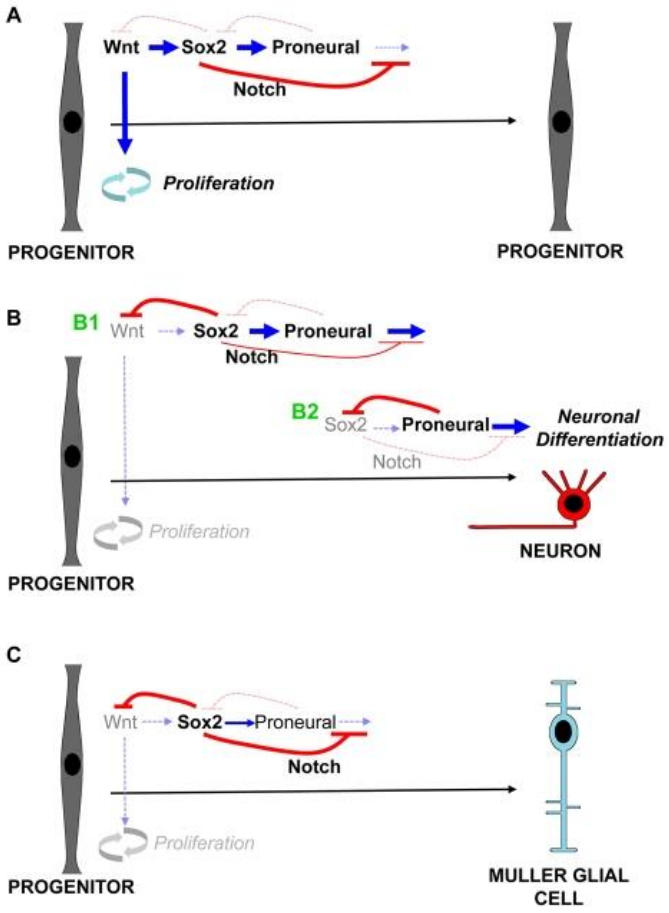

Figure. 2.3 Wnt-Sox2-Notch pathway during retinal cell differentiation.
(A) Wnt signaling activation in a neuroepithelial cell activates Sox2 and the proneural genes, but indirectly through Sox2 and Notch, which blocks proneural activity and neuronal differentiation. Wnt also independently maintains proliferation, which leads to progenitor maintenance and expansion. (B) The build-up of Sox2 switches off Wnt, inhibiting proliferation (B1), and then the accumulation of proneural activity switches off Sox2, relieving the inhibition of neuronal differentiation and leading to neurogenesis (B2). (C) Alternatively, if Sox2 levels remain high it will limit proneural activity and neuronal differentiation will be blocked, but Wnt signaling will also be inhibited, leading to cell cycle exit and glial differentiation (used with permission (Agathocleous et al, 2009)).

2.4.2 Müller glial cells (MGCs)

The other stem cell population in the neural retina are the MGCs, the radial glia in the retina, which are among the last born cells. MGCs express specific markers including *pax6*, a hallmark of retinal precursor cells, and give rise to rod photoreceptors in an uninjured zebrafish retina (Bernardos et al, 2007). Several regeneration studies have shown that MGCs respond to retinal injury and undergo dedifferentiation, increase expression of glial fibrillary acidic protein (Gfap), Alcama, a neuroepithelial cell marker. MGCs have been demonstrated to be able to give rise to almost all retinal cell types post lesion (Nagashima et al, 2013).

Specific signaling pathways have been identified which regulate MGC differentiation and injury response. Gliogenesis is driven in a sequential manner after neurogenesis is completed. Elevated Notch signaling drives glial cell specification and differentiation (Bernardos et al, 2005). MGCs in the central retinal asymmetrically divide to give rise to rod precursor and progenitor cells. These precursors express distinct markers such as *neurod*, *rx1*, and *crx* (Stenkamp, 2011).

2.5 Ocular defects in FASD

FAS is frequently associated with visual system developmental defects. About 90% of FAS patients show retinal defects (Stromland, 1987; Stromland & Pinazo-Duran, 2002). Epidemiological studies showed association of FAS with defects in almost all parts of the eye. Severe cases had microphthalmia, coloboma, and cataract. FAS/FASD patients with milder defects showed strabismus, high refractive errors, and reduced visual acuity. Human epidemiological studies showed persistently poor vision that remained unchanged even in follow-up measurements. The children with FAS also frequently display optic nerve hypoplasia and abnormal electroretinograms (ERGs) (Muralidharan et al, 2013).

Initial studies using various vertebrate animal models documented significant reduction in ganglion cell number and changes in optic nerve axon structure, specifically showing delayed or defective myelination following ethanol exposure (Stromland & Pinazo-Duran, 2002). This also correlated with defective or delayed gliogenesis seen in embryos after

ethanol exposure. Subsequent studies dissected mechanisms underlying ethanol-induced retinal defects. Clinical examinations identified that scotopic (low intensity light-rod photoreceptor response) ERGs showed more deficiencies than photopic (high intensity light-cone photoreceptor response) ERGs (Hug et al, 2000). Experimental evidence supporting this finding showed reduced rhodopsin expression in mouse embryos exposed to ethanol (Katz & Fox, 1991). Effects of ethanol on photoreceptors and their ERGs were dose-dependent (Matsui et al, 2006). Using zebrafish as a model, Bilotta *et al*. (Bilotta et al, 2002), showed that a significant reduction in eye diameter and optomotor response in fish treated with ethanol during somitogenesis (12-24 hpf). This developmental period includes optic primordium specification and neurogenesis events. Treatment during time windows before or after 12-24 hpf did not cause as severe defects as those produced by treatment during 12-24 hpf. In addition, higher ethanol concentrations during this sensitive period induced severe lamination defects and cyclopia (Arenzana et al, 2006). The authors also suggested that retinal defects may be caused by impaired migration of prechordal plate during retinal morphogenesis because mutants showing similar eye defects exhibit prechordal plate migration defects (Blader & Strahle, 1998). Experiments on *Xenopus* embryos showed that ethanol exposure during early development caused abnormal expression of eye morphogenetic genes such as *pax6* and *tbx3*, which could result from increased in *shh* expression levels (Yelin et al, 2007).

Studies on zebrafish showed that the presence of ethanol during retinal neurogenesis (24-48 hpf) induced persistent microphthalmia caused by increased cell death in the retina and lens as compared to untreated embryos (Kashyap et al, 2007). A significant reduction in the number of each retinal cell types including photoreceptors and ganglion cells was seen. The investigators designed rescue experiments to target signaling pathways that may be affected by ethanol, including Shh and RA signaling. Their experiments showed that ethanol mediated defects were not rescued by these treatments, suggesting that alternative pathways caused retinal defects during this developmental period. However, photoreceptor differentiation could be rescued by RA treatment (Kashyap et al, 2011).

Transmission of visual signals from photoreceptors occurs via the interneurons, namely the bipolar and horizontal cells. Studies on mouse embryos showed significant, persistent, and

dose-dependent reduction in the number of bipolar cells (PKC-α positive) (Deng et al, 2012). Other experiments showed significant reduction in the dendritic receptive field of horizontal cells. However, since bipolar and horizontal cells are among the last cell types to differentiate and cell types that continue differentiation postnatally, defects in bipolar and horizontal cells may be secondary effects of earlier defects in the retina. To summarize, ethanol induced defects on retinal development are dose and developmental stage dependent, leading to a severe, persistent defects in the structure and function of all retinal cell types. Additional experiments are necessary to understand specific signaling mechanisms affected by ethanol underlying retina and lens defects.

2.6 Alcohol and nutritional deficiencies

Vitamin deficiencies were linked to a number of diseases, which are particularly prevalent in populations of low socioeconomic conditions. Alcohol consumption also aggravates malnutrition by interfering with the absorption, digestion and utilization of nutrients consumed. Chronic alcoholics tend to have a poor nutritional status and vitamin deficiencies, including vitamins A (retinol), B_1 (thiamine), B_2 (riboflavin), B_6 (pyridoxine), C (ascorbic acid), E, and B_9 (folate) (Lieber, 2003). Thus, a potential contributing factor causing an increased FASD incidence in lower socioeconomic populations is severe nutritional deficiency, aggravated further by chronic alcohol consumption.

RA is a derivative of vitamin A (retinol), and RA signaling plays a crucial role during embryonic development. During retinal morphogenesis, RA performs distinct functions. RA is a morphogen for retinal dorsoventral patterning and RA induces terminal differentiation of unspecified photoreceptor progenitors and precursors into rod and cone photoreceptors in the ONL of the retina (Hyatt et al, 1996a; Prabhudesai et al, 2005; Rhinn & Dolle, 2012). Several alcohol/aldehyde dehydrogenases (ADHs/ALDHs) tightly regulate RA biosynthesis, and RA-degrading enzymes control its catabolism during development. Retinaldehyde dehydrogenase enzymes are expressed in the dorsal (Raldh2) and ventral (Raldh3) regions of the zebrafish retina during retinal morphogenesis. Early *in vitro* studies showed competitive inhibition of ADHs by ethanol (Mezey & Holt, 1971). It has been hypothesized that ethanol-induced reduced RA biosynthesis may produce RA

signaling deficits during development, leading to embryonic malformations, but particular developmental signaling targets of ethanol remain unidentified (Duester, 1991).

Folic acid (FA) is an essential vitamin that participates in nucleic acid synthesis and repair (Kamen, 1997). FA also plays a crucial role as a cofactor in 1-carbon metabolism as tetrahydrofolate, which is needed in DNA and histone methyl transfers. Recent studies identified ROS scavenging properties of FA (Ibrahim et al, 2012; Joshi et al, 2001). FA deficiency, consequently, produces a wide range of birth defects including severe ocular defects, such as microphthalmia, delayed lamination, and optic cup abnormalities (Maestro-de-las-Casas et al, 2013). Embryonic ethanol exposure affects FA metabolism, including reduced maternal-to-fetal folate transfer and reduced expression of folate metabolizing enzymes (Hutson et al, 2012). Ethanol-induced FA deficiency could alter histone and DNA methylation patterns as seen in ethanol-treated cell culture models (Mason & Choi, 2005; Singh et al, 2009; Zhou et al, 2011a). Importantly, studies showed that FA supplementation rescued overall ethanol-induced morphological defects, particularly cardiac defects (Ballard et al, 2012; Sarmah & Marrs, 2013; Serrano et al, 2010). Prenatal FA supplementation significantly reduces the risk of neural tube defects, congenital heart defects, and cleft lip/palate, and thus, FA is a recommended dietary supplement for pregnant mothers (Taruscio et al, 2011).

2.7 Alcohol and genetic interactions

A number of intrinsic and extrinsic factors influence the severity of FASD. Early studies indicated that genetic background regulates FASD susceptibility. (Abel, 1998; Abel & Hannigan, 1995). Various genetic association studies have highlighted a correlation between FAS and certain polymorphisms in alcohol dehydrogenase (ALDH), of which specific alleles have been described each with differing affinities for alcohol (Crabb et al, 2004; Green & Stoler, 2007). Certain populations, such as the Native Americans, seem to lack the FAS-protective ALDH polymorphism, suggesting a mechanism for the increased FASD prevalence in those populations. (Ehlers et al, 2004). Certain other populations, in a male Jewish population study, showed that individuals carrying different alleles for ADH cleared ethanol at significantly higher rates than other individuals (Neumark et al, 2004).

This suggests the influence of genetic background on the environmental factors affecting development.

We are interested in dissecting the cellular and molecular mechanisms underlying ethanol-induced retinal defects. Identification of rescue nutritional factors will provide insights in development of preventive approaches. Furthermore, identification of ethanol targets specific signaling pathways involved during retinal development will facilitate therapeutic measures for this frequent and devastating disorder.

CHAPTER 3. MATERIALS AND METHODS

3.1 Zebrafish husbandry

Zebrafish (*Danio rerio*; Hamilton; TL and AB strain *Tg(nrd:eGFP)*(Obholzer et al, 2008), *Tg(TP1bglob:eGFP)* (referred to as *Tg(TP1:GFP)*)(Parsons et al, 2009), *Tg(TP1:mCherry)* (Parsons et al, 2009), *Tg(gfap:GFP)mi2001* (referred to as *Tg(gfap:GFP)*) (Bernardos & Raymond, 2006), *Tg(Tcf/Lef-miniP:dGFP)* (Shimizu et al, 2012) transgenic lines) were raised and housed under standard laboratory conditions (Westerfield, 2000) in accordance with Indiana University Policy on Animal Care and Use. The embryos were treated with 1-phenyl-2-thiourea (0.003%) from 6 hpf (shield) onward in order to prevent melanogenesis.

3.2 Embryo treatments

Zebrafish embryos were exposed to ethanol by incubation in the embryo medium containing ethanol (100 mM (0.6% v/v) and 150 mM (0.9% v/v), referred to as 100 EtOH and 150 EtOH respectively) for different periods between 2 and 24 hpf in petri dishes wrapped with Parafilm® and maintained at 28.5°C. Ethanol treatment dishes were placed in chambers with 2% ethanol to minimize ethanol volatilization. After treatment, embryos were rinsed with pre-warmed embryo medium and incubated in embryo medium until the desired stage was achieved and fixed using 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS).

RA (0.1 mM) and 4-diethylaminobenzaldehyde (DEAB, 1 mM) stock solutions (Sigma, St. Louis, MO, USA) were dissolved in dimethyl sulfoxide (DMSO). Citral (1 mM; T.C.I.) stock solution was freshly prepared prior to treatment. Medium containing citral was replaced with embryo medium containing freshly diluted citral (1 μM and 10 μM) every hour for 8 hours (16-24 hpf) due to lability of citral in water. FA (1 mM, Sigma) stock

solution was made fresh prior to treatment and diluted in embryo medium. RA and FA treatments were performed as previously described (Marrs et al, 2010; Sarmah & Marrs, 2013). Other concentrations of RA and FA have been tested previously, and 1 nM RA and 75 μM FA were chosen as optimal concentrations for treatments that minimized their toxic effects and displayed maximum rescue phenotypes. Zebrafish embryos were treated with 350 nM and 500 nM GSK3β inhibitor compound (LSN 2105786; Eli Lilly and Co.) from 32-48 hpf and 48-72 hpf.

3.3 Immunofluorescence

Whole-mount immunostaining was performed as previously described (Clendenon et al, 2012) using primary antibodies against HuC/D (Sigma, 1:1000), zpr-1 (ZIRC, 1:1000), zpr-3 (ZIRC, 1:500), zrf-1 (ZIRC, 1:1000), Alcama (zn-5; ZIRC, 1:500), acetylated tubulin (Sigma, 1:500), and phospho-histone-3 (Millipore, 1:500). Alexa Fluor 488-conjugated anti-mouse and anti-rabbit, Alexa Fluor-555 conjugated anti-mouse, Alexa-fluor-647 conjugated anti-mouse, and Texas Red conjugated anti-rabbit secondary antibodies (Molecular Probes) were used at 1:200 dilutions. Alexa Fluor 488-conjugated phalloidin (Molecular Probes) was used at a 1:100 dilution. Nuclear staining was performed using TO-PRO-3 iodide at a 1:1000 dilution incubated for 1 hour.

3.4 Apoptosis imaging

Acridine orange staining was performed to visualize apoptotic nuclei by incubating the dechorionated embryos with 5 μg/mL acridine orange for 3 min, followed by several washes in embryo medium. Live embryos were imaged immediately using a confocal microscope.

3.5 In situ hybridization (ISH)

Whole mount ISH of zebrafish embryos was performed as previously described (Sarmah et al, 2010). Digoxigenin-labeled (dig-labeled) riboprobes for *rx1* (*retinal homeobox gene 1*), *shh* (*sonic hedgehog*), *crx* (*cone-rod homeobox*), *otx5* (*orthodenticle homolog 5*),

neurod (*neurogenic differentiation*), *rho* (rhodopsin), *opn1sw1* (UV opsin), *opn1lw1* (red opsin), *axin2*, and *her6* (*hes1b, hairy-related 6*) were synthesized using a Dig RNA labeling kit (Roche). Plasmid for probes to detect *rx1*, *shh*, *crx*, *otx5*, *neurod*, *rho*, *opn1sw1*, *opn1lw1*, and *her6* were generously provided by Drs. Pamela Raymond (University of Michigan), Stephen Ekker (Mayo Clinic Cancer Center), Qin Liu (University of Akron) and Yuk Fai Leung (Purdue University). The *axin2* plasmid was obtained from Addgene.

3.6 Fluorescence in situ hybridization (FISH):

Whole mount FISH was performed using dig-labeled riboprobes for the genes, *cdkn1c* (*p57 kip2*, *cyclin-dependent kinase inhibitor 1c*; T7-polymerase binding site containing primers; Table 1). Dig-labeled riboprobes were synthesized using Dig RNA labeling kit. Embryos were fixed overnight in 4% PFA in PBS at 4°C, washed in PBS containing 0.1% tween-20 (PBST), dehydrated stepwise to methanol, and stored at -20°C. After rehydration, embryos were permeabilized with proteinase K, re-fixed and washed. They were incubated in hybridization buffer for 2 hours and the riboprobe was added for overnight incubation at 70°C. Following stepwise washing to 2X SSC, 0.05X SSC and PBT at 70°C, the embryos were placed in blocking solution (1X maleic acid buffer, 2% BSA and 2% normal goat serum) for 2 hours at room temperature. The embryos were incubated in anti-Dig POD (1:1000; Roche) in blocking solution overnight at 4°C. Finally, the embryos were washed with PBS and developed with tyramide signal amplification kit. Alexa Fluor-488 labeled tyramide (1:100) with hydrogen peroxide was added to the embryos for 60-90 mins at room temperature. The embryos were washed three times for 10 minutes with PBST, and imaged using a confocal microscope (Brend & Holley, 2009).

3.7 Microscopy

Laser scanning confocal images were acquired using a Zeiss Observer Z1 LSM 700 confocal microscope (40X 1.1 NA W objective; Carl Zeiss Microscopy). Several x-y focal plane images were captured to produce a z-stack or image volume. All embryos were deyolked and imaged from the ventral side, and z-sections were analyzed, always including the optic nerve for consistency. Differential interference contrast images of live embryos

were obtained using Zeiss observer Z1 (20X 0.8 NA objective) with an Orca AG CCD camera (Hamamatsu Photonics). Brightfield dissecting microscope images were acquired using a color Leica DFC290 camera mounted on a Leica MZ12 stereomicroscope (Leica). Brightfield images for histological and ISH sections was performed using the Axiovision camera ICc1 mounted on the Zeiss observer Z1 (10X 0.3 NA; 20X 0.8 NA objectives).

3.8 Image analyses and cell counting

3D reconstruction of confocal slices was used to measure optic nerve widths using Image J software. The optic nerve widths were measured at the inner plexiform layer, which was determined using TO-PRO-3 staining. The measurement of zpr-1 intensity in the ONL was performed by highlighting the ONL using TO-PRO-3 nuclear stain channel in Carl Zeiss ZEN imaging software and exporting the highlighted images to the zpr-1 channel. The total intensity in the highlighted region was measured using Image J software.

Cell counting was performed for cell proliferation and cell apoptosis assays using ZEN software. ONL, INL, and CMZ regions in the cell proliferation counts were identified using TO-PRO-3 nuclear stain.

H&E stained histological sections containing optic nerve were used for image analyses. CMZ area and retinal area was measured using Image J software. CMZ area was demarcated to the lamination in the retina. *Tg(TP1:mCherry),* Notch-reporter transgenic line was used to perform cell counts of nuclear-localized mCherry-positive cells representing the Notch-active cells. A single optic nerve-containing section containing optical section was used in 72 hpf control and ethanol treated embryos.

3.9 Quantitative PCR analysis

RNA isolation followed by quantitative PCR (qPCR) analyses were performed as previously described (Sarmah & Marrs, 2013) for 72 hpf treated and untreated embryos or retinas. Primers for specific genes and their sequences are listed in Table 1. Independent experiments in triplicates were performed using *rsp15* endogenous control using the 7300 Real Time PCR system (Applied Biosystems).

3.10 Statistical analysis

Control and ethanol treated groups were compared using student's t-test (Graphpad software). One-way ANOVAs were used to test the group effect for each outcome, followed by pre-specified comparisons against the controls and comparisons against the rescue groups as appropriate for each analysis. A 5% significance level was used for each test (Biostatistics Services, IUSM).

Table 3.1 Primer sequences used in the study

Underlined sequence indicates T7/T3 polymerase binding promoter consensus sequence.

Primer name	Sequence (5'-3')	Reference
RT-*kip2*-F	CTTCAGTCCTCAGAAACAGACGGAAG	(Ramachandran et al, 2012)
RT-*kip2*-R	CATCCGCTCTGCAGATAAACACAGGTG	
kip2 - T7F	TGAATTGTAATACGACTCACTATAGGGCG GATAAAGTACAAAACAAGAGAGCTC	
kip2 - T3R	AAGCTCGAAATTAACCCTCACTAAAGGGC ACTTTGATTCAAAGGTACAACGTGAGC	
rx1 - F	GGACCAGGATTCGTTGCTCA	(Laranjeiro & Whitmore, 2014)
rx1 - R	ATCCCTAAGGGGTGGCAGAT	
rho - F	ACTTCCGTTTCGGGGAGAAC	
rho - R	GAAGGACTCGTTGTTGACAC	
Red opsin - F	AGATGCAATTTATGCAGCCCG	
Red opsin - R	CATCGAGGGGCAATGTGGTA	
axin2 - F	GGACACTTCAAGGAACAACTAC	(Yin et al, 2011)
axin2 - R	CCTCATACATTGGCAGAACTG	

CHAPTER 4. ETHANOL-INDUCED RETINAL DEVELOPMENTAL DEFECTS

4.1 Introduction

4.1.1 Ethanol exposure during early development induced retinal cell differentiation defects

Ethanol exposure in vertebrate animal models recapitulates retinal defects seen in FASD patients. Experiments using mice showed that *in utero* ethanol exposure induced specific defects in rod photoreceptor sensitivity and dark adaptation (Katz & Fox, 1991). Studies on zebrafish embryos showed reduced optic nerve hypoplasia (ONH), retinal lamination defects, and reduced photoreceptor outer segment growth, due to ethanol exposure during gastrulation through neurulation stages (2-24 hpf) (Arenzana et al, 2006; Bilotta et al, 2002; Matsui et al, 2006). Ethanol exposure during zebrafish retinal neurogenesis (24-48 hpf) also induced persistent microphthalmia (Kashyap et al, 2007). Shorter periods of ethanol exposure (12-24 hpf) were sufficient to induce microphthalmia, similar to that produced by longer treatments (2-24 hpf) (Bilotta et al, 2002). However, cellular details of ethanol effects on retinal cell specification, differentiation, and potential protective measures remain unclear.

Dose, duration, and timing of ethanol exposure greatly affect the severity of FASD, which is also influenced by intrinsic (genetic background) and extrinsic (nutrition and environment) factors. Here, experiments are presented that characterize effects of ethanol on cell differentiation pathways that produce retinal defects.

4.2 Chronic ethanol exposure affects retinal neurogenesis, gene expression, and tissue organization in the developing zebrafish retina

4.2.1 Ethanol exposure severely affects most retinal cell types

To dissect ethanol-induced retinal defects carefully, ethanol effect on different retinal cell types was characterized. Zebrafish embryos were treated with 100 and 150 mM (0.6 and 0.9% v/v) ethanol from 2-24 hpf were returned to regular embryo medium and grown until the desired stage was reached. This timing (2-24 hpf) was chosen to include gastrulation through somitogenesis, which is equivalent to around first 3 weeks of human pregnancy, a period when the mother may be unaware of the pregnancy, increasing the risk of prenatal ethanol exposure. At 48 hpf, the embryos showed microphthalmia (Fig 4.1A). Immunostaining of the mature axons with acetylated tubulin antibody stains the optic nerve in the retina (Fig 4.1D). Ethanol treated embryos showed significantly reduced optic nerve widths compared to control embryos indicating hypoplasic optic nerves (Fig 4.1E). Embryos were compared for lamination defects by staining for the synaptic layers (plexiform layers) for actin at 48 hpf (Fig 4.1C). At 48 hpf, the ethanol treated embryos did not show complete laminae as seen in the controls, but by 72 hpf, the ethanol treated retinas had laminated, indicated ethanol exposure delayed the lamination process (Fig 4.1B, Fig 4.2A).

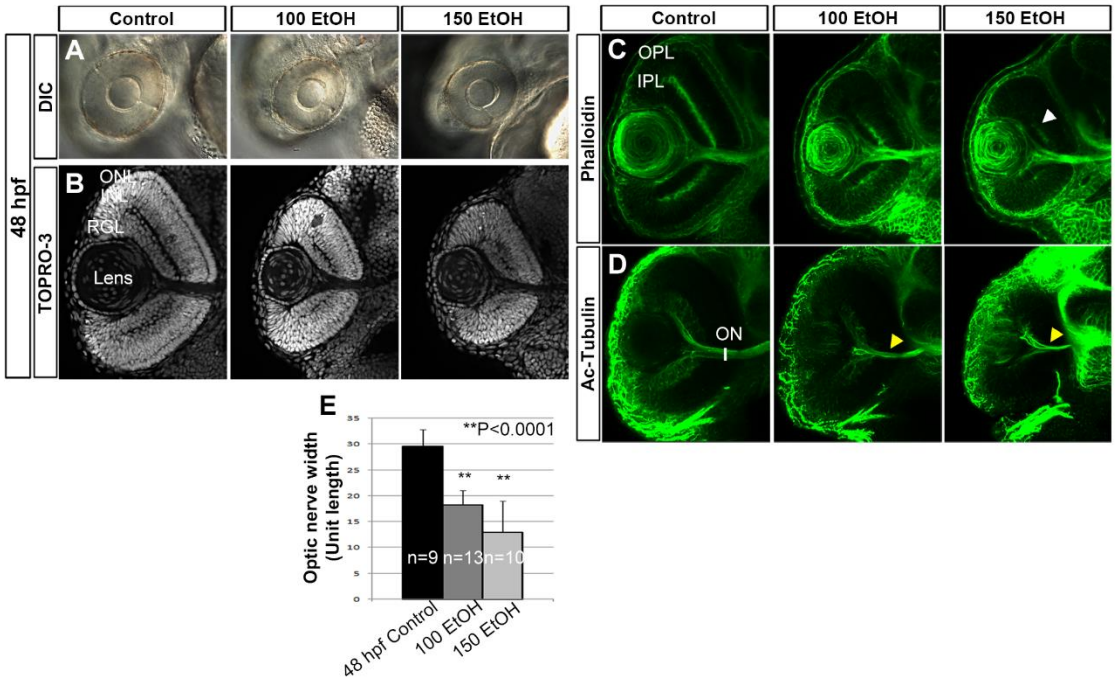

Figure 4.1 Ethanol exposure disrupts tissue organization at 48 hpf.
(A) DIC images demonstrating ethanol-induced microphthalmia at 48 hpf. (B, C) TO-PRO-3 (B) and phalloidin (C) staining at 48 hpf, showed formation of three distinct nuclear layers (RGL, INL and ONL; B) and two plexiform layers (IPL and OPL; C) in control embryos, and severely disrupted lamination in ethanol-treated embryos (white arrowhead). (D, E) 3D renderings of acetylated-tubulin stained embryos showed severe ONH following ethanol treatment as compared to untreated controls (yellow arrowheads, D). (E) Optic nerve widths quantification of 100 mM and 150 mM ethanol treated embryos showed significant reduction compared to untreated embryos. Anterior to the top; Ventral view; RGL, retinal ganglion cell layer; ONL, outer nuclear layer; INL, inner nuclear layer; OPL, outer plexiform layer; IPL, inner plexiform layer; ON, optic nerve. Error bars indicate standard deviation.

Staining using antibodies against specific terminally differentiated photoreceptor markers at 72 hpf, showed strong reduction in red-green double cone (zpr-1) and rod photoreceptor (zpr-3) differentiation (Fig 4.2 B-D). ISH with opsin riboprobes showed decrease in rhodopsin and red opsin expression (Fig 4.3 A, B). This was also reflected in the qPCR analysis of RNA obtained at 72 hpf. The ethanol embryos showed significant reduction in

Rhodopsin (100 and 150 mM ethanol-treated embryos, 0.29 and 0.097-fold change, respectively; $p<0.05$) and Red opsin (100 and 150 mM ethanol-treated embryos, 0.52 and 0.215-fold change, respectively; $p<0.05$) in comparison to untreated embryos (Fig. 4.3D). Conversely, UV opsin expression seemed to be increased after ethanol treatment (Fig 4.3C). Ethanol treated embryos also showed severe defects in MGC glial fibrillary acidic protein (Gfap) distribution as detected using zrf-1 antibody (Fig 4.2E). These results suggest that ethanol treatment perturbed retinal cell differentiation processes specifically for RGCs, photoreceptors, and MGCs.

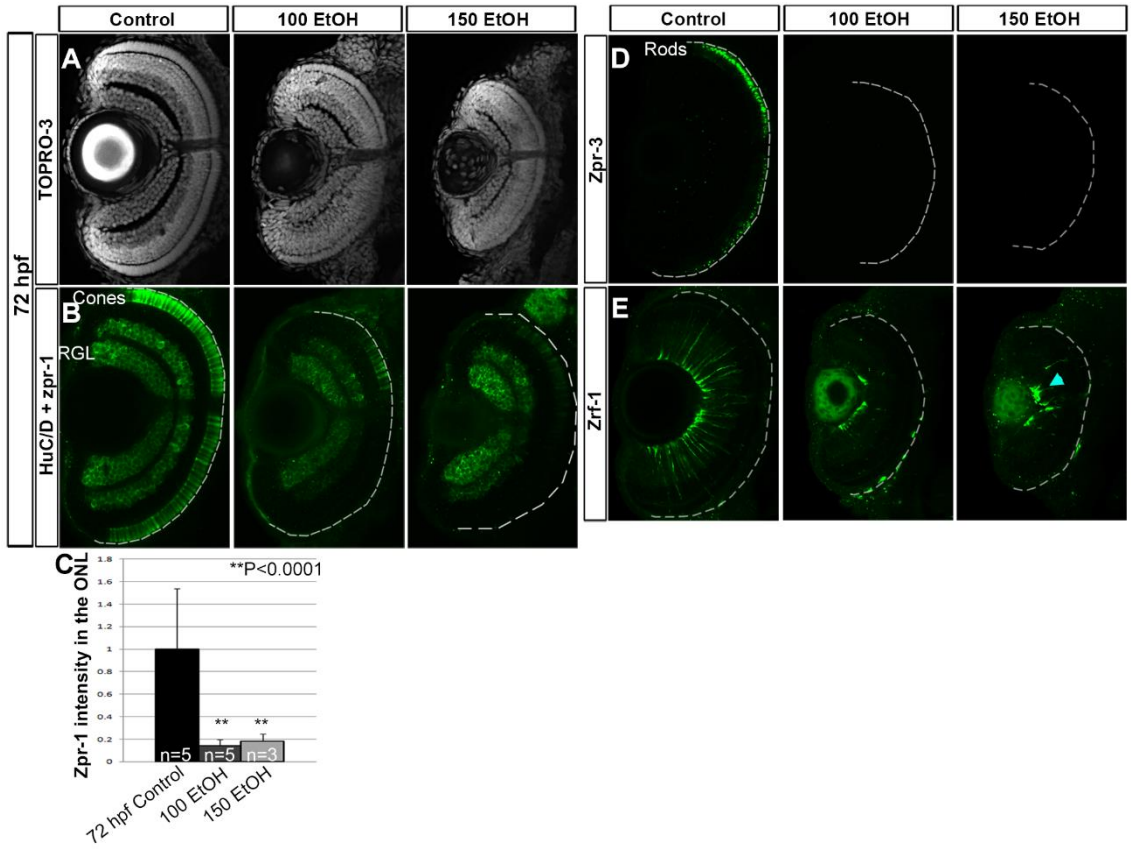

Figure 4.2 Ethanol exposure perturbs photoreceptor and MGC differentiation at 72 hpf. (A-D) At 72 hpf, TO-PRO-3 staining showed delayed lamination in ethanol-treated embryos (F); zpr-1 and zpr-3 staining showed reduced differentiation of double cones (G) and rods (I). (H) Quantification of total zpr-1 intensity in ONL showed a significant ethanol-induced reduction in double cone terminal differentiation. (E) Zrf-1 antibody staining in the retina revealed aberrant MGC morphology after ethanol treatment (blue arrowhead). Anterior to the top; Ventral view; White dashed lines indicate RPE; RGL, retinal ganglion cell layer; ONL, outer nuclear layer; INL, inner nuclear layer; OPL, outer plexiform layer; IPL, inner plexiform layer; ON, optic nerve. Error bars indicate standard deviation.

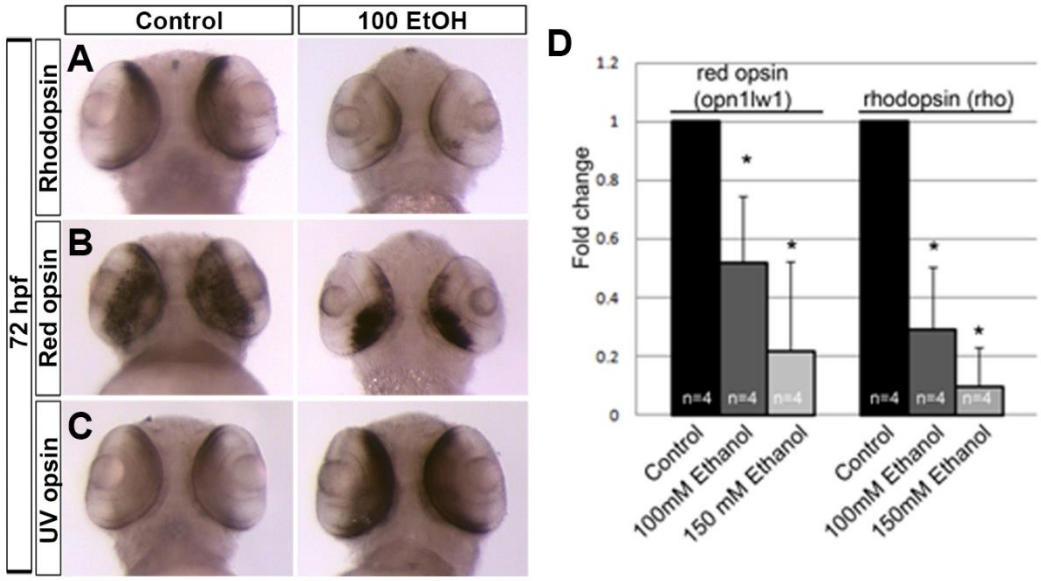

Figure 4.3 Ethanol exposure alters opsin expression.
(A-C) At 72 hpf, ethanol treated embryos showed a reduced expression of rhodopsin (expressed in rods, A) and red opsin (red cones, B) and an increased expression of UV opsin (C). Anterior to the top; dorsal view; (D) qPCR analysis comparing red opsin and rhodopsin expression levels at 72 hpf.

4.2.2 Ethanol treatment changes critical transcription factor expression

Using ISH, ethanol effects on the expression patterns of genes involved in retinal neurogenesis induction, cell specification, and differentiation. The expression of retinal neurogenesis induction gene, *shh*, remained unchanged at 30 hpf (Fig. 4.4A). However, expression of retinal cell specification gene, *rx1*, increased after ethanol treatment (Fig. 4.4B). Expression of transcription factors involved in retinal cell differentiation, *crx*, *otx5*, and *neurod*, was reduced in the retina in ethanol treated embryos in comparison to control retinas, at 48 hpf (Fig. 4.4 C-E).

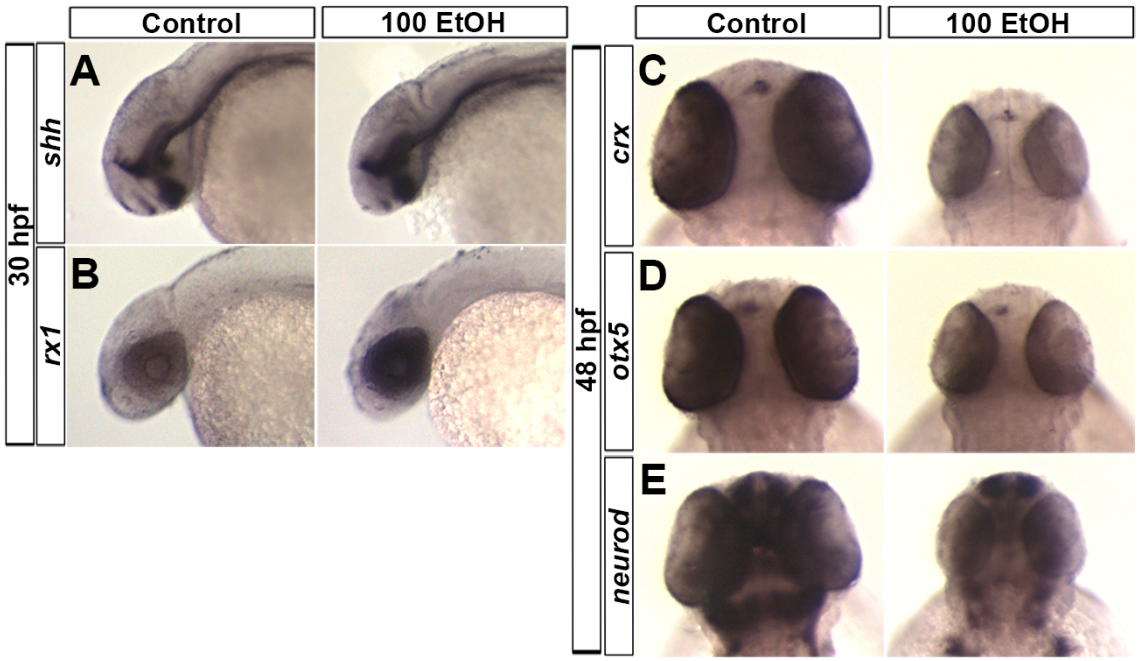

Figure 4.4 Ethanol exposure alters expression of retinal transcription factors.
(A, B) ISH at 30 hpf showing expression pattern of *shh* and *rx1*. Ethanol exposure (100 mM) did not change *shh* expression pattern (A), but the embryos showed an increased expression of *rx1* (B). Anterior to left. (C-E) At 48 hpf, ethanol-treated embryos showed a reduced expression of transcription factors including *crx* (C), *otx5* (D) and *neurod* (E) in the retina compared to the control embryos.

4.3 Ethanol-induced retinal cell death accounts for microphthalmia

To determine the causes underlying ethanol-induced microphthalmia, three possibilities were examined: (i) reduced number of cells initially induced for retinal differentiation, (ii) increased retinal cell death, and (iii) reduced retinal cell proliferation. Expression domains of retinal cell specification marker, *rx1*, were comparable in control and ethanol-treated embryos, indicating no reduction in initial specification eye field (Fig. 4.4B). Induction of retinal differentiation was examined using *crx* ISH (retinal cell differentiation marker, at 34 hpf), HuC/D antibody staining (early neuronal marker) at early stages of ganglion cell differentiation (32 hpf), and zpr-1 antibody staining (red-green double cone photoreceptor marker) at early stages of photoreceptor differentiation (60 hpf). These experiments

showed no difference in retinal cell specification and initial differentiation marker expression domains or timing after ethanol exposure (Fig. 4.5A-C).

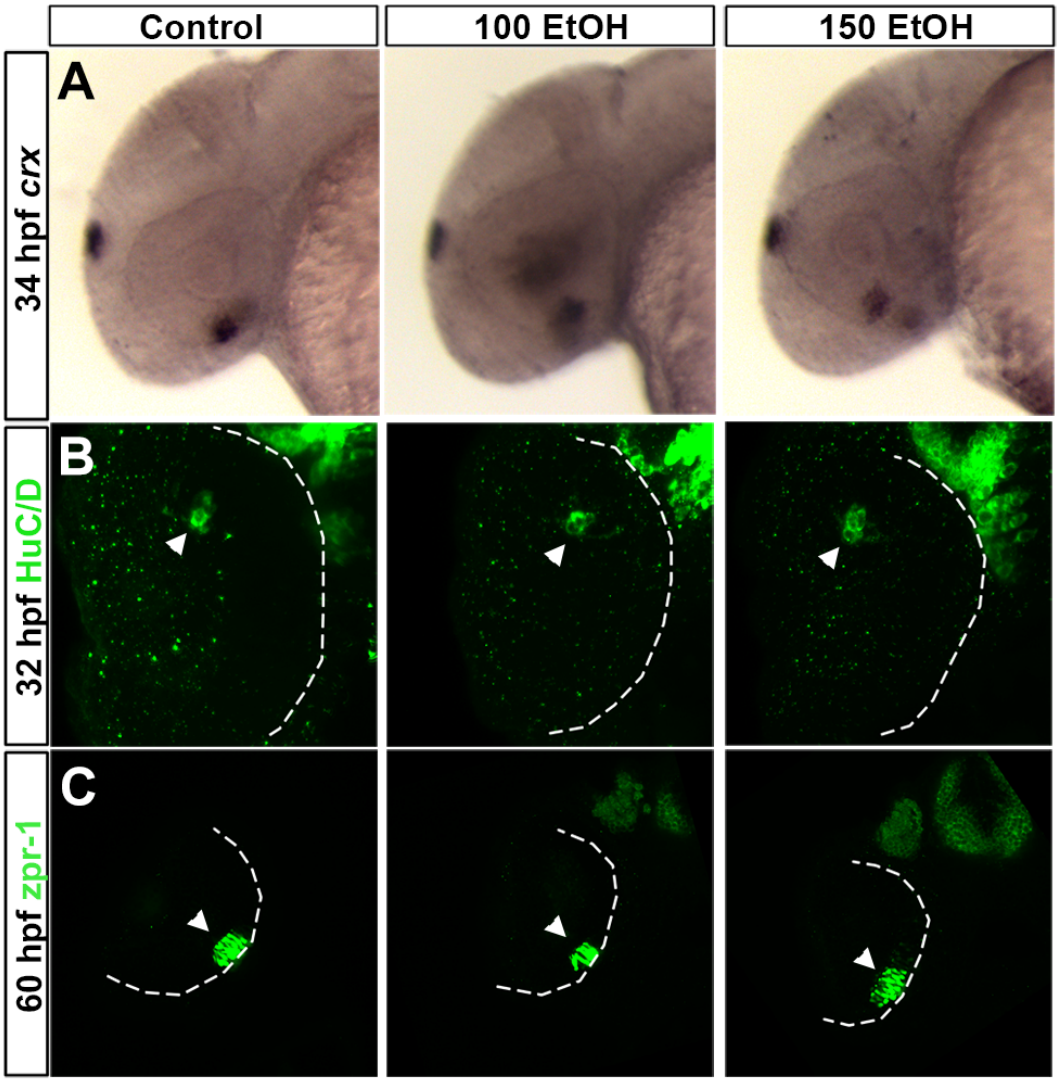

Figure 4.5 Ethanol exposure did not change the cell differentiation induction in ventral retina.
(A-C) ISH using *crx* riboprobes (A, Anterior to left; la teral view) and immunostaining detecting HuC/D protein (B) showed little or no difference in retinal ganglion cell differentiation after ethanol exposure. (C) zpr-1 antibody showed similar photoreceptor differentiation in the control and ethanol treated embryos (white arrowheads). Anterior to the top; Ventral view; White dashed line indicate future RPE. Error bars indicate standard deviation.

Acridine orange staining was used to measure apoptosis in embryonic retinas. Following completion of ethanol treatment, the number of acridine orange-positive cells per unit area did not differ from untreated control embryos at 27 hpf (Fig. 4.6B). However, at 36 hpf, there was a significant increase in the number of acridine orange-positive cells per unit area in the retina of ethanol-treated embryos (Fig. 4.6 A, B). To analyze cell proliferation in the retina, the mitosis marker phospho-histone-3 (H3) was examined using immunofluorescence. The number of phosphoH3-positive nuclei did not change following ethanol treatment at 24 and 36 hpf in the ethanol-treated retinas. However, there was a significant increase in the number of proliferating retinal cells per unit area after ethanol treatment at 48 and 72 hpf (Fig. 4.7 A, B). Mitotic cells in the retinas of ethanol treated embryos were mostly found in the ONL and INL at 48 hpf. These mitotic cells were predominantly found in the CMZ in the ethanol-treated retinas at 72 hpf (Fig.4.7B).

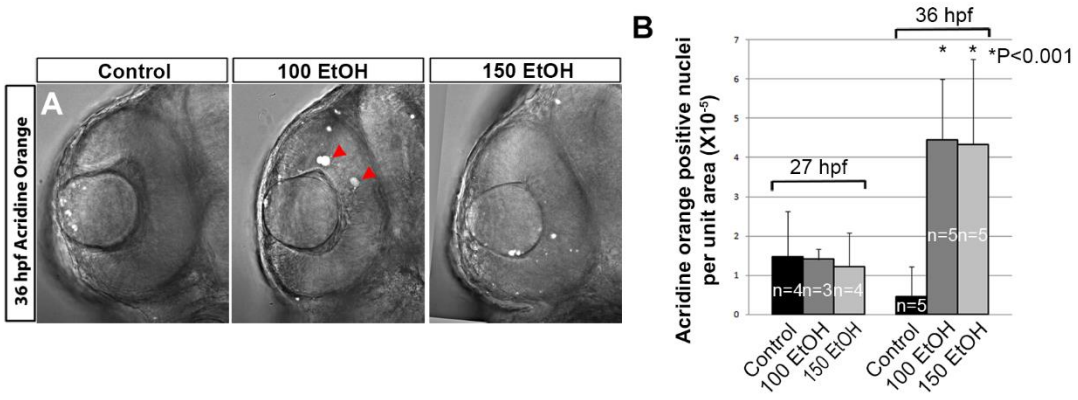

Figure 4.6 Ethanol induced cell apoptosis analysis.
(A) Acridine orange staining showed increased cell death after ethanol exposure (red arrowheads). (B) Quantification of acridine orange positive cells in the retina per unit area at 27 hpf and 40 hpf, showing little or no change in number of apoptotic cells at 27 hpf and a significant increase in cell death at 40 hpf in ethanol treated embryos. Anterior to the top; Ventral view. Error bars indicate standard deviation.

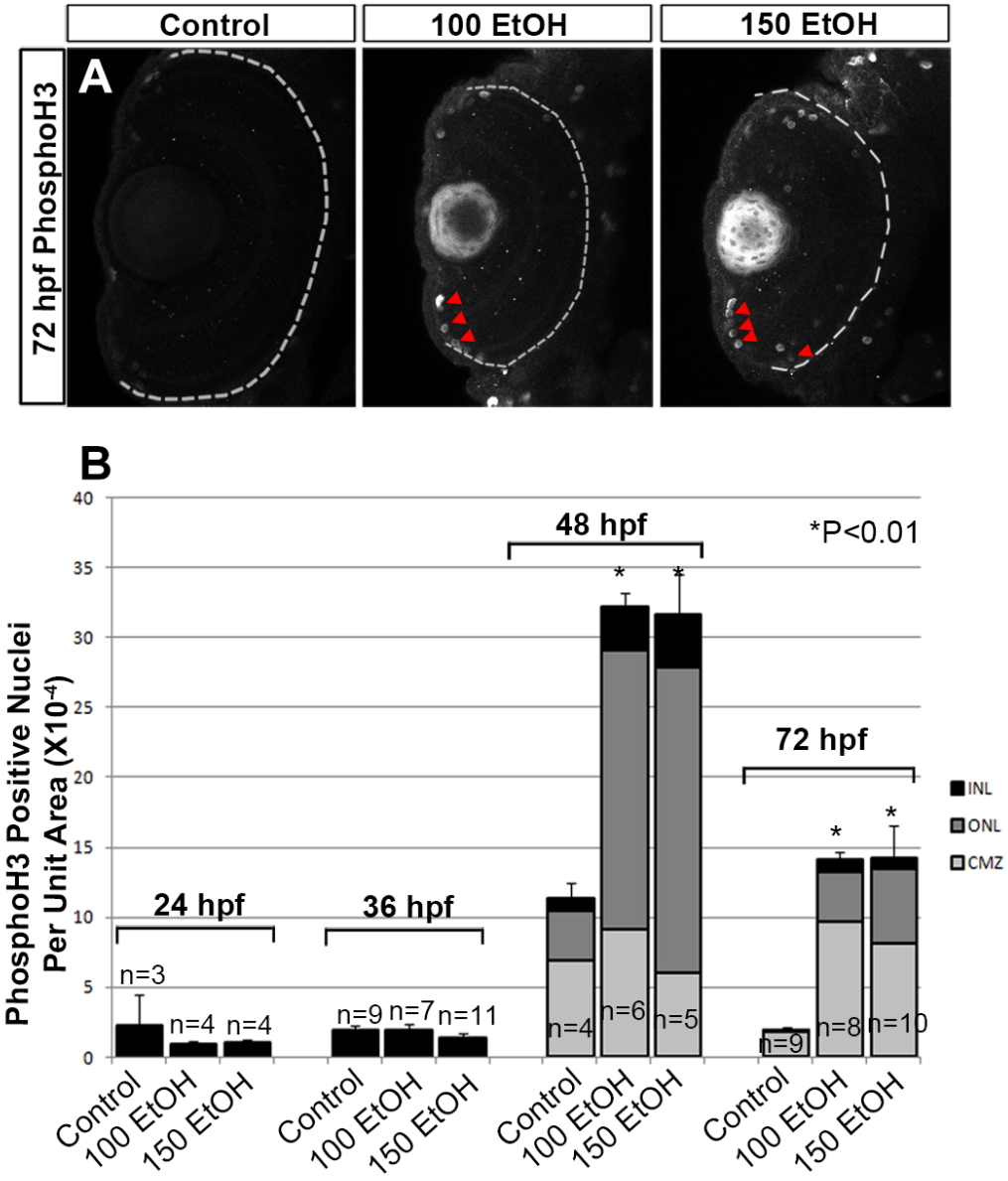

Figure 4.7 Ethanol exposure induced cell proliferation.
(A) PhosphoH3 staining showed an increase in the number of dividing cells after ethanol exposure (red arrowheads). (B) Quantification of phosphoH3 positive nuclei per unit area of the retina showed no difference in the number of proliferating at 24 hpf between control and ethanol treated retinas, but a significant increase in the number of cells undergoing division in the ONL and INL at 48 and 72 hpf. As 24 hpf retinal structure is not laminated, the cell counts at this stage represent whole retinal area. Anterior to the top; Ventral view; White dashed lines indicate RPE. Error bars indicate standard deviation.

4.4 Identification of ethanol sensitive time windows

In order to determine a sensitive time for ethanol induced defects, we split the total ethanol exposure time into two treatments, before and after 16 hpf (Fig 4.8A). Retinal morphogenetic processes and signaling events occur after 16 hpf. Treatment from 2-16 hpf showed comparable optic nerve width and photoreceptor differentiation as control embryos (Fig 4.8 B-F). However, treatment during the later time period from 16-24 hpf showed significant ONH and photoreceptor differentiation defects (Fig 4.8 B-F).

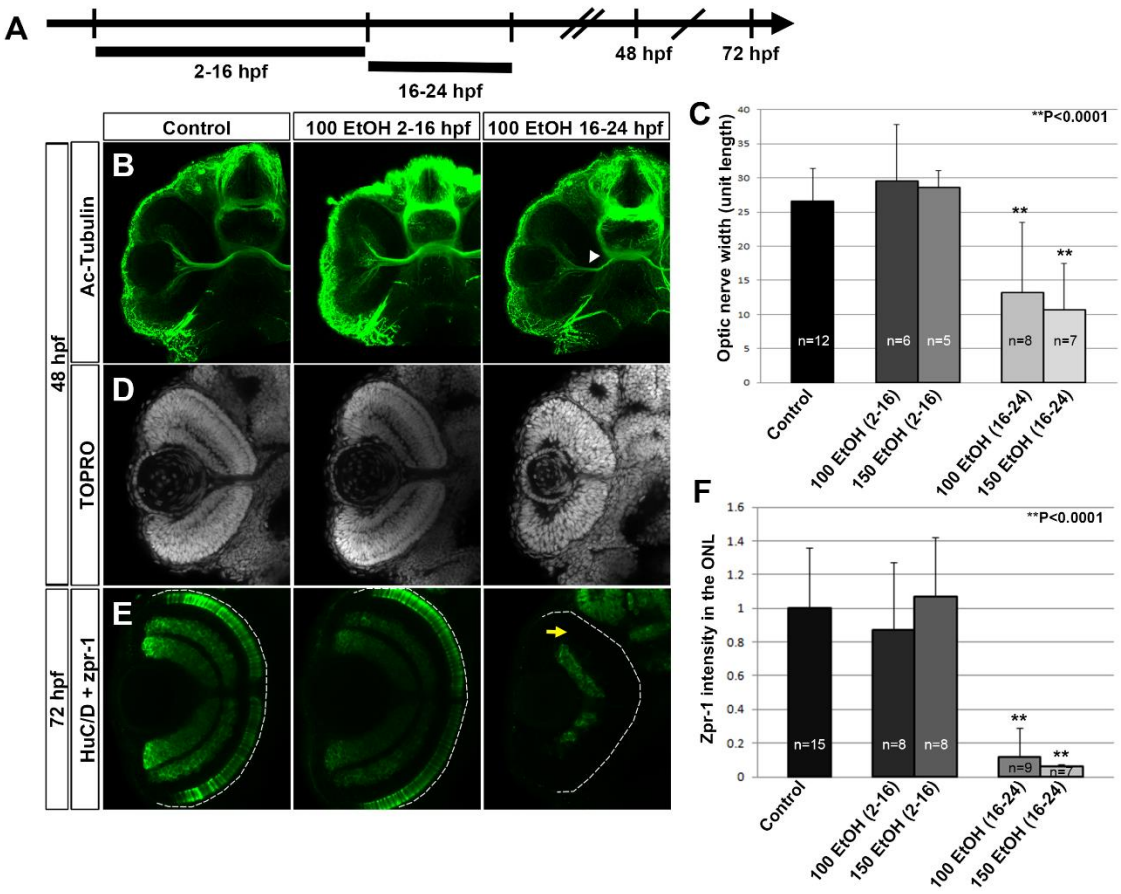

Figure 4.8 Ethanol exposure during specific time windows.
(A) Timeline showing windows of ethanol treatment from 2-16 hpf, and 16-24 hpf. (B-F) Ethanol treatment from 2-16 hpf showed near normal optic nerve, but exposure from 16-24 hpf showed ONH (white arrowhead, B). (C) Quantification of optic nerve width after ethanol treatment: ethanol alone from 2-16, and 16-24 hpf. Embryos treated with ethanol alone from 16-24 hpf showed a significantly reduced optic nerve width. (D) TO-PRO-3-iodide staining showing retinal lamination: ethanol alone from 2-16 hpf, near normal lamination; ethanol alone from 16-24 hpf, reduced retinal lamination. (E) HuC/D and zpr-1 staining showed photoreceptor differentiation at 72 hpf: ethanol alone from 2-16 hpf, near normal photoreceptor; ethanol alone from 16-24 hpf, reduction of photoreceptor (yellow arrow). (F) Quantification of total zpr-1 intensity in the ONL of the retina showed a significant decrease in photoreceptor marker expression after ethanol treatment (16-24 hpf). White dashed lines indicate RPE. Error bars indicate standard deviation.

4.5 Discussion

Early development, including gastrulation and neurogenesis, is particularly sensitive to environmental teratogens, like ethanol, which produce severe defects in the embryo (Gilbert-Barness, 2010; Lipinski et al, 2012). Ethanol is able to cross the placental barrier, allowing direct access to the developing embryo (Guerri & Sanchis, 1985). Ethanol-induced defects are greatly influenced by dose, duration and genetic background (May & Gossage, 2011). Ethanol effects on development may be influenced by comorbid environmental and nutritional factors. Zebrafish is a useful model to examine ethanol effects on development, and this model allows control over many variables including nutritional supplements (Ali et al, 2011). Previous zebrafish FASD model studies demonstrated loss of visual function at higher concentrations of ethanol (1-2% v/v) using electroretinography due to reduced rod photoreceptor function, leading to scotopic vision loss (Bilotta et al, 2002; Matsui et al, 2006). Other studies showed that persistent microphthalmia resulted from ethanol exposure when treated during retinal neurogenesis (24-48 hpf), showing slight rescue of photoreceptor differentiation by RA treatment (Kashyap et al, 2007; Kashyap et al, 2011). However, these studies did not dissect particular retinal cell specification and differentiation events.

Our laboratory previously showed that treating zebrafish embryos with ethanol (0.6 and 0.9% v/v) produced reproducible defects in gastrulation cell movements, cell adhesion and heart morphogenesis (Marrs et al, 2010; Sarmah & Marrs, 2013; Sarmah et al, 2013). Abnormal retinal cell differentiation events may be due to a combination of increased oxidative stress, increased cell death, disrupted cell signaling pathways, and altered epigenetic modifications induced by ethanol exposure (Muralidharan et al, 2013). This study dissects retinal developmental defects produced by ethanol exposure during midblastula transition, gastrulation, neurulation and somitogenesis (2-24 hpf) and highlights an ethanol-sensitive critical time window during retinal morphogenesis (16-24 hpf).

4.5.1 Early ethanol exposure severely perturbs retinal cell differentiation

Our experiments show that ethanol treatment during zebrafish early development affected expression patterns of critical genes involved in retinal development and morphogenesis, which may contribute to ocular defects. Measurement of these cellular defects allowed us to compare effects of different treatment regimens on specific cell differentiation events. Previous studies used eye diameter and optomotor response assays to compare several ethanol exposure time window treatments, showing significant microphthalmia in embryos treated with ethanol during gastrulation and retinal morphogenesis (6-24 hpf) and similar defects when treated during retinal morphogenesis (12-24 hpf), highlighting this ethanol-sensitive developmental period (Bilotta et al, 2002). Increased susceptibility to ethanol during retinal morphogenetic stages is possibly due to disruption of multiple developmental pathways including effects on epigenetic modifications and critical gene expression occurring at these times. Our studies showed that severe defects were produced by exposure during early retinal morphogenesis (16-24 hpf), resembling defects produced when ethanol was present during the entire gastrulation and retinal morphogenesis periods. Microphthalmia could result from disrupting various processes, including initial retinal differentiation induction, cell death, and cell proliferation. Ethanol increased cell death and cell proliferation rates, but no difference was detected in the induction of ganglion or photoreceptor cells differentiation, indicating that increased cell death was a major contributing to the microphthalmia phenotype. Apoptosis rates were not significantly higher immediately following ethanol treatment (at 24 hpf), but were significantly increased at later stages of cell specification and neurogenesis. Cell proliferation rates, however, were higher at later stages (at 48 and 72 hpf). The lag between cell death and cell proliferation suggests a cause and effect relationship indicating that cell proliferation is compensatory. This suggests that ethanol effects on gene expression mechanisms during retinal specification and differentiation lead to increased apoptosis, rather than inducing acute cellular toxicity. Perhaps, the effects on ethanol prevent progression of normal terminal differentiation leading to cell death, accompanied by compensatory cell proliferation.

4.5.2 Effect of ethanol on retinal photoreceptor progenitor and precursor cells

In an uninjured teleost retina, retinal stem cells reside in the CMZ, which generate new retinal tissue (Wehman et al, 2005). Slowly dividing progenitors in the INL generate rapidly dividing precursors in the ONL (Julian et al, 1998; Otteson et al, 2001; Raymond & Rivlin, 1987). MGCs are also stem cells in the central retina since they proliferate in response to injury, producing rod precursors (Yurco & Cameron, 2005). Upregulation of *rx1* gene expression and increased phosphoH3 stained cells in specific laminae of the retina following ethanol treatment suggests that damage due to ethanol exposure triggers expansion of retinal progenitor populations. Initial ethanol responses at 48 hpf showed an increased cell proliferation in the ONL and INL, indicating an activation of progenitor and precursor cells. At later stages (72 hpf), ethanol-induced increase in cell proliferation was seen in the CMZ, which may represent ongoing proliferation of retinal stem cells that may be needed to replenish the progenitor and precursor populations. Analysis of these cell populations will provide valuable insights into ethanol teratogenesis mechanisms.

4.6 Conclusion

In summary, this study illustrates novel cellular mechanisms of ethanol teratogenicity during ocular development. Additional research examining epigenetic changes, apoptosis and signaling pathway disruptions will allow us to identify specific targets of ethanol during development and facilitate design of therapeutic and preventive measures.

CHAPTER 5. RESCUE OF ETHANOL-INDUCED RETINAL DEFECTS BY NUTRITIONAL SUPPLEMENTS

5.1 Introduction

Proposed mechanisms underlying ethanol-induced ocular defects include increased cell death, developmental delay, and reduced cell differentiation (Kashyap et al, 2007). Developmental defects may be due to ethanol-induced RA signaling disruption, reactive oxygen species (ROS) generation, and epigenetic defects (Brocardo et al, 2011; Kot-Leibovich & Fainsod, 2009; Marrs et al, 2010; Singh et al, 2009; Zhou et al, 2011a). In addition, low socioeconomic populations show increased FASD incidence, which correlates with nutritional deficiencies. Reduced absorption and increased excretion of essential vitamins in adults caused by ethanol consumption aggravates malnutrition (Lieber, 2003). Several studies showed nutritional compounds, including retinoids, folate, choline, and vitamin E partially rescued ethanol-induced developmental defects (Heaton et al, 2011; Kot-Leibovich & Fainsod, 2009; Marrs et al, 2010; Mitchell et al, 1999; Sarmah & Marrs, 2013; Thomas et al, 2010; Wang et al, 2009; Yelin et al, 2005). RA and FA were very effective in rescuing various developmental defects (Ballard et al, 2012; Marrs et al, 2010; Sarmah & Marrs, 2013; Yelin et al, 2005).

Retinoids are critical signaling factors needed during development. RA signaling plays a crucial role during antero-posterior patterning. During retinal development, it is involved in retinal dorso-ventral patterning. Spatial expression of RA synthesizing enzymes and RA degrading enzymes regulate the RA levels in the tissue. This cues and activates specific gene expression driving cell type differentiation and patterning. Several studies have shown the effect of RA supplementation on ethanol-induced defects. Genetic association studies also show the effect of genetic variation in the ALDH and the ADH genes and alcohol susceptibility (Ehlers et al, 2004; Neumark et al, 2004). The developing forelimb

also requires RA for initial induction of forelimb outgrowth, and reductions of RA, or inhibition of RA receptor results in postaxial ectrodactyly (loss of digits) similar to prenatal ethanol exposure (Johnson et al, 2007).

FA is an essential nutrient that has to be supplied through diet. Since folate-dependent 1-carbon metabolism is the subject of genetic variability, several common polymorphisms of genes encoding folate-dependent enzymes have also now been identified as risk factors for a variety of clinical conditions including several cancers, Alzheimer's, and Down syndrome (Butterworth, 1993; Clarke et al, 1998; James et al, 1999; Kamei et al, 1993; Slattery et al, 1999; Zhang et al, 1999). A study using Drosophila treated with Methotrexate (MTX), a potent inhibitor of the enzyme dihydrofolate reductase, showed that led to severe effects on female fecundity, abnormal egg development, and developmental defects in newborns, including with ocular abnormalities. (Affleck et al, 2006). Experimental studies in mice using folate-deficient diet for females during gestation showed that the offspring developed severe retinal abnormalities including anophthalmia and microphthalmia (Maestro-de-las-Casas et al, 2013). Not only does folate-deficiency itself lead to birth defects, comorbid conditions with poverty and alcohol consumption lead to increased malnutrition and vitamin deficiencies increasing the prevalence of fetal alcohol spectrum disorder (FASD) in low socio-economic populations.

This study examines the effect of RA and FA supplementation on ethanol-induced retinal defects. Experiments were used to identify time windows that show rescue and potential insights into mechanisms underlying ethanol-induced retinal defects.

5.2 Ethanol-induced retinal defects can be rescued by RA supplementation

Previous studies showed that 1 nM RA supplementation rescued cardiac and other phenotypic defects in zebrafish embryos (Marrs et al, 2010; Sarmah & Marrs, 2013). To test RA rescue of ethanol-induced retinal defects, embryos were co-treated with ethanol and 1 nM RA from 2-24 hpf and compared with embryos treated with ethanol alone (Fig. 5.1A). *rx1* expression was restored to near normal levels by RA supplementation (Fig. 5.1B). Co-treatment of RA with ethanol moderately rescued ONH (Fig. 5.1 C, D) and completely rescued photoreceptor differentiation (Fig. 5.1 E, F). These findings highlight

that exogenous RA can restore retinal defects and indicate that ethanol exposure produced RA-signaling defects in the retina. Effects of two known RA biosynthesis inhibitors, citral and DEAB, were compared to ethanol to determine whether similar retinal defects are produced by directly disrupting RA synthesis (Fig. 5.2 A-C). Embryos treated with these inhibitors displayed ONH at 48 hpf and red-green cone photoreceptor differentiation defects at 72 hpf. The similarity of retinal defects produced with RA biosynthesis inhibitors and ethanol supports the idea that ethanol affects RA levels in the retina.

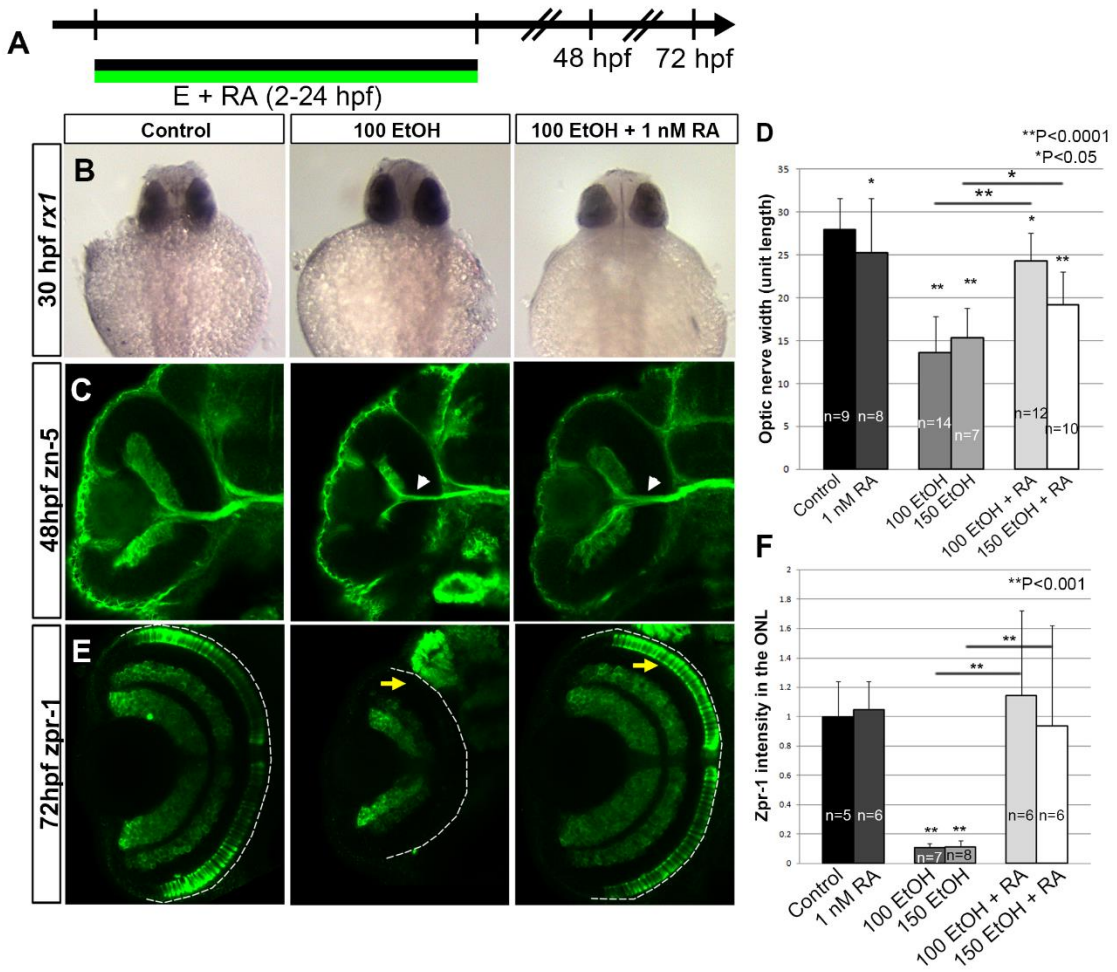

Figure 5.1 RA supplementation rescues ethanol-induced retinal defects.
(A) Timeline showing RA and ethanol co-supplementation from 2-24 hpf. (B-E) Treatment with low concentrations of RA (1 nM) rescued ethanol-induced retinal defects. (B) *rx1* gene expression level was restored by RA and ethanol co-treatment. (C) ONH (white arrowheads) seen in ethanol exposed embryos using zn-5 antibody staining ganglion cells, showed rescue after RA and ethanol co-treatment. (D) Quantification of optic nerve width showing a significant reduction after ethanol treatment which was restored by RA co-treatment. (E) Restoration of terminally differentiated double cone photoreceptor (yellow arrows) after RA co-treatment as shown by zpr-1 staining. (F) Quantification of total zpr-1 intensity in the ONL showed a significant reduction after ethanol treatment which was restored to control levels following RA co-treatment. White dashed lines indicate RPE. Error bars indicate standard deviation.

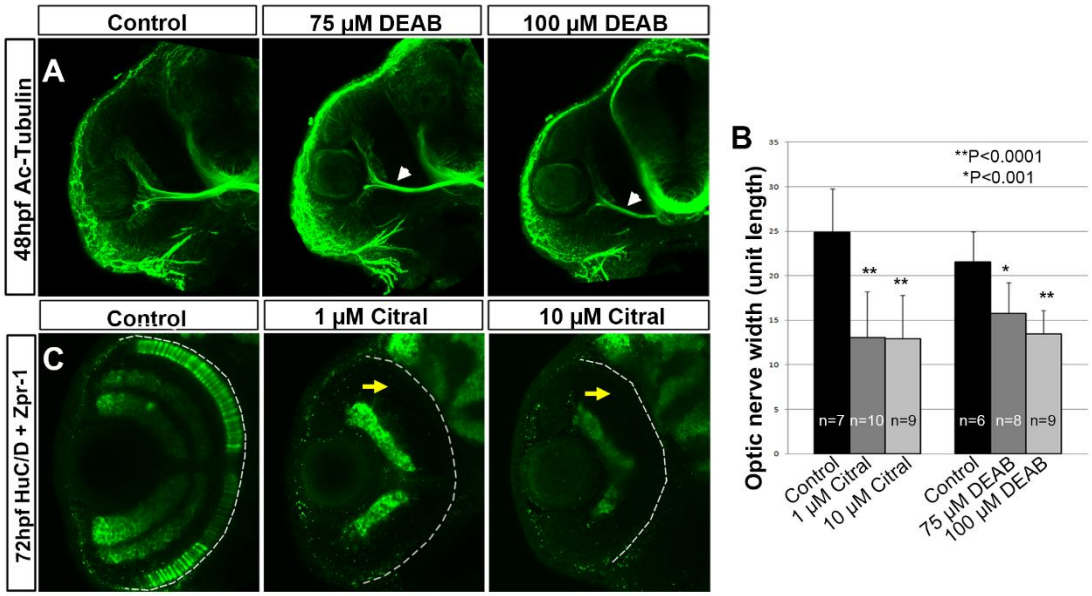

Figure 5.2 RA synthesis chemical inhibitor treatment.
(A-C) RA signaling inhibitors DEAB and citral, induced retinal defects similar to ethanol. (A) ONH after treatment with DEAB (75 µM and 100 µM, white arrowheads). (B) Quantification of optic nerve size reduction after DEAB and citral treatment showed significant reduction compared to untreated embryos. (C) Terminal double cone photoreceptors differentiation using zpr-1 staining showed reduction/absence of differentiated photoreceptors (yellow arrows) after citral treatment (1 µM and 10 µM). White dashed lines indicate RPE. Error bars indicate standard deviation.

RA signaling in the zebrafish retina begins when *raldh3* expression is activated in the ventral retina around 18 hpf (Ma et al, 2010). To test whether ethanol-induced retinal defects coincide with this activation of RA signaling, embryos were treated with ethanol during different time windows, before and after the onset of *raldh3* expression. The ethanol treatment period in previous experiments (2-24 hpf) was subdivided into two windows, 2-16 hpf and 16-24 hpf, the latter encompassing *raldh3* expression initiation of the previous treatment period (Fig. 5.3A). If ethanol-induced defects are due to reduced RA activity, then ethanol treatment only during RA biosynthesis should affect the cell differentiation processes, producing similar retinal defects as those seen in embryos treated with ethanol from 2-24 hpf, and not affect retinal development as severely as by treating during an

earlier period prior to RA biosynthesis (2-16 hpf). As expected, embryos treated with ethanol from 2-16 hpf showed only minimal retinal defects. However, embryos treated with ethanol during the 16-24 hpf time window showed thinner optic nerve widths and decreased red-green cone photoreceptor differentiation, as compared to control embryos (Fig. 5.3 B-F). Similar to embryos treated with ethanol from 2-24 hpf, RA co-supplementation with ethanol during 16-24 hpf showed dramatic rescue of the optic nerve widths to near control levels, and were significantly different from embryos treated with only ethanol during this period (Fig. 5.3C). Embryos co-treated with ethanol and RA showed rescue of photoreceptor differentiation that was significantly different from ethanol-treated embryos (Fig. 5.3F). Less extensive rescue of ONH and photoreceptor differentiation in the 150 mM ethanol and RA co-treated embryos reflects more severe defects caused by higher ethanol concentrations.

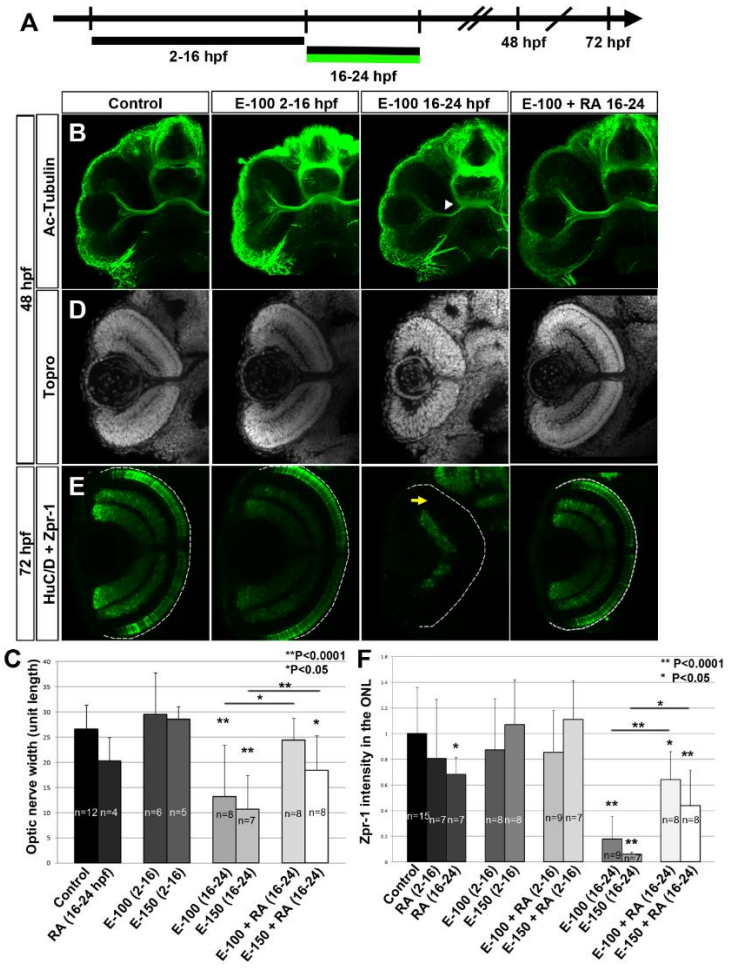

Figure 5.3 Critical time window of ethanol exposure coincides with RA synthesis induction. (A) Timeline showing windows of ethanol treatment from 2-16 hpf, 16-24 hpf and RA treatment from 16-24 hpf. (B-E) Ethanol treatment from 2-16 hpf showed near normal optic nerve, but exposure from 16-24 hpf showed ONH (white arrowhead, B). (C) Quantification of optic nerve width after ethanol treatment: ethanol alone from 2-16 and 16-24 hpf; ethanol +RA from 16-24 hpf. Embryos treated with ethanol alone from 16-24 hpf showed a significantly reduced optic nerve width, this was rescued by RA co-treatment. (D) TO-PRO-3-iodide staining showing retinal lamination: ethanol alone from 2-16 hpf, near normal lamination; ethanol alone from 16-24 hpf, reduced retinal lamination; ethanol +RA from 16-24 hpf, normal lamination. (E) HuC/D and zpr-1 staining showed photoreceptor differentiation at 72 hpf: ethanol alone from 2-16 hpf, near normal photoreceptor; ethanol alone from 16-24 hpf, reduction of photoreceptor (yellow arrow); ethanol +RA from 16-24 hpf, near normal photoreceptor. (F) Quantification of total zpr-1 intensity in the ONL of the retina showed a significant decrease in photoreceptor marker expression after ethanol treatment (16-24 hpf) and subsequent rescue by RA co-treatment. White dashed lines indicate RPE. Error bars indicate standard deviation.

5.3 Ethanol-induced disruption of RA-dependent pathways during retinal morphogenesis

To further validate ethanol effects on RA-dependent developmental pathways during retinal morphogenesis, embryos were treated with ethanol during the gastrulation and somitogenesis periods, 2-24 hpf, and supplemented with RA only during the 2-16 or 16-24 hpf time windows (Fig. 5.4A). If the retinal defects are produced by RA inhibition only during the sensitive time window of 16-24 hpf, then RA supplementation during that time period alone should rescue the ethanol-induced defects. Embryos treated with ethanol from 2-24 hpf and RA from 2-16 hpf exhibited ONH, similar to embryos treated with ethanol alone (2-24 hpf) (Fig. 5.4 B, C; E100 2-24 vs. E 100 2-24 + RA 2-16, $p = 0.3350$). Embryos treated with ethanol from 2-24 hpf and RA from 16-24 hpf showed significant improvement of optic nerve defects, compared to ethanol-treated embryos (Fig. 5.4 B, C). Presence of RA during the critical time window of 16-24 hpf was sufficient to completely rescue photoreceptor differentiation to normal levels (Fig. 5.4 D, E). This indicates a strong disruption of RA-dependent developmental events during retinal morphogenesis due to ethanol exposure.

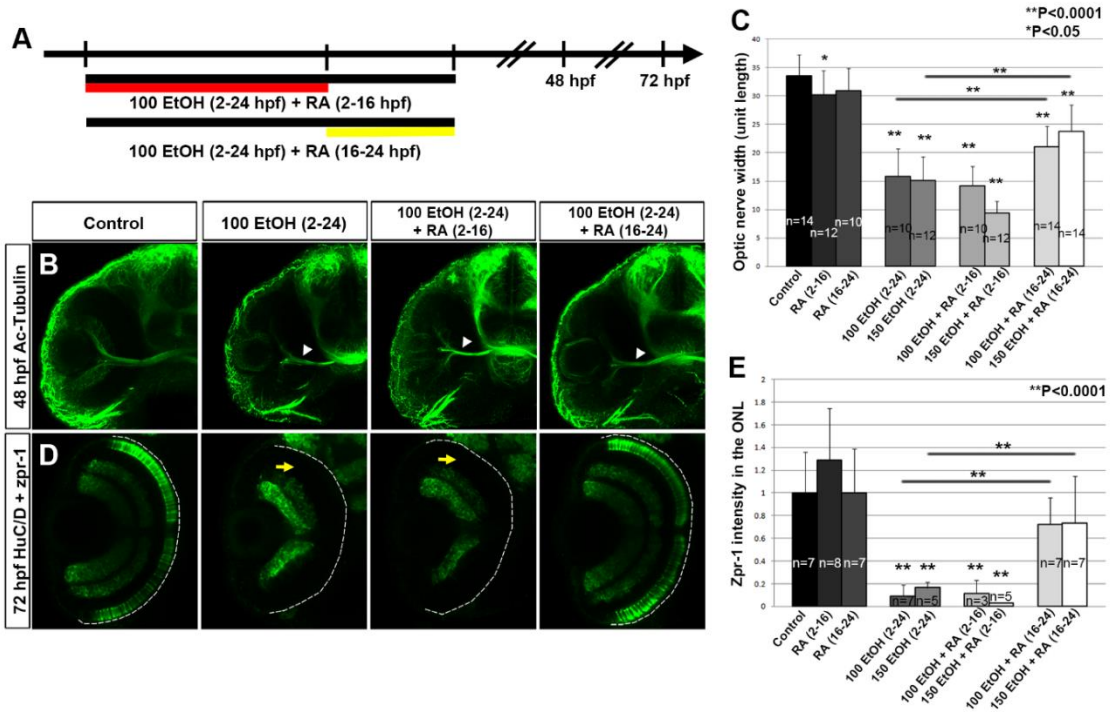

Figure 5.4 RA independent retinal defects and RA rescue after ethanol exposure.
(A) Timeline showing treatment with ethanol (2-24 hpf) and RA supplement (2-16 or 16-24 hpf). (B) Embryos treated with ethanol (2-24 hpf) +RA (2-16 or 16-24 hpf) showed severe ONH. (C) Quantification of optic nerve width showed significant reduction in ethanol treated embryos and no rescue by RA supplement during 2-16 hpf. A slight improvement was seen in RA-supplemented embryos from 16-24 hpf. (D, E) zpr-1 staining intensity analysis showed reduction of photoreceptor differentiation in the embryos treated with ethanol alone (2-24 hpf) and ethanol(2-24 hpf) +RA(2-16 hpf) (yellow arrows); near normal differentiated photoreceptors in embryos treated with ethanol(2-24 hpf) +RA(16-24 hpf). White dashed lines indicate RPE. Error bars indicate standard deviation.

5.4 Ethanol effects on retinal neurogenesis and photoreceptor development

If ethanol exposure produced a persistent alteration of RA biosynthesis and signaling, then RA supplementation at later stages (after ethanol exposure) should rescue retinal developmental defects caused by earlier ethanol exposure. To test this idea, embryos were ethanol-treated from 2-24 hpf, and RA was supplemented from 24-48 hpf (Fig. 5.5A). These embryos showed a significant rescue of ONH and photoreceptor differentiation (Fig. 5.5 B-D, F). Interestingly, supplementation with RA during photoreceptor differentiation

(48-72 hpf) improved expression of terminal red-green cone photoreceptor differentiation marker (Fig. 5.5 E, F), suggesting a trend of photoreceptor differentiation rescue even at this later stage.

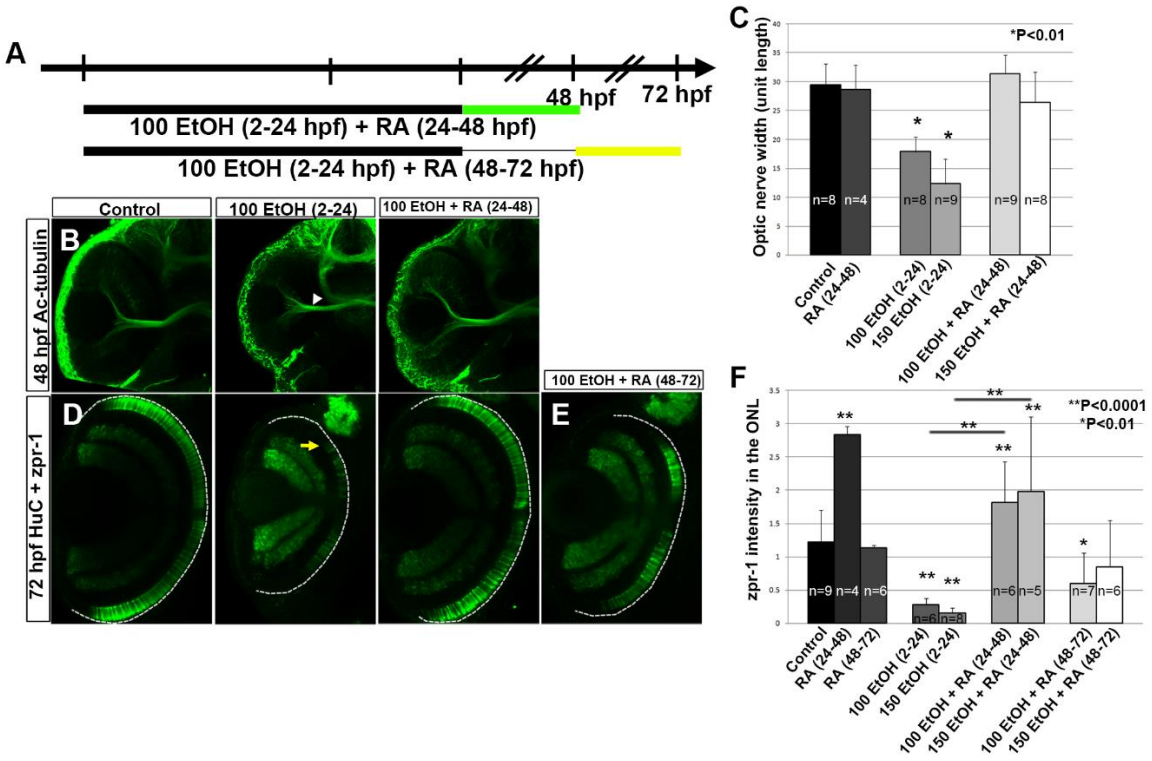

Figure 5.5 RA supplementation after ethanol exposure can restore retinal defects.
(A) Timeline showing treatment with ethanol (2-24 hpf) and RA (24-48 hpf and 48-72 hpf. (B) Acetylated-tubulin staining showed ONH in ethanol treated embryos, which was rescued by RA supplement (24-48 hpf). (C) Quantification of optic nerve width showed significant rescue of ethanol treated embryos with RA supplement. (D-F) Terminal photoreceptor differentiation assay using zpr-1 staining showed reduced photoreceptor differentiation in embryos treated with ethanol (2-24 hpf; yellow arrow); near normal differentiated photoreceptors in embryos treated with ethanol (2-24 hpf) +RA (24-48 hpf); slight rescue of photoreceptor differentiation in ethanol(2-24 hpf)+RA(48-72 hpf) treated embryos. White dashed lines indicate RPE. Error bars indicate standard deviation.

5.5 Prevention of ethanol-induced retinal defects by FA co-supplementation

To test the protective effects of FA on ethanol-induced retinal defects, embryos were co-supplemented with ethanol and 75 µM FA from 2-24 hpf (Fig. 5.6A). At 48 hpf, ethanol-treated embryos showed a significant reduction in optic nerve width, which was rescued by FA supplementation (Fig. 5.6 B, C). Photoreceptor differentiation defects induced by ethanol exposure (2-24 hpf) were also rescued by FA co-supplementation, as revealed by zpr-1 staining at 72 hpf (Fig. 5.6 D, E). Similarly, ethanol and FA co-treatment during the ethanol-sensitive time window from 16-24 hpf rescued ONH and photoreceptor differentiation effects at 48 and 72 hpf, respectively (Fig. 5.7 A-E). These findings indicate that FA protects ocular morphogenetic events from ethanol toxicity.

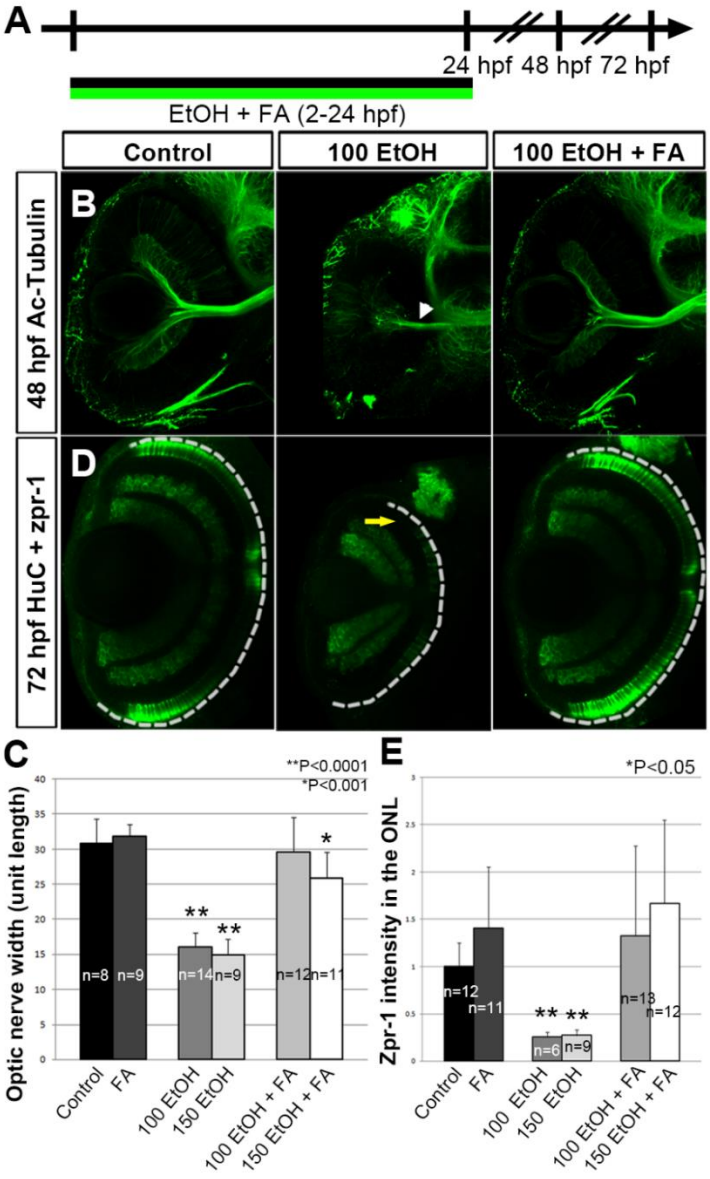

Figure 5.6 FA co-supplementation prevented ethanol-induced retinal defects.
(A) Timeline demonstrating FA co-supplementation (2-24 hpf). (B-E) Treatment with ethanol (2-24 hpf) produced ONH (white arrowhead; B), which was rescued by FA co-supplementation. (C) Optic nerve widths measured in acetylated-tubulin stained embryos showed rescue after FA co-supplementation. (D) Terminal photoreceptor differentiation marker expression, zpr-1, in the ONL showed rescue after FA co-treatment (yellow arrow). (E) Quantification of zpr-1 intensity in the ONL. White dashed lines indicate RPE. Error bars indicate standard deviation

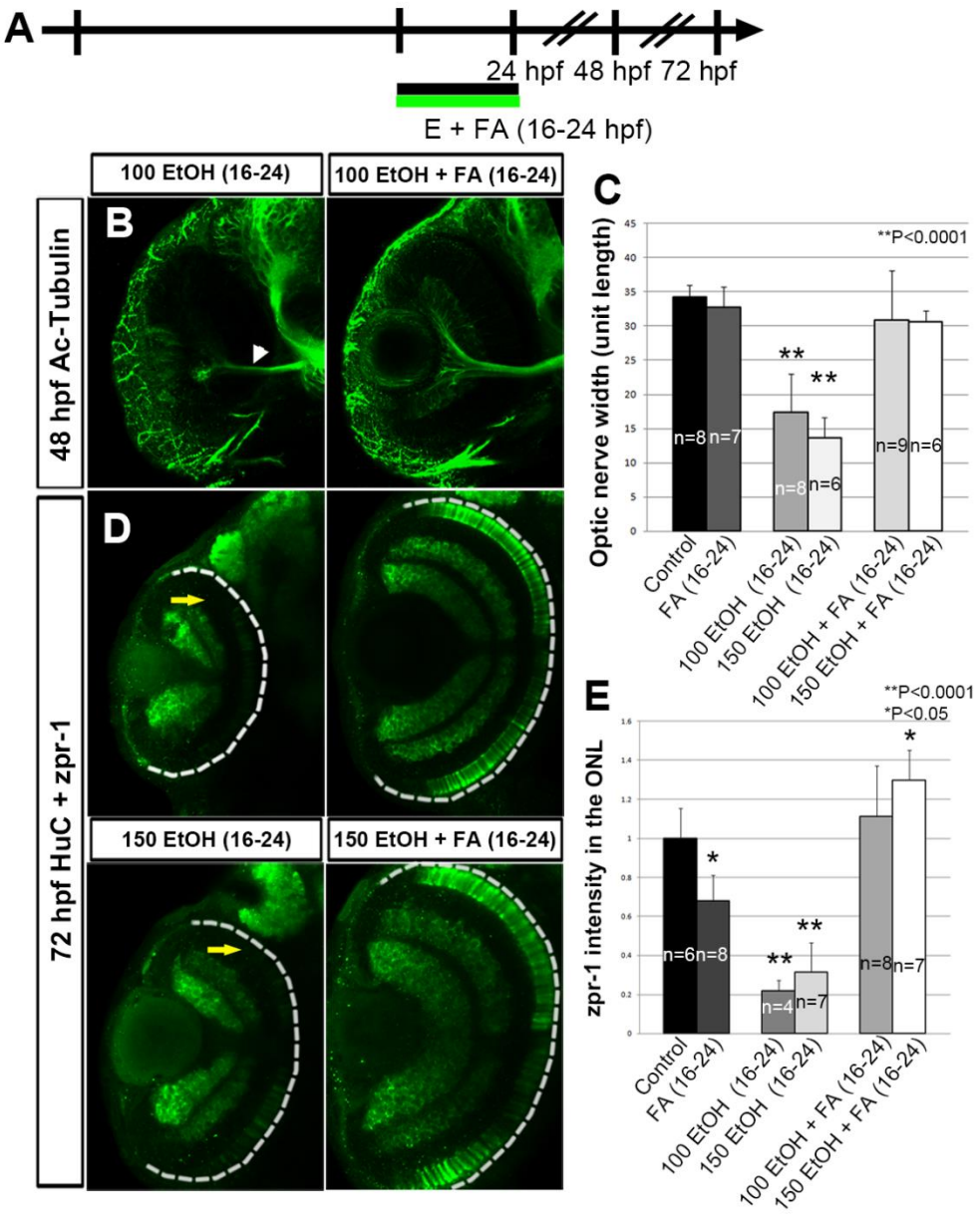

Figure 5.7 FA co-supplementation can rescue ethanol-induced defects during retinal morphogenesis.

(A) Timeline showing FA co-treatment during ethanol-sensitive time-window (16-24 hpf). (B-E) FA supplementation (16-24 hpf) rescued ethanol-induced ONH and retinal lamination defects. (B, C) Acetylated-tubulin stained optic nerve width measurements showed significant ethanol-induced decrease, which was restored by FA treatment. (D, E) zpr-1 staining showed photoreceptor differentiation defect was restored by FA co-treatment. White dashed lines indicate RPE. Error bars indicate standard deviation

Comparison of FA and RA co-supplementation regimens during the ethanol-sensitive time window (16-24 hpf) show statistically normal optic nerve and photoreceptor differentiation after FA co-supplementation, as compared to partial rescue after RA co-supplementation (Fig. 5.3 C, F; Fig. 5.7 C, E). This indicated a surprisingly better rescue of ONH and photoreceptor differentiation by FA than RA during the ethanol-sensitive time period. FA supplementation during both gastrulation (2-16 hpf) and retinal morphogenesis (16-24 hpf) showed moderate rescue of ONH and photoreceptor differentiation defects in comparison to ethanol-treated embryos (2-24 hpf) (Fig. 5.8 A-E). Similar experiments with RA pretreatment (Fig. 5.4 A-E) showed that the 16-24 hpf time point was critical for RA rescue. However, in both RA and FA rescues, presence of ethanol during 2-24 hpf caused severe retinal defects, which were only partially rescued by supplementation during 2-16 or 16-24 hpf alone. To determine whether FA treatment following ethanol exposure could reverse ethanol-induced defects, ethanol-treated embryos were supplemented with FA during retinal neurogenesis (24-48 hpf) and photoreceptor differentiation stages (48-72 hpf; Fig. 5.9A). FA treatment following the ethanol-exposure period (2-24 hpf) during retinal neurogenesis (24-48 hpf) or photoreceptor differentiation stages (48-72 hpf) did not rescue retinal cell differentiation defects (Fig. 5.9 B-F). These findings reveal differences between RA- and FA-dependent pathways after retinal morphogenesis, as their supplementation during the different stages after ethanol treatment showed varying abilities to remedy ethanol-induced eye defects (Fig. 5.10).

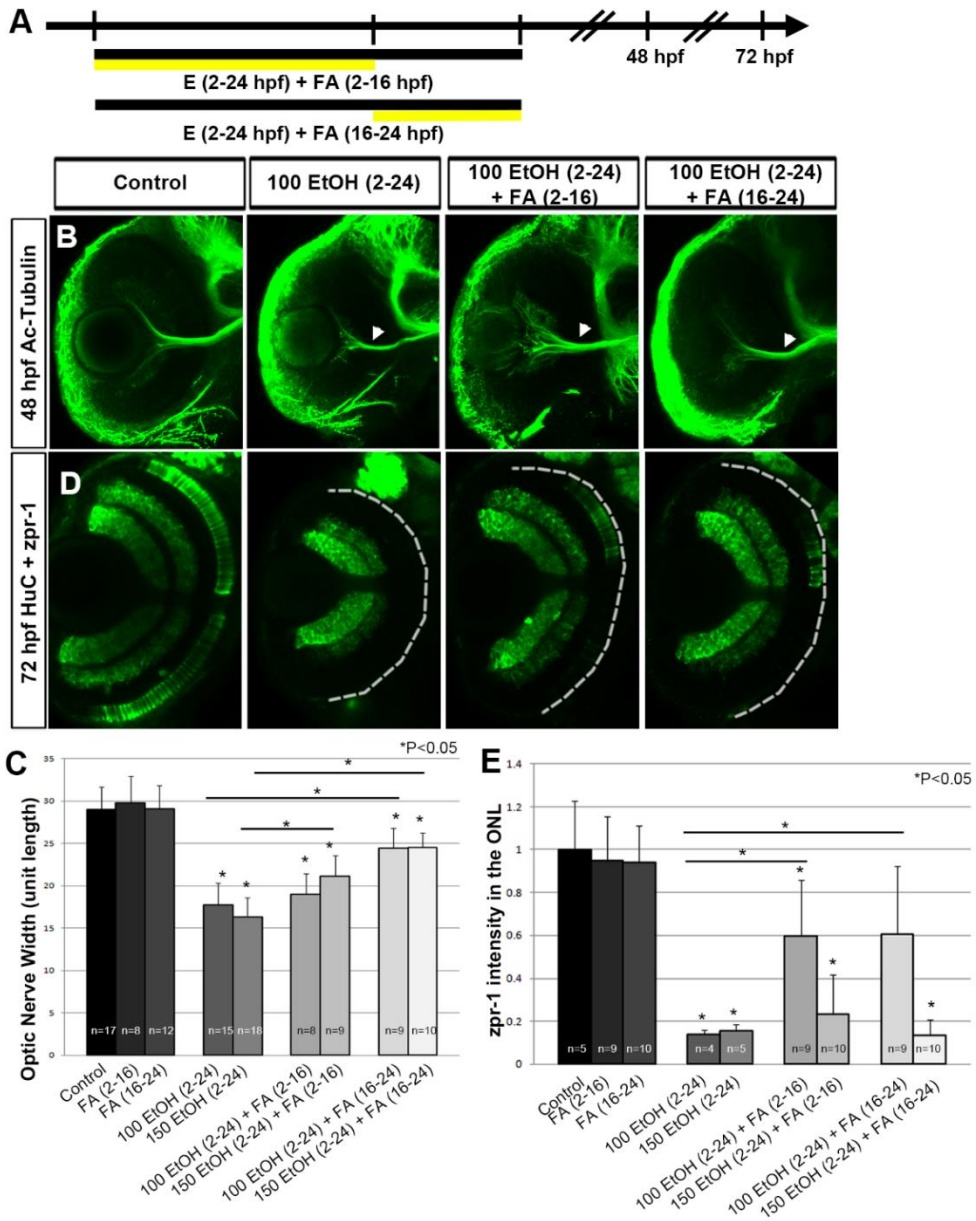

Figure 5.8 FA rescue of ethanol-induced retinal defects.
(A) Timeline showing treatment with ethanol (2-24 hpf) and FA supplement (2-16 or 16-24 hpf) (B) Embryos treated with ethanol (2-24 hpf) +FA (2-16 or 16-24 hpf) showed partial rescue of ONH (C) Quantification of optic nerve width showed slight rescue FA supplement during 2-16 hpf and 16-24 hpf. (D, E) zpr-1 staining intensity analysis showed reduction of photoreceptor differentiation in the embryos treated with ethanol alone (2-24 hpf) and partial rescue in ethanol (2-24 hpf) +FA(2-16 hpf) and ethanol(2-24 hpf) +FA(16-24 hpf). White dashed lines indicate RPE. Error bars indicate standard deviation.

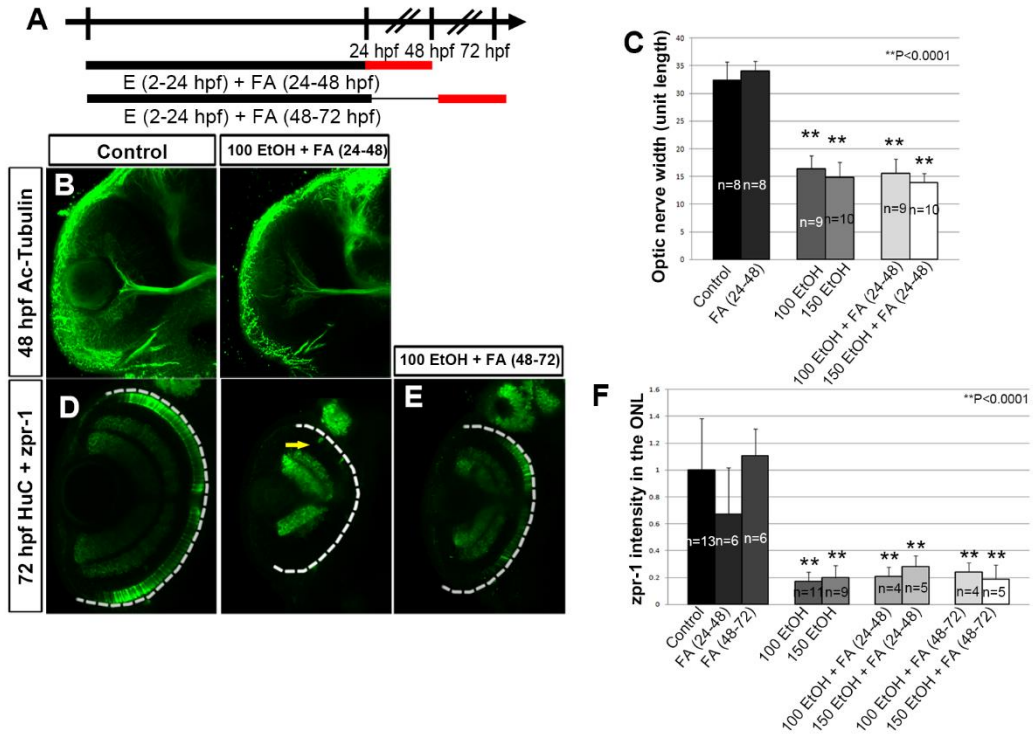

Figure 5.9 FA supplementation after ethanol exposure does not restore ethanol-induced retinal defects.

(A) Timeline showing FA treatment (24-48 and 48-72 hpf) after ethanol exposure (2-24 hpf). (B-F) (B) Acetylated-tubulin staining showed ONH in ethanol (2-24 hpf) + FA (24-48 hpf) embryos. (C) Quantification of optic nerve width after FA supplementation (24-48 hpf). (D-F) Reduction/absence of photoreceptor differentiation was detected in zpr-1 stained embryos supplemented with FA from 24-48 hpf (D) and 48-72 hpf (E). (F) Quantification of total zpr-1 intensity in the ONL did not show rescue after FA supplementation (48-72 hpf). White dashed lines indicate RPE. Error bars indicate standard deviation.

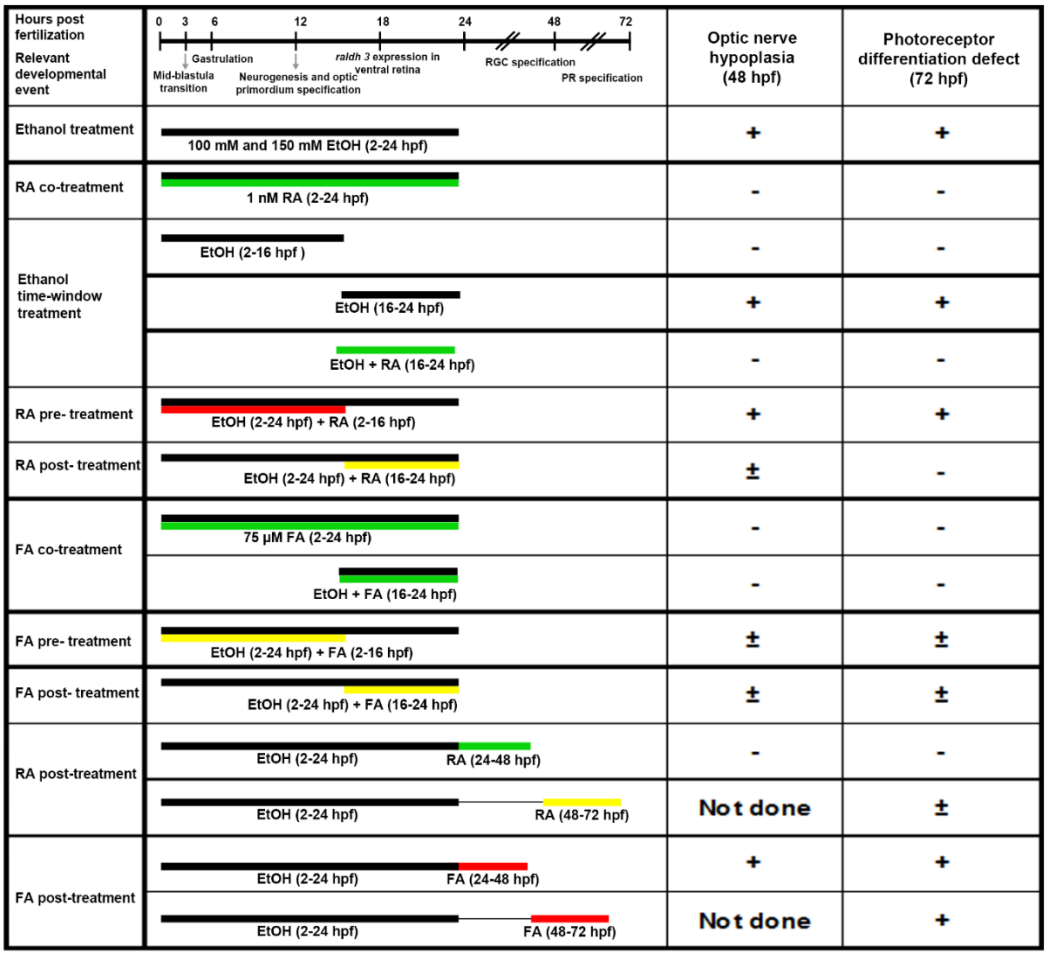

Figure 5.10 Schematic representation summarizing the various treatments.
The developmental timeline on the top shows numbers in hours post fertilization (not to scale). Relevant developmental events occurring during that time are highlighted. Black bars represent ethanol exposure. Green bars represent significant rescue of retinal defects, including ONH and photoreceptor differentiation defects. Yellow bars represent partial rescue of retinal defects. Red bars represent little or no rescue. Observed defect is indicated by positive sign (+); No observed defect is indicated by negative sign (-); and partial defect is indicated by (±). Not done, experiment was not done because the treatment period was after the required time for the assay.

5.6 Discussion

Rescue strategies designed in this study identified severe and persistent disruption of RA biosynthesis and signaling by ethanol exposure. Experiments tested the effect of nutritional

supplements such as RA and FA during and after ethanol exposure. Significantly, this is the first study showing significant rescue of ethanol-induced retinal morphogenesis and differentiation defects using FA. Our experiments highlight the potential influence of nutritional status on teratogen exposure effects.

Significantly, RA and FA co-supplementation could rescue retinal defects during this early retinal morphogenesis period. FA co-supplementation was more effective than RA, even during this critical time window (16-24 hpf) that coincides with RA biosynthesis when *raldh3* expression is initiated. One-carbon metabolism and antioxidant functions of FA may protect the retina from developmental defects during the early retinal morphogenesis time window. Additional experiments will be needed to identify common developmental mechanisms protected by RA and FA supplements.

5.6.1 Effect of ethanol on RA signaling during retinal development

Several investigators proposed inhibitory effects of ethanol on RA biosynthesis. Experiments using *Xenopus* embryos showed phenotypic similarities between ethanol treatments and RA signaling inhibitors or *raldh* gene knockdown (Yelin et al, 2005). Ethanol exposure also produced downregulation of RA-responsive *hox* gene expression and early limb genes (Johnson et al, 2007; Kot-Leibovich & Fainsod, 2009). Rescue of many craniofacial features and cardiac defects by RA supplementation in zebrafish strongly suggests that RA deficiency is induced by ethanol exposure in developing embryo (Marrs et al, 2010; Sarmah & Marrs, 2013).

The present study demonstrates rescue of ethanol-induced retinal defects by co-supplementing RA at low concentrations. If RA synthesis inhibition was partially responsible for the FASD phenotype, then ethanol sensitive developmental stages should coincide with the onset of RA signaling in the retina at 18 hpf. Indeed, a critical time window for ethanol sensitive retinal defects coincides with RA signaling (16-24 hpf). RA rescue of photoreceptor differentiation and optic nerve defects during 16-24 hpf critical time window showed that ethanol-induced defects were masked by RA supplement. However, the retinal defects induced by ethanol were not limited to this developmental period. Treating embryos with ethanol from 2-24 hpf and co-supplementing with RA

during 16-24 hpf did not rescue all ethanol-induced defects, indicating retinal developmental pathways other than RA signaling were also affected by ethanol. Persistent RA deficiency and signaling was indicated by rescue of optic nerve defects by RA treatment (24-48 hpf) after ethanol treatment from 2-24 hpf, and rescue of photoreceptor differentiation defects by RA treatment (24-48 and 48-72 hpf) after ethanol treatment from 2-24 hpf.

RA signaling influences photoreceptor differentiation and patterning in the retina. Studies showed that exogenous RA promoted rods and red cones and decreased blue and UV cones without significantly affecting green cones, and vice versa when RA signaling was inhibited using specific chemical inhibitors (Hyatt et al, 1996a; Prabhudesai et al, 2005). Thus, opsin expression patterns after ethanol treatment, showing downregulation of rhodopsin and red opsin and upregulation of UV opsin expression, indicate that ethanol treatment produced decreased RA levels during photoreceptor differentiation. Restoration of red-green double cones by RA supplementation at later stages (24-48 and 48-72 hpf) suggests persistent reduction in RA signaling due to ethanol exposure during early stages (2-24 hpf). This reveals a possibility of reducing the severity of retinal defects caused by ethanol exposure by ending ethanol intake and improving nutrition during later stages of development.

5.6.2 Effect of RA supplementation on retinal photoreceptor progenitor and precursor cells

A recent study on zebrafish embryos showed that ethanol exposure prevented cell cycle exit in the retina (Chung et al, 2013). Photoreceptor differentiation after exogenous RA application indicates that there are persistent precursor and progenitor cells in the ethanol-treated embryo retina that can differentiate. The differentiation status of these precursors still needs to be defined. However, presence of RA is sufficient to induce their terminal differentiation. Together, these data indicate an increased number of precursor cells in the ONL and INL lacking appropriate signals for terminal differentiation. Abnormal terminal differentiation may lead to the increased apoptosis observed at 40 hpf. Our results provide evidence that ethanol induced persistent defects in retinal stem and progenitor cell

populations, which prevent complete regeneration of normal retinal tissue. RA may be an important missing signal, supplementation of which leads to restoration of photoreceptor differentiation. Interestingly, RA supplementation during the period when increased apoptosis was observed (24-48 hpf) could restore retinal defects to near normal levels in comparison to later supplementation (48-72 hpf).

5.6.3 Preventive role of FA in FASD

Roles for FA in 1-carbon metabolism as a cofactor for methyl-group transfers and its antioxidant properties function in a wide range of developmental processes. In the present study, FA co-supplementation with ethanol during 2-24 hpf and 16-24 hpf rescued retinal defects. FA supplementation (2-16 and 16-24 hpf) during ethanol exposure (2-24 hpf) could partially rescue ethanol-induced defects. Similar experiments with RA showed no rescue during 2-16 hpf and partial rescue from 16-24 hpf, potentially due to activation of RA biosynthesis starting at 18 hpf. Unlike RA supplementation, FA rescue was effective during 2-16 hpf and 16-24 hpf time windows. This indicates that rescue mechanisms underlying RA and FA are different. FA supplementation (24-48 hpf or 48-72 hpf) following ethanol treatments (2-24 hpf) did not rescue retinal defects, showing that in contrast to RA supplementation, FA supplementation could protect retinal morphogenesis only during ethanol treatment. Although the molecular details underlying FA rescue is unclear, exogenous FA may prevent abnormal epigenetic processes and reactive oxygen species generation disrupted due to ethanol exposure (Brocardo et al, 2011; Zhou et al, 2011a). Additional studies will be required to dissect the differences between RA and FA supplementation and explore the potential protective and therapeutic benefits.

Previous study from our laboratory showed prevention of cardiac defects induced by ethanol exposure by FA co-supplementation (Sarmah & Marrs, 2013). FA co-supplementation rescued defects in both myocardium and endocardium, whereas RA co-supplementation with ethanol did not rescue endocardial cushion formation defects. During eye development, where RA plays a critical role in the differentiation and maturation of photoreceptors, RA was able to remedy retinal developmental defects even after ethanol exposure ended. These findings highlight organ-to-organ differences in ethanol-sensitive

signaling and timing, illustrating complexity of the underlying molecular basis of FASD. Despite this complexity, nutritional status heavily influences birth defect type and severity.

5.7 Conclusion

Apart from identifying specific ethanol-induced retinal defects. Our experiments also reveal therapeutic potential of specific molecules that stimulate developmental signaling pathways. These nutritional compound supplementation experiments also shed light on the influence of maternal nutritional background on embryonic development and prevention of birth defects.

CHAPTER 6. ETHANOL EFFECTS ON RETINAL STEM CELL POPULATIONS

6.1 Introduction

As discussed earlier, vertebrate retina comprises of six main cells types including, retinal ganglion cells (RGCs), bipolar cells, amacrine cells, horizontal cells, photoreceptors, and the major glial cell type, Müller glial cells (MGCs). Different retinal cells are born progressively as development proceeds: commencing with RGCs and ending with bipolar cells, rods, and MGCs. Retinal precursors exit cell cycle periodically and sequentially, as various signaling pathways regulate neurogenesis and gliogenesis (Cepko, 2014; Cepko, 1999; Harris, 1997). In zebrafish, retinal growth continues radially throughout the life of the fish. Specific cell populations have been identified as the multipotent retinal stem cells residing in the ciliary marginal zone (CMZ) (Centanin et al, 2011; Stenkamp, 2007). Cells from the CMZ can give rise to most retinal cell types including MGCs. MGCs, in turn divide asymmetrically in the inner nuclear layer (INL) to produce rod precursor, which rapidly divide to form neurogenic cluster. These rod progenitor cells migrate to outer nuclear layer (ONL) and terminally differentiate to form rod photoreceptors (Nelson et al, 2008; Raymond et al, 1995; Raymond & Rivlin, 1987).

Retinal development is extremely sensitive to teratogen exposure (Tandon & Mulvihill, 2009). Previous studies using zebrafish as model for fetal alcohol spectrum disorder (FASD) showed that embryos treated with ethanol during early gastrulation through somitogenesis (2-24 hours post-fertilization, hpf) show persistent and severe retinal cell differentiation defects (Muralidharan et al, 2015). Ethanol exposure caused reduced photoreceptor terminal differentiation marker expression and optic nerve hypoplasia in comparison to control embryos. Mature rod photoreceptors that arise from MGCs are almost absent. Cell differentiation initiation was minimally affected but small eye phenotype largely visible by 48 hpf indicating that the central retinal neurogenesis is complete and the cells arising

from the CMZ are largely affected. Specific laminae of the retina including the CMZ, INL and ONL showed increased proliferation in the ethanol treated embryos. Ethanol-induced cell cycle exit failure has been implicated in the retina and CNS. (Chung et al, 2013). Also, RA and FA co-supplementation with ethanol during early gastrulation and somitogenesis rescued photoreceptor differentiation defects and optic nerve hypoplasia (Muralidharan et al, 2015). The cellular and molecular mechanism underlying rescue by RA and FA supplementation remains unclear.

Here, we dissect ethanol effects on retinal stem cell compartments and various signaling pathways, in order to understand the genesis of persistent ethanol-induced retinal defects. Ethanol exposure altered gene expression patterns in the CMZ. We identify ethanol-induced Wnt signaling disruption in the retinal CMZ as a crucial mechanism underlying ethanol-induced retinal cell differentiation defects. This evidence highlights the critical role of Wnt signaling in FASD eye defects and points to identifying therapeutic targets for this devastating birth defect.

6.2 Ethanol-induced expansion of the cells in the CMZ

Zebrafish embryos were treated from 2-24 hpf with 100 and 150 mM ethanol (0.6 and 0.9% v/v, respectively). Studies show that zebrafish embryos treated with ethanol concentration as high as 166.67 mM (1% v/v) from 6-24 hpf, have an equilibrated ethanol concentration inside the embryo that ranges from 40-58 mM (Lovely et al, 2014). As previously shown, 100 and 150 mM ethanol-treated embryos display severe retinal cell differentiation defects (Muralidharan et al, 2015). Ethanol-induced differences in the CMZ composition was examined using specific markers labeling multipotent retinal stem and progenitor cells in the CMZ.

Histological analysis at 4 dpf and 18 dpf indicates a persistent microphthalmia in ethanol treated embryos (Fig 6.1 A-C, 6.2 A-F). At these later stages, ethanol treated embryos also show persistent changes in the CMZ relative to the controls. In the 4 dpf H&E stained sections, CMZ cells in ethanol treated embryos also showed distinct nuclear morphological differences as compared to controls. The compact nature of the nuclei in the control CMZ was not as evident in the ethanol treated embryos. Consequently, CMZ area in proportion

to the retinal area showed a significant increase in comparison to control embryos (Fig 6.3 D). This is more evident in the schematic drawn to scale showing the ventral half of the retina, which showed an expansion of the CMZ in comparison to neural retina at 4 dpf (Fig 6.3A-C).

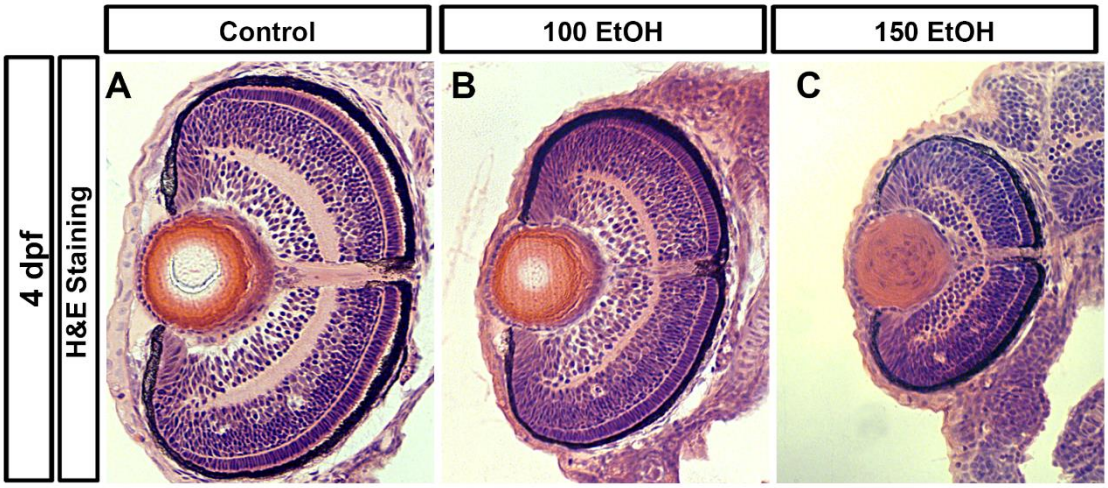

Figure 6.1 Histology of ethanol treated embryos at 4 dpf.
(A-C) H&E staining of control (A), 100 mM Ethanol (B), and 150 mM ethanol treated embryos show persistent defects at 4 dpf.

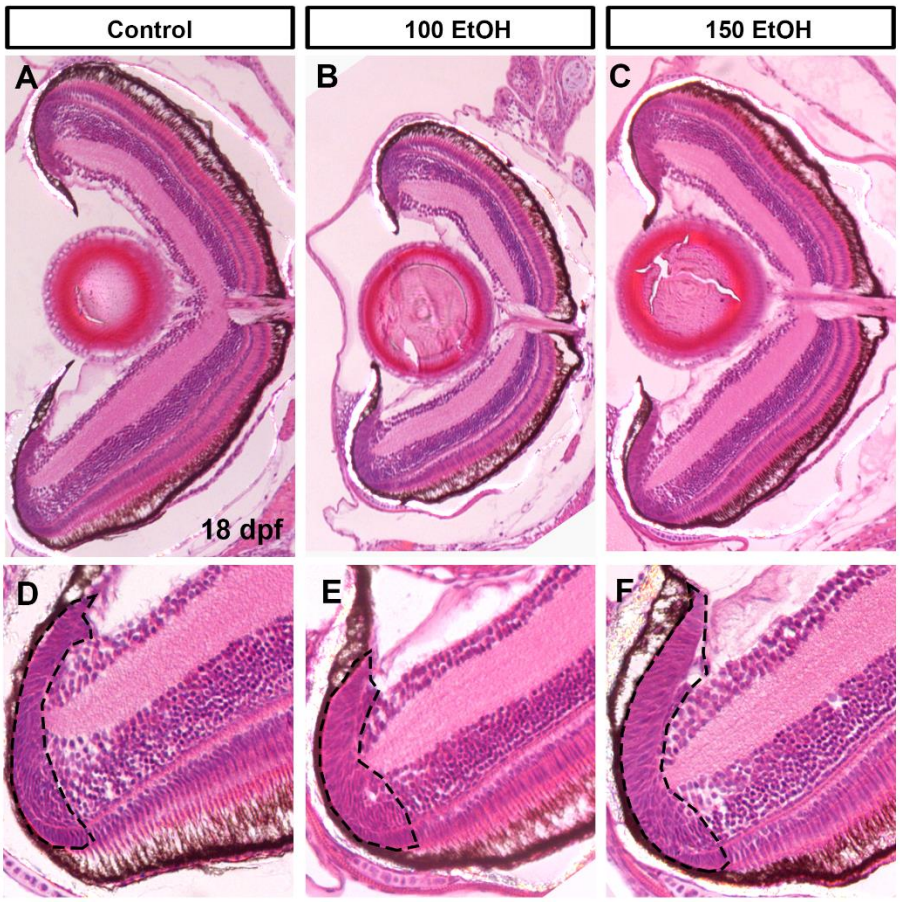

Figure 6.2 Histology of ethanol treated embryos at 18 dpf.
(A-C) H&E staining of control (A), 100 mM Ethanol (B), and 150 mM (C) ethanol treated embryos show persistent defects at 18 dpf. (D-F) Magnified view of the CMZ in the control (D) and the ethanol treated embryos (E, F) showing slightly expanded CMZ. Dashed lines indicate CMZ demarcated by lamination.

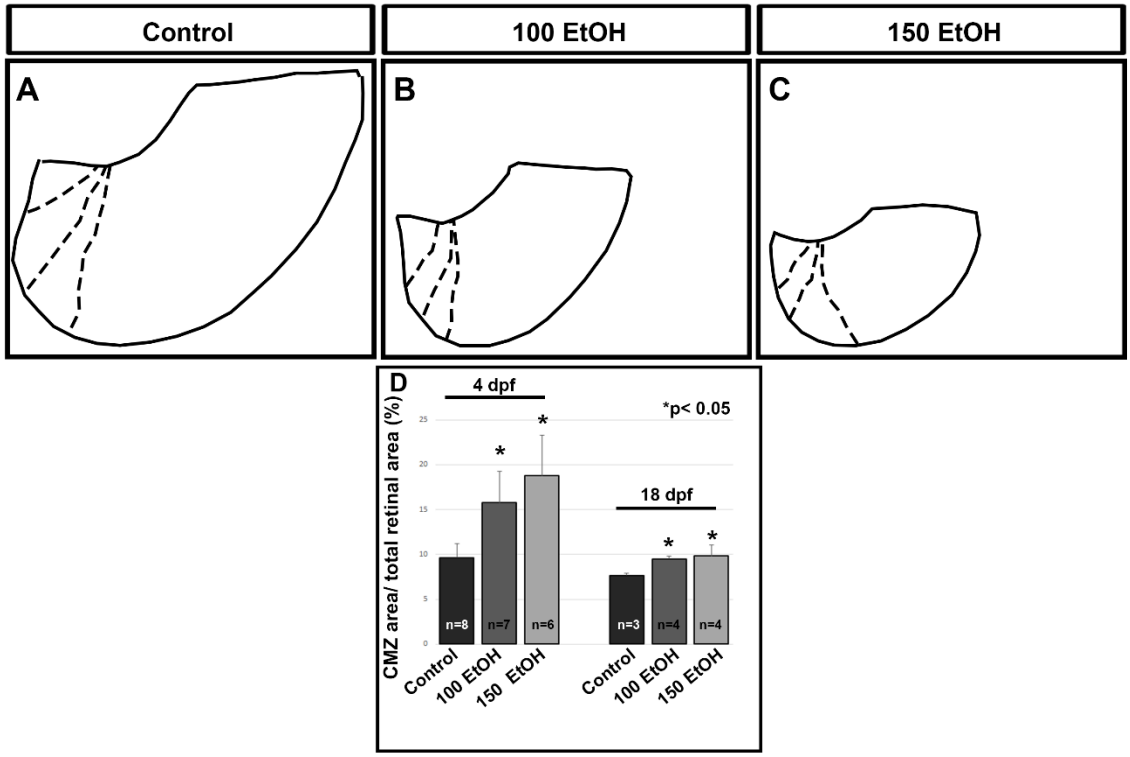

Figure 6.3 Schematic representation of the CMZ.
(A-C) Schematic drawn of the ventral half of the retina in proportion to the histology sections at 4 dpf. The solid line indicates the retinal area ending at the optic nerve and the dashed lines indicate the various parts of the CMZ as can be distinguished by the nuclear morphology. (D) The ratio of the CMZ area to the retinal area showing an increase in CMZ in proportion to the retina persistently at 4 and 18 dpf. Error bars indicate standard deviation.

In order to dissect the cellular changes in the CMZ, specific gene expression patterns were examined using immunostaining, ISH, and qPCR. Alcama, a neuroepithelial marker labeling the multipotent retinal stem cells and RGCs in the retina, staining at 48 hpf showed an expanded expression domain in the CMZ cells after ethanol treatment (Fig 6.4 A-C). *rx1* expression, which is mostly restricted to multipotent retinal stem cell populations in the CMZ at 48 hpf, was analyzed using ISH. Ethanol exposure lead to reduced *rx1* expression domain at 48 hpf (Fig 6.4 D-F). Cyclin dependent kinase inhibitor (*cdkn1c* or $p57^{kip2}$; a cell cycle exit marker) expression in control embryos is restricted to central CMZ which express cell cycle exit markers was greatly reduced after ethanol treatment (Fig 6.4 G-I). This was also reflected in the quantitative PCR analyses for *rx1* and $p57^{kip2}$, which

showed a significant decrease in transcript levels at 72 hpf in ethanol treated embryos (Fig 6.4J). These results show an altered composition of the CMZ.

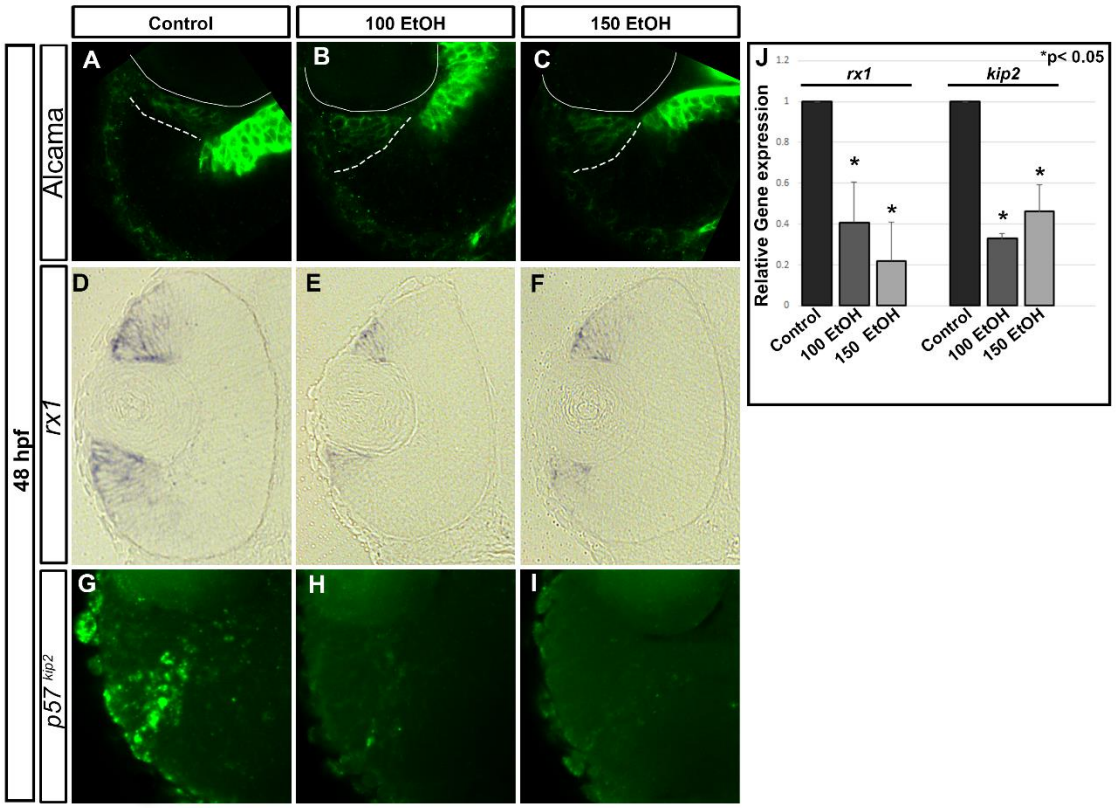

Figure 6.4 Ethanol exposure altered CMZ composition.
(A-C) Alcama staining of ethanol treated embryos (B, C) showed expansion in comparison to control embryos (dashed lines, A) at 48 hpf in peripheral CMZ. (D-F) *rx1* ISH sections showed reduced expression after ethanol treatment in peripheral CMZ. (G-I) *p57 kip2* FISH experiment showed reduced expression in central CMZ. (J) qPCR showed reduced transcript levels of *rx1* and *p57kip2* genes at 72 hpf after ethanol treatment in comparison to control retinas. Error bars indicate standard deviation.

6.3 Ethanol exposure arrests cells in precursor state

Notch signaling regulated neuronal vs glial fates during retinal development (Furukawa et al, 2000; Gaiano & Fishell, 2002; Vetter & Moore, 2001). Differentiating MGCs also express Gfap (Bernardos et al, 2007). To examine ethanol-induced MGC defects, double

transgenic line, *Tg(TP1:mCherry)*, expressed in Notch active cells, and *Tg(gfap:GFP)*, expressing GFP under GFAP promoter were used. At 72 hpf, GFP expression and active Notch signaling can be seen in central retina of control embryos (Fig 6.5A). However, after ethanol exposure at 72 hpf, retinas show fewer GFP positive and fewer Notch-active cells, indicating reduced MGC differentiation (Fig 6.5 A-C).

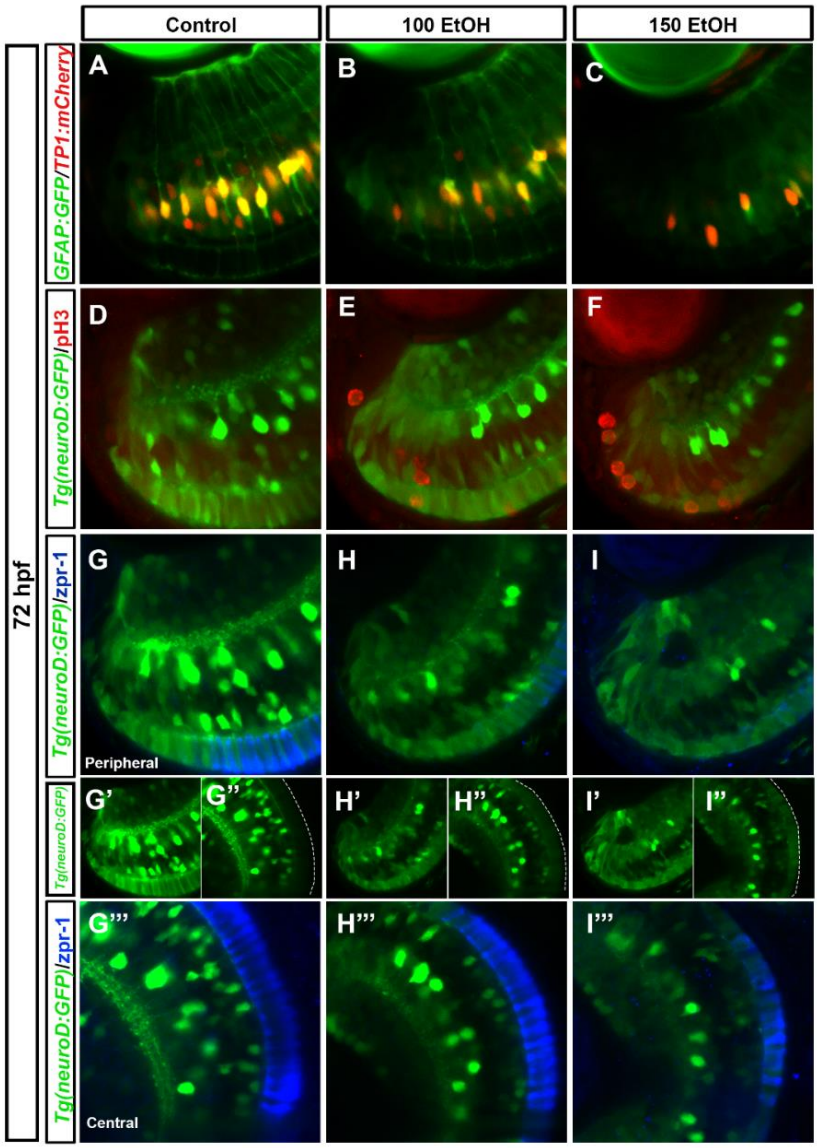

Figure 6.5 Effect of ethanol on MGC and central retinal precursor cell populations.
(A-C) Double transgenic line *Tg(gfap:GFP)* and *Tg(TP1:mCherry)* showed reduced notch-active and Gfap-positive cells after ethanol exposure at 72 hpf. (D-F) PhosphoH3 immunostaining on *Tg(nrd:GFP)* embryso showed an increased number of mitotically active cells in the ONL and INL many of which co-labeled with *neurod*- positive cells. (G-I’’’) zpr-1 immunostaining on *Tg(nrd:GFP)* embryos showed fewer zpr-1 positive cells in the peripheral retina after ethanol exposure.(G-I). In the central retina, zpr-1-positive cells were double labelled with *neurod* after ethanol exposure (G’’’-I’’’) compared to *Tg(nrd:GFP)* expression showing expanded *neurod* expression in the ONL (G’,G’’,H’,H’’, I’,I’’). White dashed lines indicate RPE.

Rod photoreceptor lineage arises from asymmetric MGC division in the INL, which migrate to the ONL. These rod precursors are proliferative and cells of the cone and rod lineage express *rx1*, *crx* and *neurod* (Ochocinska & Hitchcock, 2009; Stenkamp, 2011). To examine rod photoreceptor precursor cells, *Tg(nrd:GFP)*, embryos expressing GFP under *neurod* promoter, were stained using phosphoH3 antibody (Fig 6.5 D-F). Ethanol treated embryos showed many double positive embryos in the INL and ONL, indicating the increased number of proliferative neural precursor cells. In control embryos, nascent and immature photoreceptor cells in the ONL, in the central CMZ showed *neurod* expression, which transitioned into *neurod* and zpr-1 (detecting red-green double cones) double positive cells and finally mature photoreceptors are zpr-1 positive with minimal *neurod* expression. Unlike the control embryos, ethanol treated embryos showed cells in the central CMZ and ONL, that co-labeled for *neurod* and zpr-1, indicating that these poorly differentiated cells are immatures or nascent cones (Fig 6.5 G-I, G'''-I'''). Inset images show *neurod* expression throughout the ONL of ethanol treated embryos (Fig 6.5 H'-I', H''-I'').

Precursor and progenitor cell populations were examined using *crx* and *rx1* riboprobes for expression in the central retina after ethanol treatment (Fig 6.6 A-F). At 48 hpf, *crx* expression in the control embryos was mostly reduced from mature central retinal and present in the newly differentiated cells in the peripheral neural retina and ONL (Fig 6.6A). In ethanol exposed embryos, *crx* expression was seen throughout the central retina evenly through the central CMZ (Fig 6.6 B, C). Similarly, at 72 hpf, *rx1* expression pattern is restricted to the peripheral CMZ and newly differentiating photoreceptors and almost completely absent from the central retina (Fig 6.6D). Embryos treated with 100 mM ethanol showed *rx1* expression in the photoreceptor layer throughout the central retina indicating the presence of immature photoreceptors (Fig 6.6E). Embryos treated with 150 mM showed a smaller region of *rx1* expression in the central retinal indicating fewer photoreceptors precursors (Fig 6.6F). These results indicate that retinal cells remain in a precursor state and are incompletely differentiated after embryonic ethanol exposure.

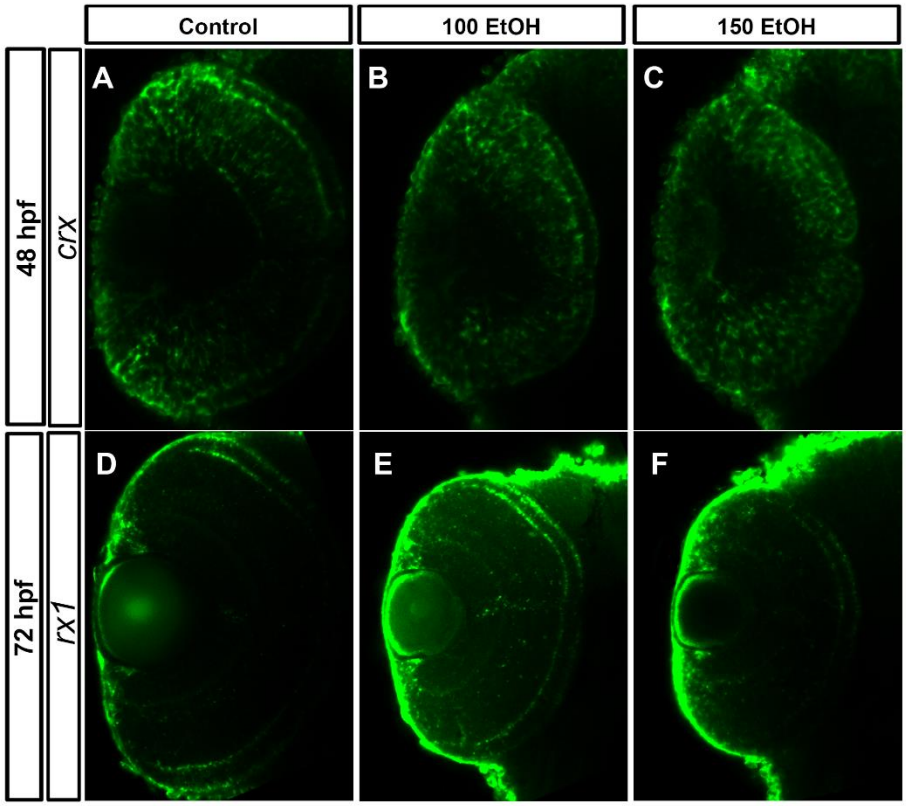

Figure 6.6 Ethanol induced altered expression of *crx* and *rx1* genes.
(A-C) *crx* expression stained using Fluorescent in situ hybridization was expanded in the central retina after ethanol treatment at 48 hpf. (D-F) *rx1* expression also showed an expansion in the ONL at 72 hpf due to ethanol exposure.

6.4 Ethanol exposure disrupted Wnt and other differentiation pathways

Wnt signaling plays a crucial role in the maintenance of retinal stem cell population (Meyers et al, 2012). Ethanol effects on Wnt, Notch and proneural signaling pathways involved in retinal stem cell maintenance and cell differentiation was examined using specific reporter lines, including *Tg(Tcf/Lef-miniP:dGFP)*, Wnt activity-reporter line; *Tg(TP1:GFP)*, *Tg(TP1:mCherry)*, Notch activity-reporter lines; and *Tg (nrd:GFP)*, proneural (*neurod*) gene expression transgenic line. Ethanol treatment from 2-24 hpf showed minimal difference in Wnt-active cells in the retinal at 24 hpf. (Fig 6.7 A-C). At 48 hpf, peripheral CMZ contains Wnt active cells in untreated retains. Most Wnt-active

cells are also Alcama-positive. However, few cells are Wnt-negative and Alcama-positive. After ethanol exposure, fewer Wnt-active cells were detected in the CMZ. Not only were there fewer Wnt/Alcama double positive cells in ethanol-treated embryos, but the total number of Alcama-positive cells in the CMZ was significantly higher than control embryos (Fig 6.8 A-D).

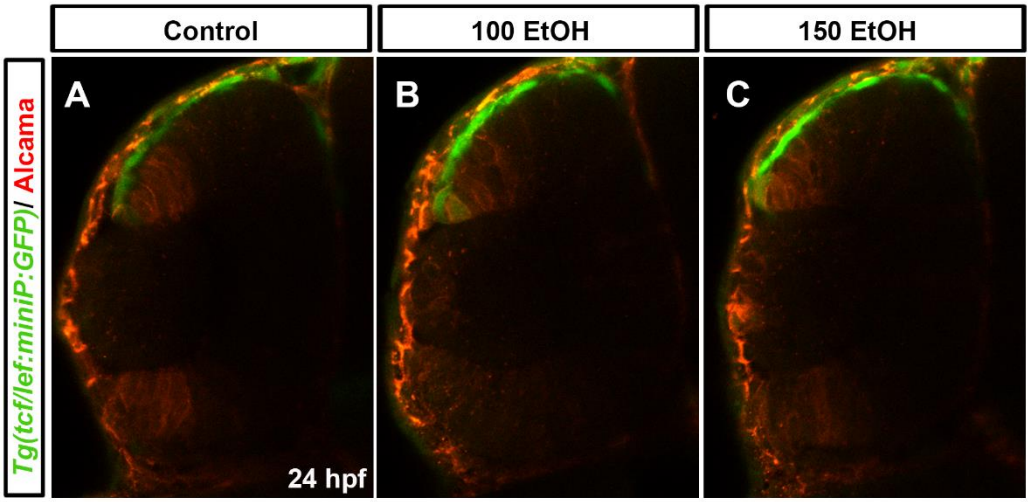

Figure 6.7 Ethanol exposure showed minimal effect on Wnt signaling at 24 hpf.
(A-C) Wnt-active cells in the dorsal CMZ of the retina in *Tg(Tcf/Lef-miniP:dGFP)* embryos showed minimal difference in ethanol treated embryos in comparison to controls at 24 hpf.

This finding was also validated by *axin2* ISH, which is a downstream target of Wnt signaling. In control embryos, *axin2* positive cells can be observed in the peripheral CMZ. However, this *axin2* expression was extremely reduced after ethanol treatment (Fig 6.8 E-G). *axin2* gene expression levels in the retina using qPCR showed a significant decrease ($p<0.05$) after ethanol treatment (100 mM ethanol and 150 mM ethanol- treated embryos showed 0.294 and 0.562- fold change respectively). This shows that ethanol exposure led to reduced Wnt reporter activity, Wnt target gene expression, and more cells exhibit neuroepithelial properties in the CMZ.

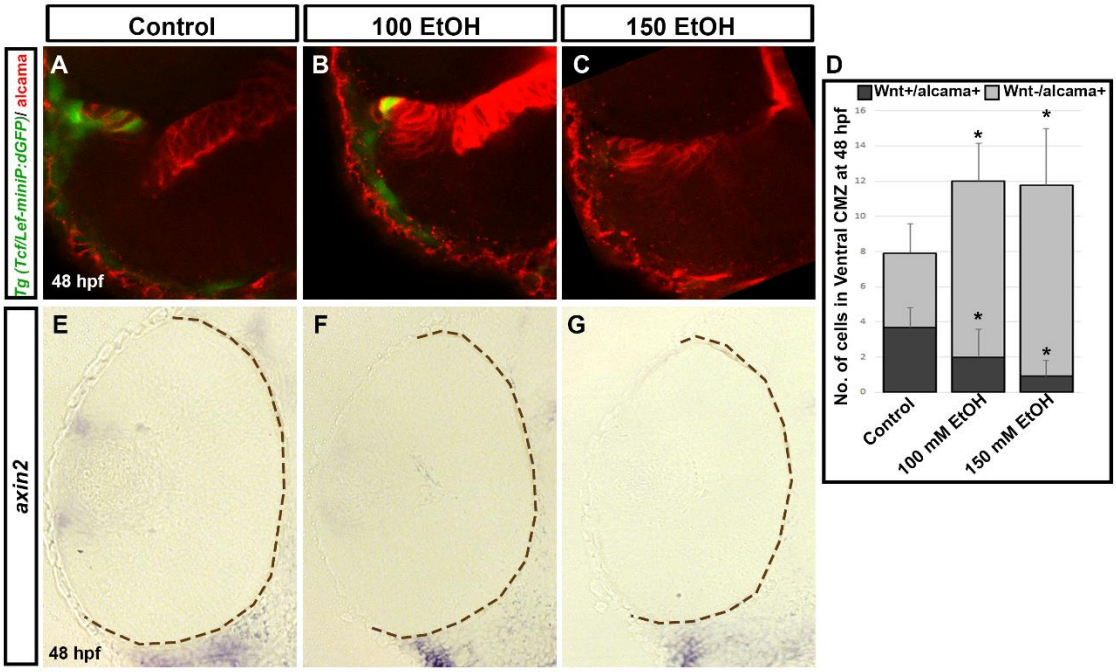

Figure 6.8 Effect of ethanol exposure on Wnt signaling at 48 hpf.
(A-C) *Tg(Tcf/Lef-miniP:dGFP)* embryos showed active Wnt-active cells (green) in the peripheral CMZ labelled with Alcama (red). (D) Quantification of Wnt+/Alcama+ and Wnt-/Alcama + cells in the peripheral CMZ. (E-G) *axin2* ISH showed decreased *axin2* expression in the peripheral CMZ after ethanol exposure. Dashed lines indicate RPE. Error bars indicate standard deviation.

Our working model suggests that Notch signaling is downstream of Wnt signaling in the CMZ. Using transgenic Notch reporter line, Notch activity was examined after ethanol exposure. Ethanol exposure caused reduced Notch-activity at 48 and 72 hpf in comparison to controls (Fig 6.9 A-C; G-I). The number of cells at 72 hpf was significantly fewer in comparison to control embryos (Fig 6.9M). However, in the central CMZ, ethanol treated embryos showed increased number of Notch-active cells (Fig 6.9 J-L). *her6*, a downstream target of Notch signaling, showed that is expressed in the CMZ in a compact layer in the control embryos was expanded in 100 and 150 mM ethanol treated embryos (Fig 6.9 D-F). The proneural gene, *neurod*, expression also showed differences after ethanol exposure. At 48 hpf and 72 hpf, bright *neurod*-positive cells in the INL, which are a subset of differentiated amacrine cells, could be seen in control embryos (Ochocinska & Hitchcock,

2009). The ethanol treated retinas showed fewer *neurod*-positive cells in the INL (Fig 6.10 A-F). *neurod*-positive cells in the ONL seemed to be expanded in ethanol treated embryos compared to controls (as discussed earlier) (Fig 6.5 G-I; Fig 6.10 D-F). Similar to Notch signaling, *neurod* expression showed an expansion in the central CMZ of the ethanol treated embryos further emphasizing proneural and immature nature of these cells (Fig 6.10 G-I). TO-PRO-3 stained image of the CMZ at 72 hpf was used to demarcate the CMZ area, the unlaminated portion of the peripheral retina (Fig 6.9N).

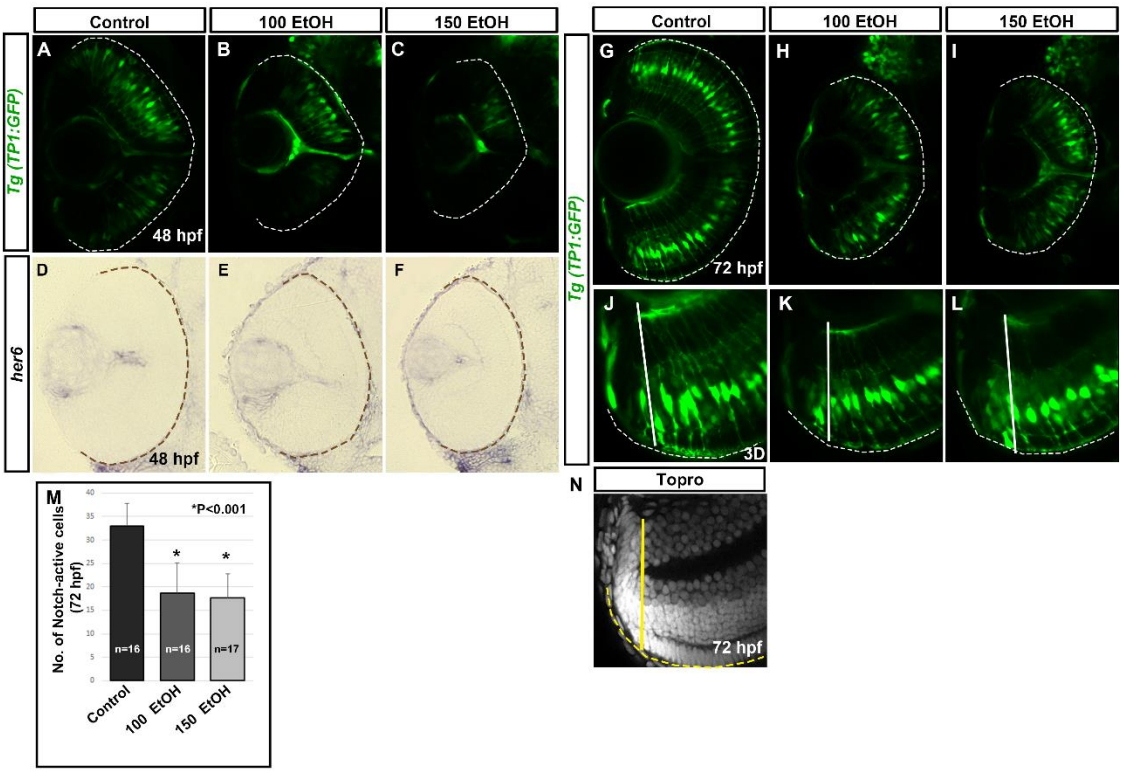

Figure 6.9 Ethanol effects on Notch signaling.
(A-C) *Tg(TP1:GFP)* embryos showed reduced Notch activity after ethanol exposure at 48 hpf. (D-F) *her6* expression in the CMZ showed an expansion in expression domain after 100 mM ethanol exposure (E), and reduced expression after 150 mM ethanol exposure (F). (G-I) *Tg(TP1:GFP)* embryos showed reduced expression after ethanol exposure at 72 hpf. (J-L) Notch-active cells in central CMZ were increased after ethanol exposure. (M) Quantification of Notch-active cells using *Tg(TP1:mCherry)* showed a significant decrease after ethanol treatment in comparison to control embryos. Solid lines demarcate the CMZ from the neural retina based on retinal lamination using TO-PRO-3 staining as indicated (N, yellow). Dashed lines (white, brown and yellow) indicate RPE.

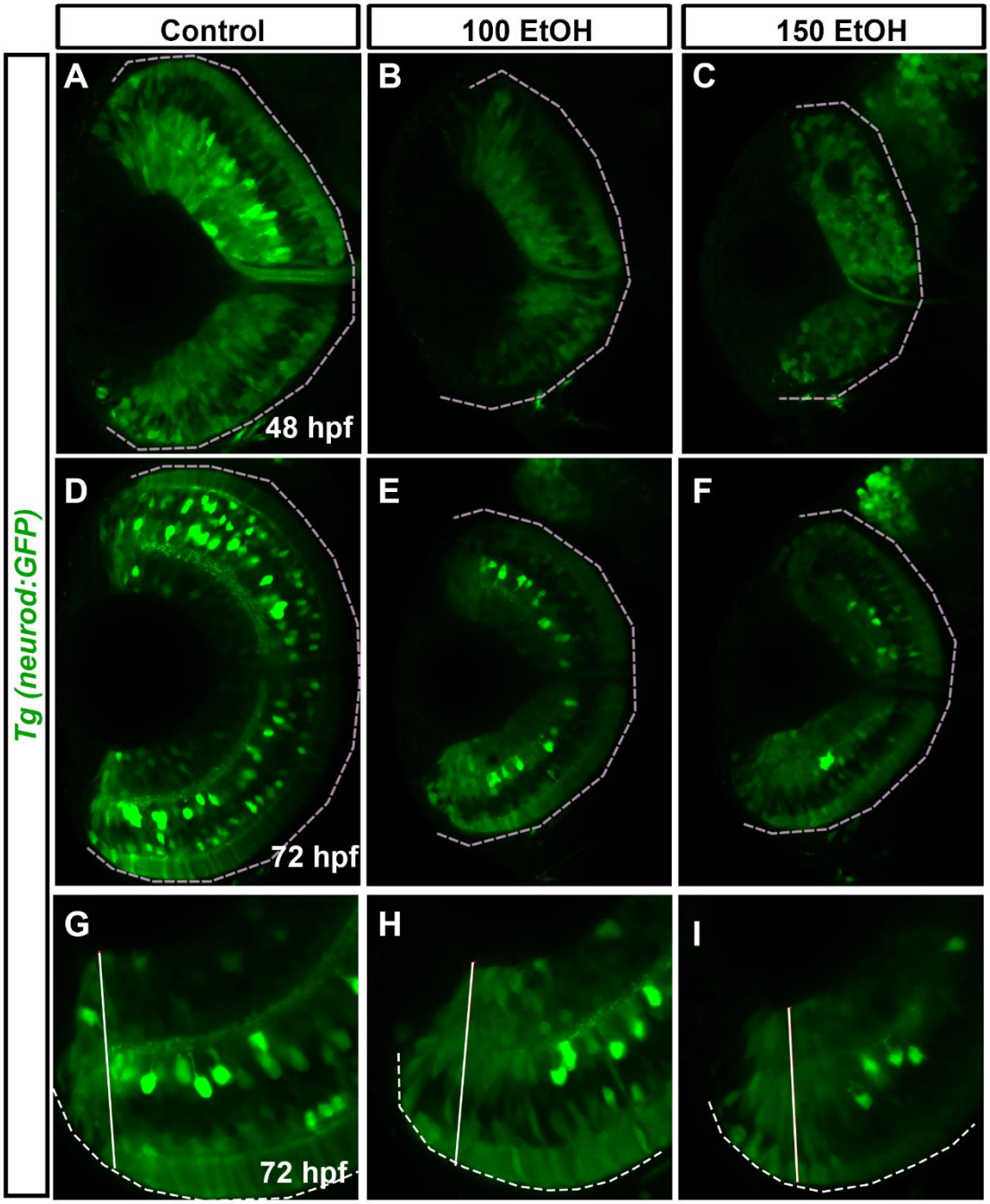

Figure 6.10 Ethanol effects on *neurod* expression.
(A-F) *Tg(nrd:GFP)* embryos showed reduced *neurod* expression after ethanol exposure at 48 hpf (A-C) and 72 hpf (D-F). (G-I) Central CMZ region showed increased *neurod* positive cells after ethanol treatment at 72 hpf. Solid lines demarcate the CMZ from the neural retina based on retinal lamination using TO-PRO-3 staining as indicated (Fig 6.9N, yellow). White dashed lines indicate RPE.

6.5 RA and FA co-supplementation rescue Wnt signaling in the CMZ compartment

Previous studies showed that RA and FA co-supplementation with ethanol rescues eye phenotypes produced by ethanol exposure. We hypothesize that these rescue treatments will restore Wnt activity in the CMZ, which ultimately rescues the ethanol-induced retinal cell differentiation defects. Embryos co-supplemented with 1 nM RA or 75 µM FA with ethanol from 2-24 hpf were tested for various markers for the CMZ and precursor cells. Staining with Alcama showed that ethanol exposure caused expanded Alcama expression domain, which could by rescued by RA and FA co-supplementation (Fig 6.11 A-J). In order to examine the effect of RA or FA treatments on Wnt signaling in the CMZ, *Tg(Tcf/lef-miniP:dGFP)* embryos were co-supplemented with RA/FA and ethanol. Both RA and FA co-supplementation could rescue Wnt-active cells in the peripheral CMZ. The number of Wnt/Alcama double positive cells in the peripheral CMZ was significantly reduced after ethanol treatment which could be rescued by RA and FA co-supplementation (Fig 6.11 J). However, 150 mM ethanol and FA treated embryos did not show quite as much rescue. The number of Wnt-/Alcama+ cells showed a significant increase after ethanol treatment which were also rescued by RA and FA co-supplementation.

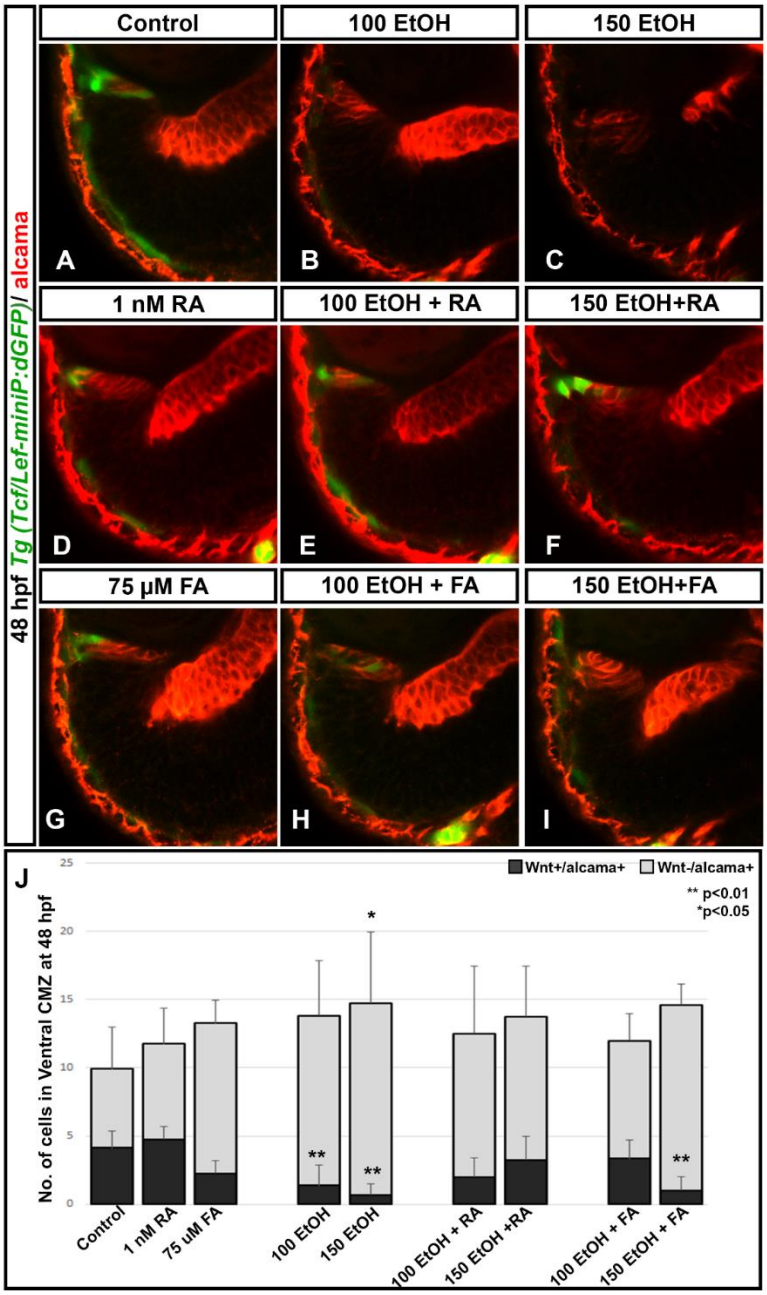

Figure 6.11 RA and FA co-supplementation rescued Wnt signaling.
(A-J) Alcama staining on *Tg(Tcf/lef-miniP:dGFP)* embryos showed that ethanol treatment reduced the number of Wnt+/Alcama+ cells in the peripheral CMZ (B, C); RA co-supplement could rescue Wnt+/Alcama+ cells (D-F); FA co-supplementation could also rescue Wnt+/Alcama+ cells in the CMZ particularly in 100 mM Ethanol + FA treated embryos (G-I). (J) Quantification of Wnt+/Alcama+ cells in the peripheral CMZ showed significant reduction after ethanol treatment which was rescued by RA and FA co-supplementation. Error bars indicate standard deviation.

6.6 RA or FA co-supplementation rescue downstream Notch activity in ethanol exposed retinas

As noted earlier, ethanol treated embryos showed fewer Notch active cells in the retina at 72 hpf. RA or FA co-supplementation of Notch reporter (*Tg(TP1:mCherry)*) embryos showed rescue of the Notch-active cells to control levels in the retina. Notch-active cells were significantly fewer after ethanol treatment, which could be rescued after RA and FA co-supplementation (Fig 6.12 A-J).

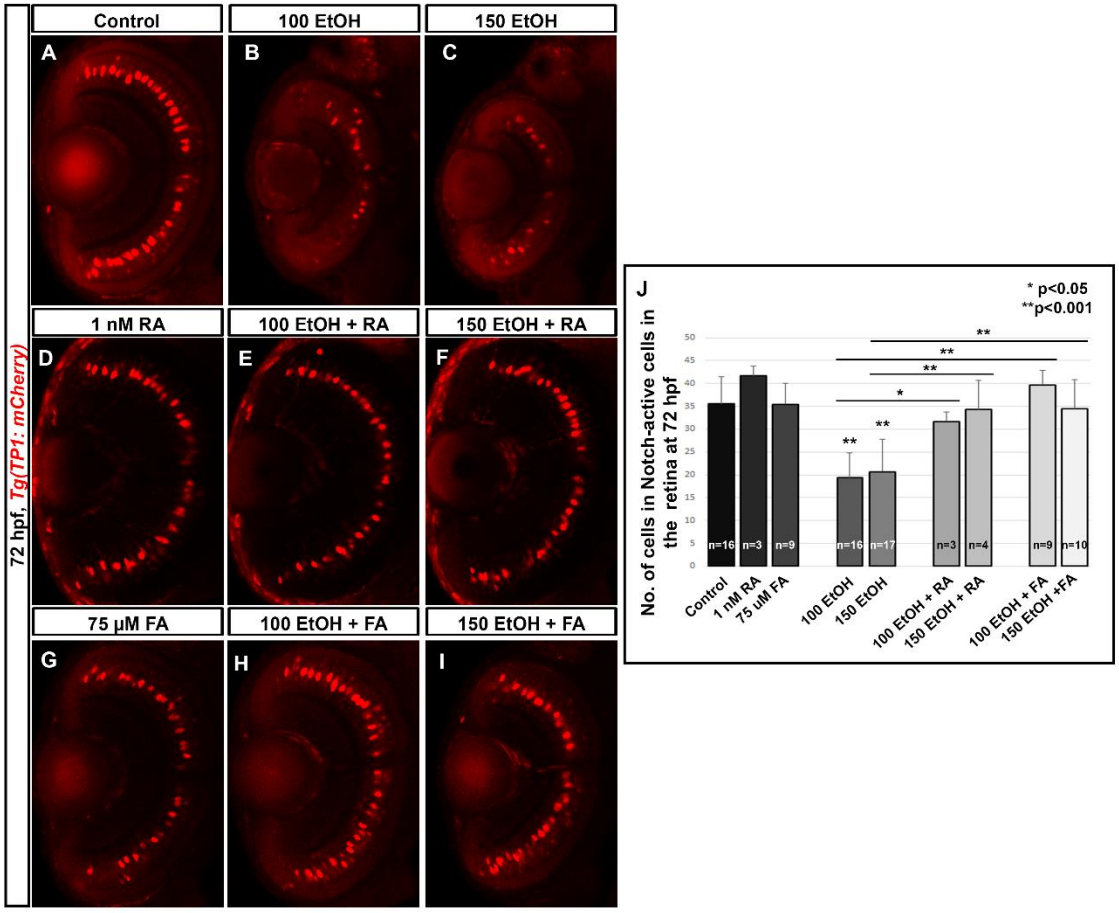

Figure 6.12 RA and FA co-supplementation rescued Notch signaling.
(A-J) RA and FA co-supplementation on *Tg(TP1:mCherry)* embryos showed rescue of Notch signaling. Ethanol treated embryos showed reduced notch –activity (red, B, C) which was restored by RA (D-F), and FA (G-I) co-supplementation. (J) Quantification of mCherry-positive (Notch-active cells) in the retina showed a significant rescue of notch signaling after both RA and FA co-supplementation. Error bars indicate standard deviation.

6.7 RA co-supplementation induced proliferation in ethanol treated embryos

Examination of cell proliferation using phosphoH3 antibody staining showed that ethanol-induced increase in proliferation at 72 hpf localized to the CMZ, ONL and INL of the retina (Fig 6.13 A-J). RA co-supplementation at 72 hpf showed a significant increase cell proliferation over the ethanol treated embryos particularly in the CMZ. Only RA supplemented embryos showed a significant increase in cell proliferation in the retina, particularly in the CMZ retinal cells. However, FA co-supplementation showed a rescue of the ethanol-induced cell proliferation. FA co-supplementation could rescue the ONL and INL cell proliferation levels but the number of mitotic cells in the CMZ remained similar to ethanol treated retinas. This suggests different mechanisms underlying RA and FA rescue. RA co-supplementation may induce proliferation of the precursor and progenitor cell populations in the central retina and the middle CMZ.

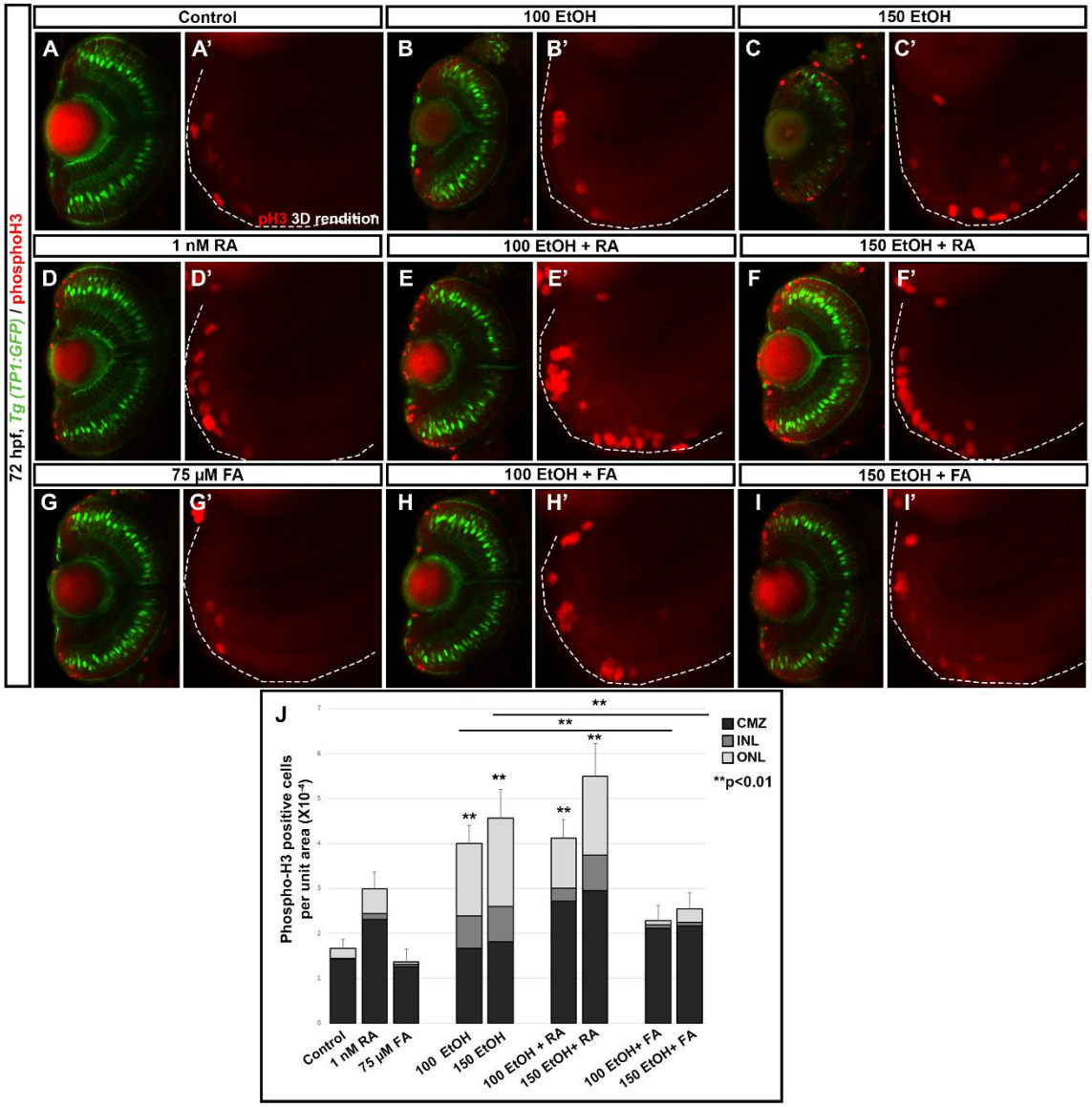

Figure 6.13 RA, but not FA, co-supplementation induced proliferation.
(A-I) phosphoH3 (red) staining of *Tg(TP1:gfp),* (green cells) embryos showed that ethanol exposure induced proliferation in the CMZ, INL and ONL. (A'-I') 3D rendition of phosphoH3 staining in the peripheral retinal region showed the increase in proliferation in ethanol treated embryos (B', C') which was increased after RA treatment (D',E', F') and rescued after FA co-supplementation (G', H', I'). (J) Quantification of phosphoH3-positive cells per unit area at 72 hpf showed statistically significant increase in phosphoH3-positive cells in after ethanol treatment which was rescued after FA co-supplementation. Dashed lines indicate RPE. Error bars indicate standard deviation.

6.8 Wnt activation after ethanol exposure restores downstream signaling and differentiation:

Due to the peripheral location of Wnt-signaling and their being a minority of cells in the stem cell compartment, we hypothesize that Wnt-signaling defects induced by ethanol exposure are responsible for persistent retina defects. To test this hypothesis, ethanol treated and untreated embryos exposed to Wnt agonist (LSN 2105786) were tested for rescue of Notch, proliferation, and photoreceptor differentiation. Since much of the difference in Wnt signaling was seen after 24 hpf, embryos were treated with 350 and 500 nM LSN 2105786 from 32-48 hpf. LSN 2105786 treatment could rescue the Wnt-activity in the CMZ (Fig 6.14 A-F). Quantification of Wnt/Alcama double positive cells in the CMZ of the treated retina showed that, in comparison to controls, ethanol treatment showed significantly reduced number of Wnt/Alcama double positive cells and increased Wnt-/Alcama+ cells. LSN 2105786 treatment could rescue the Wnt+/Alcama+ cells but did not rescue the Wnt-/Alcama+ cells to control levels (Fig 6.14 G).

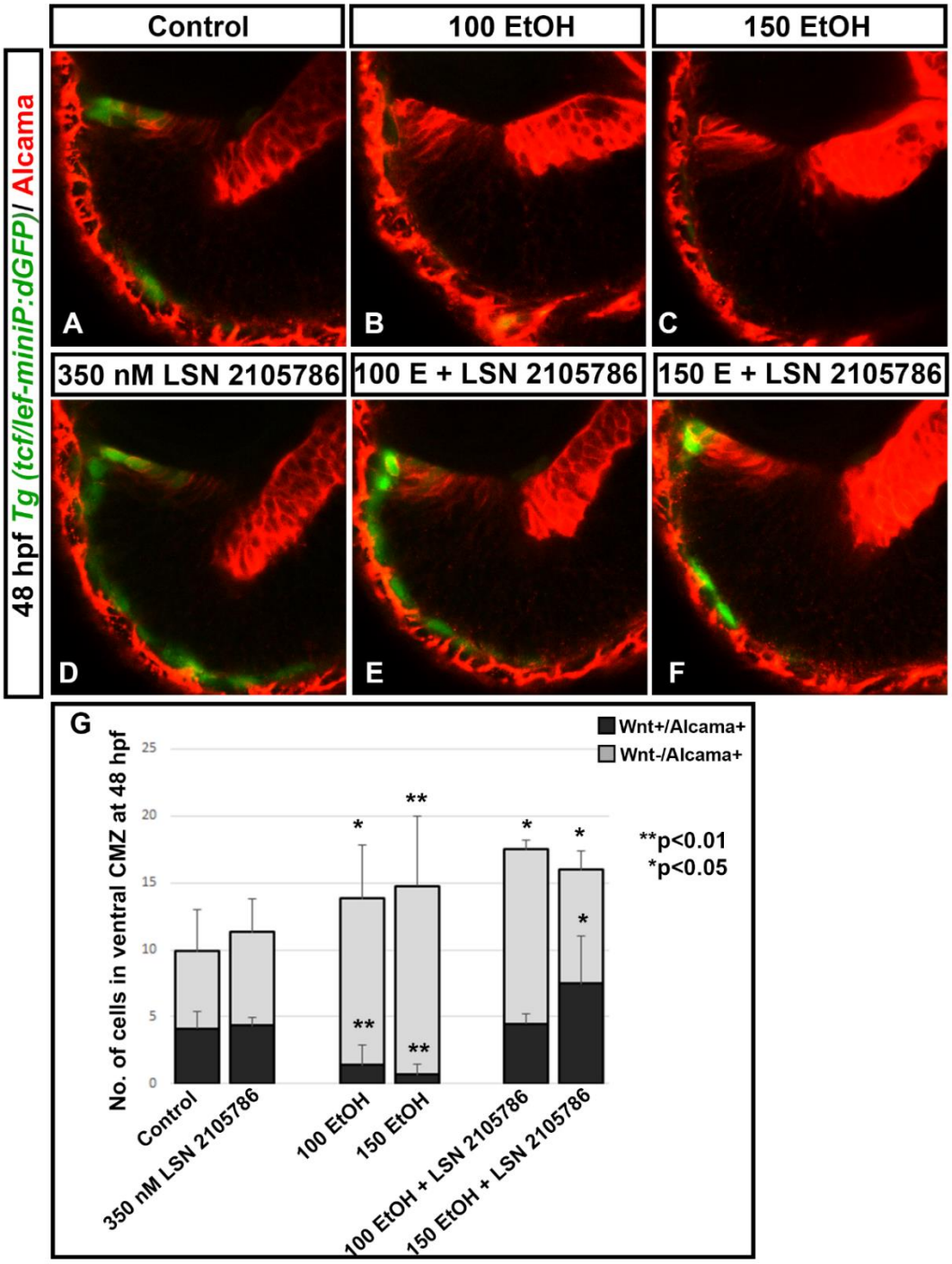

Figure 6.14 Treatment with Wnt Agonist.
(A-F) Treatment with 350 nM LSN 2105786 showed a rescue of Wnt+ cells in the peripheral CMZ, which were reduced after ethanol treatment. (G) Quantification of Wnt+/Alcama+ and Wnt-/Alcama+ cells in the peripheral CMZ. Error bars indicate standard deviation.

Downstream of Wnt activity, examination of Notch-active cells using *Tg(TP1:GFP)* and *Tg(TP1:mCherry)* (for cell counts), at 72 hpf, showed that ethanol treated embryos showed significantly fewer Notch-active cells at 72 hpf, which could be rescued to control levels by 500 nM LSN 2105786 treatment (Fig 6.15 A-G). LSN 2105786 treated embryos also showed rescue of proliferation response. Ethanol treated embryos showed significantly increased proliferation at 72 hpf in the CMZ, ONL and INL of the retina. This could be rescued by LSN 2105786 treatment from 32-48 hpf (Fig 6.15 H).

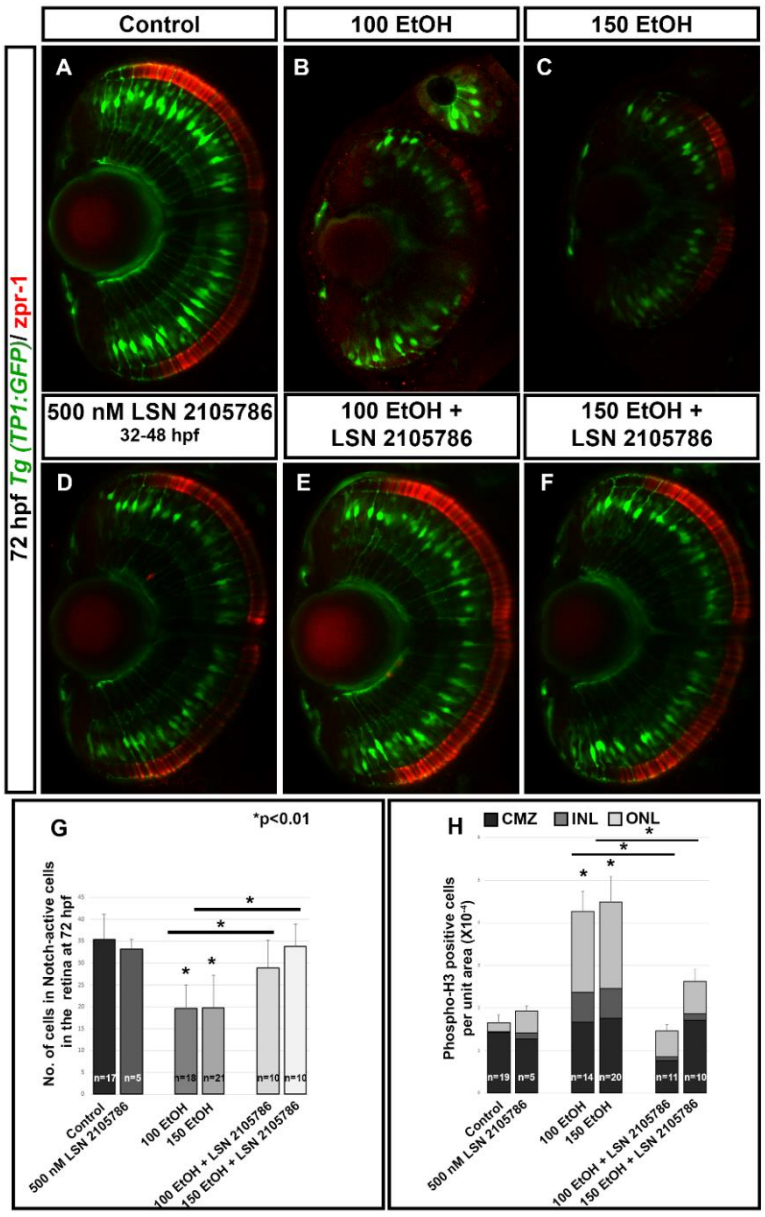

Figure 6.15 Notch signaling and proliferation rescued by Wnt agonist treatment.
(A-F) Treatment with LSN 2105786 on *Tg(TP1:GFP)* embryos showed rescue of Notch signaling after LSN 2105786 treatment. Ethanol treated embryos show reduced Notch-activity (green) which was restored by LSN 2105786 treatment. (G) Quantification of mCherry-positive, Notch-active cells, in the retina showed a significant rescue of notch signaling after LSN 2105786 treatment. (H) Quantification of phosphoH3-positive cells in the retina showed a significant rescue of cell proliferation response after LSN 2105786 treatment at 72 hpf. Error bars indicate standard deviation.

Previous study showed that, at 72 hpf, ethanol exposure caused severe photoreceptor differentiation defects, which was quantified by comparing zpr-1 staining, the red-green double cones with control embryos (Muralidharan et al, 2015). To examine the effects of Wnt signaling activation in the CMZ on photoreceptor differentiation and effective time windows, embryos were treated with ethanol from 2-24 hpf and Wnt agonist from 32-48 hpf or 48-72 hpf (Fig 6.16 A-J). At 72 hpf, treatment with 500 nM LSN 2105786 from 32-48 hpf, showed a significant rescue of zpr-1 expression intensity in the ONL to control levels. However, LSN 2105786 treatment from 48-72 hpf, showed a slight trend towards rescue. This suggests that Wnt signaling restoration a rescue of photoreceptor differentiation due to Wnt agonist treatment in ethanol treated embryos.

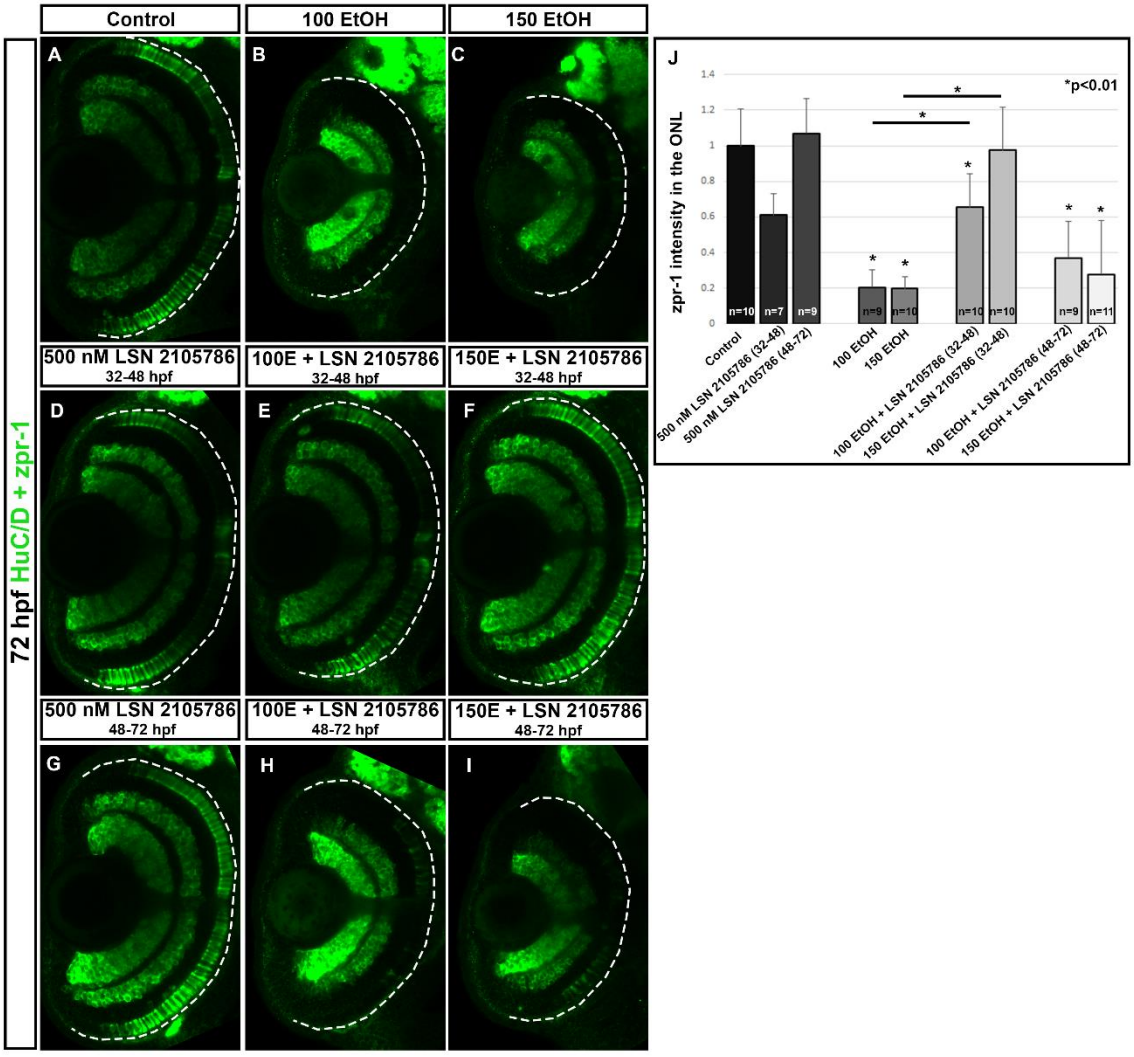

Figure 6.16 Wnt agonist treatment rescued photoreceptor differentiation.
(A-J) HuC/D and zpr-1 staining showed photoreceptor differentiation at 72 hpf: ethanol alone from 2-24 hpf, reduction of photoreceptor differentiation; ethanol + LSN 2105786 from 32-48 hpf, near normal photoreceptor. Ethanol +LSN 2105786 (48-72 hpf) slight rescue. (J) Quantification of total zpr-1 intensity in the ONL of the retina showed a significant decrease in photoreceptor marker expression after ethanol treatment (2-24 hpf) and subsequent rescue by LSN 2105786 treatment from 32-48 hpf. Dashed lines indicate RPE. Error bars indicate standard deviation.

6.9 Discussion:

Many key signaling pathway disruptions have been implicated in FASD. These include but are not limited to RA, epigenetic modifications, and oxidative stress mediated deficits (Brocardo et al, 2011; Garic et al, 2011; Marrs et al, 2010; Muralidharan et al, 2015; Muralidharan et al, 2013; Sarmah & Marrs, 2013; Serrano et al, 2010; Yelin et al, 2005; Zhou et al, 2011a). Our data shows a persistent defect in the zebrafish retina up to 18 dpf larvae, which suggests that, even in a regenerative model organism, such as zebrafish, ethanol-exposure leads to severe and persistent disruption in the multipotent retinal stem cell differentiation pathways and potentially stem cell niches. In order to examine these defects, retinal CMZ compartments were first dissected. CMZ was expanded in size, and the CMZ subdomains showed altered composition in ethanol treated embryos. Expanded retinal neuroepithelial cell marker expression domain was shown by Alcama staining (Nagashima et al, 2013). Alcama is a cell adhesion molecule and is expressed in hematopoietic and neuronal stem cell populations, among others, in mammalian systems (Wai Wong et al, 2012). Although its role in peripheral CMZ of the retina has not been evaluated, it is involved in maintenance of the stem cell niche in hematopoietic and stromal stem cells (Wai Wong et al, 2012). Expanded Alcama expression due to ethanol exposure suggests changes in adhesive interactions in the peripheral CMZ due to ethanol treatment lead to altered retinal stem cell niches. Other markers for peripheral CMZ, including *rx1*, showed reduced expression, indicating that fewer cells were expressing the retinal multipotent stem cell markers in the peripheral CMZ.

Another important stem cell population in the neural retina are the MGCs, the radial glia of the retina, which are among the latest cells born that can give rise to rod photoreceptors (Bernardos et al, 2007). These cells are greatly reduced in number after ethanol treatment in comparison to untreated controls. Studies have shown the involvement of Notch-Delta signaling in MGC specification and differentiation (Bernardos et al, 2005). Not surprisingly, Notch-active and Gfap-positive MGCs in the central retina are reduced after ethanol treatment, suggesting that defective MGC specification and differentiation due to ethanol exposure are connected to Notch signaling defects. Not only do MGCs perform various functions in maintenance of normal visual function, and as the resident multipotent

stem cells, but also provide tensile strength to the retina (MacDonald et al, 2015). Reduced number of MGCs can lead to persistent defects in the retina potentially also due to decreased tissue stability.

Previous clinical and experimental studies have shown reduced photoreceptors in FASD patients and various animal systems (Arenzana et al, 2006; Bilotta et al, 2004; Bilotta et al, 2002; Chung et al, 2013; Hug et al, 2000; Kashyap et al, 2007; Katz & Fox, 1991; Matsui et al, 2006). Our previous study showed that after ethanol treatment, the cells were present in the photoreceptor layer, but these cells express lower levels of terminal differentiation markers. Photoreceptor precursors, in the INL and ONL, and terminally differentiated nascent photoreceptors in the ONL, express specific set of transcription factors, including *neurod*, *rx1* and *crx* (Stenkamp, 2011). As the cells mature, expression of these transcriptions factors reduces. To determine the differentiation status of these ONL cells, we used *neurod*, *rx1*, and *crx* markers identifying photoreceptor precursor and progenitor populations. Ethanol treated embryos showed more photoreceptor precursor cells and immature photoreceptors in the ONL. Thus, fewer cells in the ethanol treated embryos undergo complete terminal differentiation and maturation. The cells present in the ONL of these embryos are in a precursor state. Co-labeling with phosphoH3 showed increased number of these cells are mitotically active, indicating cell cycle exit failure. Together, these results show that retinal cells in the ethanol treated embryos are in a precursor differentiation state. These cells are in photoreceptor lineage but fail to exit the cell cycle. These precursors may lack the signaling cues that normally facilitate their differentiation into mature and functional photoreceptors.

Studies on *Xenopus* retina show that Wnt signaling through Sox2 and Notch modulate proneural gene expression which determine progression from progenitor to differentiated state of cells (Agathocleous et al, 2009; Van Raay et al, 2005). Increasing evidence in zebrafish suggest that this mechanism may be conserved (Nadauld et al, 2006; Yamaguchi et al, 2005). Increased Wnt in peripheral CMZ maintains stem cell progenitor state, increased Sox2 and Notch inhibit neurogenesis and promote gliogenesis, and increased proneural gene expression promotes neuronal differentiation. Closely examining specific signaling pathways governing retinal cell differentiation process showed decreased Wnt

and Notch signaling. Reduced Wnt activity and *axin2* expression in the peripheral CMZ due to ethanol exposure points out one of the main defects in the CMZ compartment. Increased GSK3β and consequent cell death has been reported in ethanol treated fetal cerebral cortical neurons (Riar et al, 2014). Furthermore, studies in FASD rat models showed increased activation of GSK3β and reduced canonical Wnt and Notch signaling in chronic prenatal ethanol exposed cerebellum (Gundogan F, 2013; Tong et al, 2013). Ethanol-induced noncanonical Wnt signaling disruption has been reported in neural crest cell death (Garic et al, 2011). Treatment with lithium chloride, a GSK3β inhibitor, could protected against ethanol-induced neurotoxicity (Garic et al, 2011; Luo, 2010). Since many molecular pathways underlying retinal development also overlap with CNS development, ethanol-induced reduction in Wnt signaling in the retina agrees with the previous studies in the CNS development. Reduced Wnt signaling may lead to reduced downstream signaling pathway activation, including Notch and *neurod*, which is supported by rescue experiments using a Wnt agonist (GSK3β inhibitor).

In zebrafish, Wnt signaling hyperstimulation, during specific developmental time windows, led to reduced differentiation and increased proliferation. Conversely, reduced Wnt signaling led to increased differentiation and reduced proliferation (Meyers et al, 2012). However, ethanol exposure caused reduced Wnt signaling and reduced differentiation and increased proliferation. One possibility for this is that ethanol-induced disruption of Wnt may produce more progenitor cells in the middle and central CMZ, leading to an increased size of the transit amplifying compartment and thus, increased proliferation. This effect would also explain the expansion of Notch-active and *neurod*-positive cells in the central CMZ of ethanol treated embryos as compared to controls. Furthermore, ethanol-induced disruption of other signaling pathways, including RA, may lead to cell cycle exit failure and reduced terminal differentiation (Muralidharan et al, 2015). However, Wnt signaling disruption likely to be only one of the pathways affected by ethanol exposure, and these are likely to be context dependent effects of ethanol on various developmental events. Variations in development timing and tissue interactions could produce differential defects on various cell types. Thus, additional studies dissecting the specific Wnt pathway defects are needed to understand the complete genesis of retinal defects.

Zebrafish retina injury models have shown Wnt signaling controls the regenerative response (Ramachandran et al, 2011). Several studies on mammalian retinal regeneration models show that Wnt signaling stimulates MGC proliferation *in vivo* (Inoue et al, 2006; Lamba et al, 2009; Osakada et al, 2007; Sanges et al, 2013; Stoick-Cooper et al, 2007). These MGC derived cells could be differentiated *in vitro* into photoreceptors using retinoid or hedgehog containing media. (Del Debbio et al, 2010). These studies strongly suggest that Wnt signaling plays a critical role in regeneration of retinal tissue. Ethanol-induced disruption of Wnt activity may lead to persistent defects in CMZ and the central retina, preventing full deployment of regeneration mechanisms.

Several studies have shown effects of nutrient compound supplementation on ethanol-induced defects (Ballard et al, 2012; Kot-Leibovich & Fainsod, 2009; Marrs et al, 2010; Muralidharan et al, 2013; Sarmah & Marrs, 2013). Our previous experiments showed RA and FA supplementation rescues ethanol-induced retinal defects, particularly, retinal photoreceptor cell differentiation and optic nerve development (Muralidharan et al, 2015). However, specific differences were identified in RA and FA rescue mechanisms. Although RA and FA co-supplementation could rescue photoreceptor differentiation defects, RA supplementation from 24-48 hpf or 48-72 hpf, after ethanol treatment (2-24 hpf), could restore photoreceptor differentiation but FA post-treatment did not rescue photoreceptor defects. The role of RA in photoreceptor differentiation has been studies, and RA signaling is involved in the maintaining the balance of rod, red-green cones and blue and UV cones in the retina (Hyatt et al, 1996a; Prabhudesai et al, 2005). Our data suggests that ethanol treatment produces increased number of photoreceptor precursors in the ONL of the retinas, lacking retinoid differentiation signal. Here, further examination of mechanisms underlying RA and FA co-supplementation showed that, both RA and FA could restore Wnt active cells in the CMZ, and Notch-active cells in the central retina. However, a clear difference was seen in proliferation response of the cells. RA co-supplementation, but not FA co-supplementation, showed increased proliferation in the CMZ, in comparison to embryos only treated with ethanol. Together these data suggest that RA promotes proliferation and differentiation of precursor cells in the CMZ and central retina.

Ethanol effects on the most peripheral CMZ compartment reveals a defect in the stem cell niche that can lead to a persistent retinal defects. Reduced Wnt signaling in this compartment and consequent reduction of Notch activity may underlie many, if not all, ethanol-induced retinal cell differentiation defects. Restoration of the Wnt-active cells, using GSK3β inhibitor treatment, RA, and FA co-supplementation, lead to restoration of Notch-activity, precursor to differentiated cell transition, and terminal differentiation. Normal Notch signaling would also potentially lead to normal MGC differentiation leading to better retinal tissue stability and function.

This study shows that ethanol exposure affected the CMZ stem and precursor cell status leading to persistent defects in the retina. Wnt signaling was disrupted in the retinal CMZ. Furthermore, pathways downstream of Wnt, such as Notch and proneural gene expression were also affected. Rescue using Wnt agonists could restore downstream mechanisms leading to differentiation of photoreceptors. Additional research examining specific members of the signaling pathways and lineage tracing will provide detailed mechanistic insight in ethanol-induced retinal defects and help find therapies for this devastating disorder.

6.10 Conclusions

These studies show that ethanol exposure affected the CMZ composition leading to persistent defects in the retina. Specific signaling pathways such as Wnt signaling are disrupted in the retinal CMZ. Furthermore, downstream pathways such as Notch and proneural gene expression are also affected. Rescue using Wnt agonists could restore downstream mechanisms leading to differentiation of photoreceptors. The role of Wnt signaling in maintenance of CMZ and central retina during development is unclear. Additional research examining specific members of the signaling pathways and lineage tracing will provide mechanistic insight in ethanol-induced retinal defects and help identify therapies for this devastating disorder.

CHAPTER 7. FUTURE DIRECTIONS

7.1 Mechanistic perspectives and insights into therapeutic targets

This research has revealed crucial mechanisms underlying retinal cell differentiation defects. Rescue experiments with Wnt agonists suggests a therapeutic potential of Wnt signaling modulation in retinal differentiation defects. Initial studies show that nutritional compounds plays a crucial role in the severity of FASD ocular manifestation. Rescue experiments with RA and FA suggest the show rescue of many ethanol-induced retinal defects, while showing subtle differences in the rescue timing and extent. These differences suggest that the involvement of various mechanisms underlying RA and FA rescue of ethanol-induced defects. While rescue of RA points to a direct effect on the RA signaling pathway, FA rescue suggests a more general and global mechanism, such as epigenetic changes.

7.1.1 Ethanol-induced epigenetic changes

Ethanol interferes with components of the one carbon metabolism pathway, including folate, choline (a derivative of homocysteine) and SAM (methyl donor). Disrupting one carbon metabolism can lead epigenetic alterations and global changes in epigenetic profiles. Pregnant mice treated with ethanol showed significant embryo DNA hypomethylation (Garro et al, 1991). Ethanol-induced alterations DNA methylation profile during early neurulation in developing mouse embryos and neural stem cell cultures were also observed. High-throughput methyl DNA microarray analysis identified specific DNA methylation changes associated with dysregulation of neural stem cell migration and differentiation program after ethanol exposure (Zhou et al, 2011a). This suggests that alcohol exposure prevented normal DNA methylation progression, a critical sequence of events during differentiation of neural cells (Zhou et al, 2011b). Effects of ethanol on DNA and histone

methylation are not clearly understood, but these effects may be very central to the ethanol induced defects, as illustrated by the rescue effects of various epigenetic modifiers. Role of RA and FA in epigenetic modifications could be one of the mechanisms leading to rescue of the ethanol-induced altered gene expression during development.

7.1.2 Ethanol effects on retinal differentiation pathways

A number of signaling pathways are involved in retinal cell differentiation. Also, complex tissue interactions drive tightly orchestrated specification and differentiation processes. A simplified overview of the process is represented in Fig 7.1. We hypothesize that ethanol exposure disrupted Wnt signaling in the CMZ leading to stem cell maintenance and downstream differentiation defects. Furthermore, effects of ethanol on other signaling pathways such as RA, lead to retinal differentiation defects. Our experiments showed Wnt agonist treatment could rescue ethanol-induced retinal cell differentiation defects. However, cells in the central retina that are derived from the retinal neuroblasts (specified during retinal morphogenesis, 16-24 hpf) showed ethanol-induced defects. These central retinal cells are not directly derived from the CMZ cells. There may be independent effects of ethanol leading to defects in the CMZ and central retina.

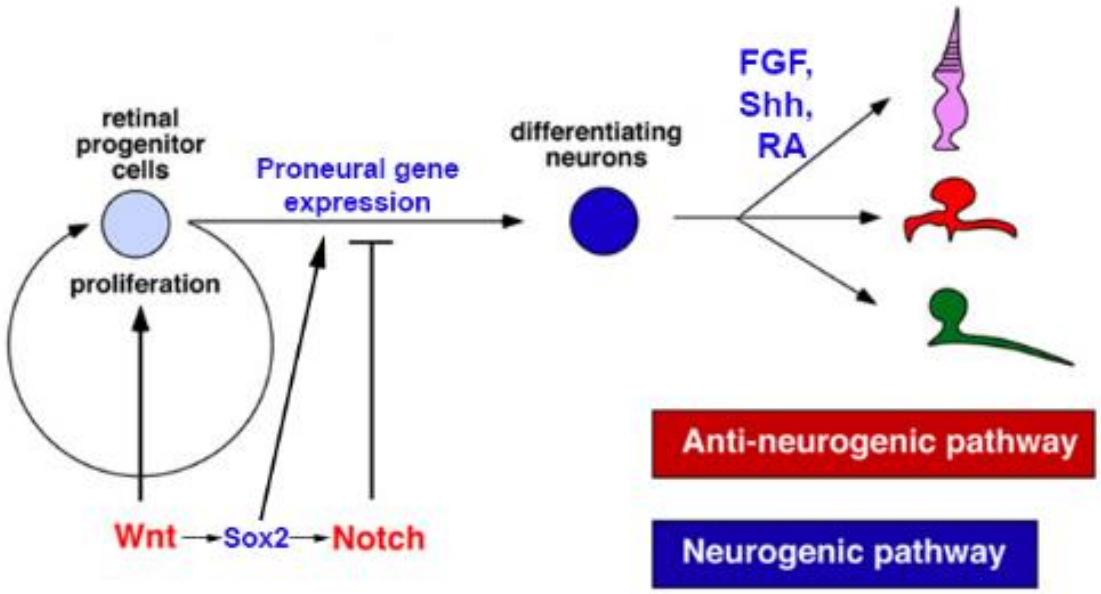

Figure 7.1 Molecular network regulating retinal neurogenesis in zebrafish.
Wnt, Sox2, and Notch signaling pathways regulate retinal neurogenesis. Other signaling pathways such as Fgf, Shh, and RA regulate progression of neurogenesis and differentiation. Modified from (Agathocleous et al, 2009; Yamaguchi et al, 2005).

Role of Wnt signaling in the central retinal differentiation is unclear. Some studies on adult zebrafish retina show that Wnt activation is necessary and sufficient to activation of regeneration mechanisms in the retina (Ramachandran et al, 2011). In zebrafish, these regenerated retinal tissues are arise from MGC dedifferentiation, proliferation, and differentiation close to the retinal lesion site. Studies on mice identify similar proliferative precursor cell population in retina, which are responsive Wnt activation (Osakada et al, 2007; Sanges et al, 2013). This suggests a role of Wnt signaling on central retinal cells in adult tissues. The role of Wnt during induction and central retinal neuroblast differentiation, lateral tissue interactions, and how these are disrupted by ethanol treatment remains unclear. Further research directed towards understanding retinal regeneration response at a lesion site using lineage tracing experiments, and response to specific signaling modulation will be insightful in understanding central retinal defects after ethanol exposure.

Future studies cans evaluate the Wnt signaling defects in the retina, and effects of Wnt signaling in retinal regeneration defects induced by early ethanol-exposure.

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VITA

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EDUCATION

2016: Doctor of Philosophy: Cell and Developmental Biology,
Department of Biology, School of Science, Purdue University (Indianapolis), Indianapolis, IN. Thesis Advisor: Dr. James A. Marrs

2010: Master of Science: Genomics,
Centre for excellence in Genomic sciences, School of Biological Sciences, Madurai Kamaraj University, Madurai, Tamil Nadu, India.

2008: Bachelor of Science: Microbiology,
Department of Microbiology, Ramnarain Ruia College, University of Mumbai, Mumbai, Maharashtra, India

PUBLICATIONS:

- Sarmah, S., Chism, G. W., Vaughan, M. A., Muralidharan, P., Marrs J. A. and K. A. Marrs. 2015. Using Zebrafish to Implement a Course-Based Undergraduate Research Experience (CURE) to Study Teratogenesis in Two Biology Laboratory Courses. Zebrafish (in press).
- Muralidharan, P., Sarmah, S., and J. A. Marrs. 2015. Zebrafish retinal defects induced by ethanol expousure are rescued by retinoic acid and folic acid supplement. Alcohol 49: 149-163.

- Sarmah S., Muralidharan P, Curtis C, McClintick J. N, Buente B, Holdgrafer D, Ogbeifun O, Olorungbounmi O, Patino L, Lucas R, Gilbert S, Groninger E, Arciero J, Edenberg H. J., and J. A. Marrs. 2013. Ethanol exposure disrupts extraembryonic microtubule cytoskeleton and embryonic blastomere cell adhesion, producing epiboly and gastrulation defects. Biology Open 2: 1013–1021**.**
- Muralidharan, P., Sarmah. S., Zhou, F. C., and Marrs, J. A. 2013. Fetal Alcohol Spectrum Disorder (FASD) Associated Neural Defects: Complex Mechanisms and Potential Therapeutic Targets. Brain Sciences. 3 (2): 964-991.

- Manuscripts in preparation:
  1. Sarmah, S., Muralidharan, P., and Marrs, J. A. 2016. Embryonic ethanol exposure produced dysregulation of BMP and Notch signaling leading to persistent atrio-ventricular valve defects in zebrafish. (Research Article, in review).
  2. Muralidharan, P., Sarmah. S., and Marrs, J. A. 2016. Wnt signaling disruption by ethanol exposure in zebrafish retinal stem cell compartment. (Research Article, in preparation).
  3. Sarmah, S., Muralidharan, P., and Marrs, J. A. 2016. Folic acid containing multivitamins as measures for prevention of congenital anomalies. Prevention of Birth Defects, SMGroup. (Book chapter, in review).
  4. Muralidharan, P., Connors, C., Mohammed, A. S., Sarmah, S., Marrs, K. A., Marrs, J. A., and Chism, G. W. 3rd . 2016. Effect of turmeric supplement on fetal alcohol spectrum disorder zebrafish model. (Research Article, in preparation)

HONORS AND AWARDS:

- Outstanding Research Student Award (doctorate) - April 2016
- FASD Study Group Travel Award, San Antonio, TX. June, 2015.
- Graduate and Professional Students Council Travel Award, Zebrafish Meeting, Madison, WI. June, 2014.
- School of Science Graduate Students Council Travel Award, Zebrafish Meeting, Madison, WI. June 2014.
- "Elizabeth Steele Creveling Memorial" Scholarship April, 2013.
- Pre-doctoral Travel Grant, from ASCB, Sept 2011.
- Fellowship from School of Science, IUPUI. 2010-2011.

PLATFORM TALKS:

- Friday Seminar Series, Department of Biology, IUPUI 20th September 2013.
- IUPUI Biology Department retreat, Bradford Woods, IN. 15th October 2013
- Research in progress Seminar, IUPUI. 21st November 2014.
- FASD study group FASt data talks, San Antonio, TX. 20th June 2015
- IUPUI Biology Department retreat, Bradford Woods, IN. 20th October 2015

POSTER PRESENTATIONS:

- American Society for Cell Biology, Denver, CO, 2011.
- Zebrafish Meeting, Madison, WI, 2012.
- IUPUI Research Day, Indianapolis, IN. 2013.
- Zebrafish Meeting, Madison, WI. 2014.
- IUPUI Research Day, Indianapolis, IN. 2015.
- Research Society for Alcoholism Meeting, San Antonio, TX. 2015.
- American Society for Cell Biology, San Diego, CA. 2015.
- IUPUI Research Day, Indianapolis, IN. 2016.

TEACHING EXPERIENCE

- Introduction to Biology, Lab instructor (K101, K102 honors) – 3 semesters
- Human Physiology, Lab instructor (N217) -5 semesters
- Cell Biology, Lab instructor (K325) – 1 semester