ABSTRACT

PICKETT, MELISSA ANNE. A Novel Non-neuronal Function of Acetylcholinesterase during the Development of the Vertebrate Gut (Under the direction of Dr. Nanette Nascone-Yoder).

Acetylcholinesterase (AChE) is a highly conserved protein well studied for its role in terminating nervous signaling through degradation of the neurotransmitter, acetylcholine. However, a growing body of evidence suggests AChE also has non-neuronal activities associated with cell adhesion and polarized cell migration, cell behaviors that underlie much of embryogenesis. Consistent with a non-neuronal role in embryonic development, we detected AChE within the non-innervated endoderm cells of the Xenopus embryo that rearrange to lengthen the embryonic gut and form the epithelial lining of the intestine. Exposing embryos to chemical AChE inhibitors, or knocking down endoderm AChE via antisense agents (morpholino), results in shortened guts with disrupted cell polarity, abnormal cytoskeletal structure, and failure of the endoderm to differentiate and rearrange into a single epithelial layer. Importantly, we were able to rescue these phenotypes with expression of either wild type AChE or a mutated, non-enzymatic form of AChE. These results not only suggest that AChE is required for gut development, but that non-enzymatic functions of the protein are essential for this process to occur. Knockdown of AChE had no affect on cell-cell adhesion in *ex vivo* assays, but did reduce endoderm cell adhesion on fibronectin, but not laminin substrates. Taken together, this suggests that a specific interaction between AChE and fibronectin may be required for cell polarization, rearrangement, and differentiation in the intestine. This work reveals a previously unrecognized role for a neurotransmitter hydrolase in coordinating organ morphogenesis.

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A Novel Non-neuronal Function of Acetylcholinesterase during the Development of the Intestine

by
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DEDICATION

To my grandparents, Cedric and Shirley Pickett, my parents, Alan and Varina Pickett, and my brother, Joe Pickett, for their endless support, love, and encouragement.

BIOGRAPHY

Melissa Anne Pickett was born on July 21, 1989 to Alan and Varina Pickett. She was raised on the family cattle ranch in Midvale, ID along with her younger brother, Joe. Melissa's dream to become a biologist started on a family camping trip when she was in the 4th grade. Her parents stopped in the small town of Hagerman, ID, where Melissa saw the remains of Idaho's state fossil, the Hagerman Horse. This was only a few years after she had read about the successful cloning of Dolly the sheep, and her imagination ran wild, dreaming of cloning the Hagerman Horse and bringing back an extinct species.

Melissa's '*Jurassic Park'*-like fantasies dissipated, but her passion for biology remained strong. After graduating from Midvale High School in 2007, Melissa attended the College of Idaho. As an undergraduate, Melissa became interested in toxicology thanks to Mark Gunderson's Animal Physiology course. The class collected Redband trout from two Idaho streams and used biomarkers to evaluate the influence of mine proximity on fish health. She joined Dr. Gunderson's lab, where she studied the influence of *in ovo* toxicant exposure on the American Alligator for the remainder of her undergraduate studies. Melissa was honored to be named the College of Idaho's *Biology Student of the Year,* and graduated *summa cum laude* with her bachelor's degree in biology in 2011. Melissa joined the Environmental and Molecular Toxicology Program at North Carolina State University to pursue her PhD in the fall of 2011. She joined Nanette Nascone-Yoder's laboratory in January 2012, where she has studied the function of acetylcholinesterase in gut development in African Clawed Frog tadpoles, and how organophosphate pesticides disrupt this process.

Melissa enjoys spending time outdoors camping, hiking, backpacking, and rock climbing. She also enjoys reading Sci-Fi and discussing the merits and flaws of *Star Trek* movie plots with her father and her labmates. She likes engaging with the general public in science outreach and has especially enjoyed volunteering in the Living Conservatory at the North Carolina Museum of Natural Sciences, where she is surrounded by beautiful butterflies and flowers.

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TABLE OF CONTENTS

Table of Contents

LIST OF TABLES .... **VIII**

LIST OF FIGURES .... **IX**

CHAPTER 1 – INTRODUCTION **1**

1) INTESTINAL DEVELOPMENT **1**

1.1) Overview of vertebrate intestinal morphogenesis *1*

1.2) Intestinal development in Xenopus laevis *3*

1.3) Cell signaling pathways required in gut morphogenesis *4*

1.4) Lessons from Toxicology *11*

2) ORGANOPHOSPHATE PESTICIDES **12**

2.1) OP structure *13*

2.2) Mechanism of Action *14*

2.3) Bioactivation and degredation of OPs *16*

2.4) OP toxicity in humans *18*

3) ACETYLCHOLINESTERASE (AChE) **22**

3.1 AChE structure *23*

3.2 AChE Classical Role – Neurotransmission and acetylcholine degradation *25*

3.3 Non-classical functions of AChE *26*

3.4 Role of AChE in development *35*

4) RATIONALE AND HYPOTHESIS **40**

REFERENCES: **44**

CHAPTER 2 – CHEMICAL INHIBITION AND KNOCKDOWN OF ACETYLCHOLINESTERASE DISRUPT INTESTINAL DEVELOPMENT IN FROGS **63**

INTRODUCTION: **63**

RESULTS: **65**

Organophosphates, Huperzine A, and AChE knockdown disrupt intestinal development *65*

Malathion exposure disrupts intestinal development in Lebidobatrachus laevis *67*

Chemical AChE inhibitors disrupt endoderm structure *68*

AChE knockdown disrupts endoderm structure *71*

Chemical AChE inhibitors disrupts mesoderm structure *72*

AChE knockdown disrupts mesoderm structure *73*

MTH exposure disrupts the enteric nervous system structure *74*

AChE acts cell autonomously and is essential in the endoderm for intestinal morphogenesis ... *75*

DISCUSSION: **77**

MATERIALS AND METHODS: **87**

Embryos and chemical exposures *87*

Morpholino Knockdown *87*

AChE Activity Assays *88*

Comparison of AChE sequences ... 89
Immunohistochemistry ... 89
REFERENCES: ... **92**

CHAPTER 3 – ACETYLCHOLINESTERASE PLAYS A NON-NEURONAL, NON-ESTERASE ROLE DURUING ORGANOGENESIS ... **117**
INTRODUCTION: ... **117**
RESULTS: ... **121**
Acetylcholinesterase is required for intestinal elongation ... 121
Acetylcholinesterase is required for ***gut endoderm*** *cell shape, arrangement, and polarization* ... 123
Acetylcholinesterase is required for cytoskeletal rearrangement in ***gut endoderm*** *cells* ... 125
Acetylcholinesterase is required for ***gut endoderm*** *cell adhesion on fibronectin but not laminin* ... 126
DISCUSSION: ... **128**
AChE is required for organogenesis ... 128
AChE has non-esterase functions in vivo ... 131
AChE is essential for cell-substrate adhesion on fibronectin ... 133
MATERIALS AND METHODS: ... **135**
Embryo Culture ... 135
Chemical exposures ... 135
Morpholino Knockdown ... 136
AChE mRNA Injections ... 136
AChE Activity Assays ... 137
Immunohistochemistry ... 138
Ex vivo cell assays ... 138
Cell-Cell adhesion ... 139
Cell-Substrate adhesion assay ... 139
Statistics ... 140
REFERENCES: ... **141**

CHAPTER 4 - DISCUSSION ... **161**
ACHE IS REQUIRED FOR ORGANOGENESIS ... **162**
ACHE HAS NON-ESTERASE FUNCTIONS REQUIRED FOR CELL POLARIZATION, CYTOSKELETAL REORGANIZATION, AND MOVEMENT THAT DRIVE INTESTINAL MORPHOGENESIS ... **166**
ACHE DIRECTS ENDODERM CELL CHANGES THROUGH INTERACTIONS WITH FIBRONECTIN. **170**
MODELS FOR ACHE'S NON-ESTERASE ROLE IN INTESTINAL DEVELOPMENT ... **175**
REFERENCES: ... **178**

CHAPTER 5 – CONCLUSIONS AND FUTURE DIRECTIONS ... **188**
REFERENCES ... **194**

APPENDICES ... **197**

APPENDIX A ... **198**
FROGS AS INTEGRATIVE MODELS FOR UNDERSTANDING DIGESTIVE ORGAN DEVELOPMENT AND EVOLUTION ... **198**

LIST OF TABLES

TABLE 1.1. ANTHROPOGENEIC TOXICANTS FOUND TO DISRUPT GUT DEVELOPMENT IN XENOPUS LAEVIS. 43

LIST OF FIGURES

FIGURE 1.1) INTESTINAL DEVELOPMENT IN *XENOPUS LAEVIS*. 60
FIGURE 1.2) GENERAL STRUCTURES OF ORGANOPHOSPHATES IN PARENTAL (A) AND BIOACTIVE OXON (B) FORMS. 61
FIGURE 1.3) MODEL OF ACHE PROTEIN STRUCTURE. 62
FIGURE 2.1) ACHE INHIBITION OR KNOCKDOWN RESULT IN INTESTINAL SHORTENING AND MALROTATION IN A DOSE DEPENDENT MANNER. 98
FIGURE 2.2) QUANTIFICATION OF INTESTINAL MALFORMATION AND EMBRYO MORTALITY IN RESPONSE TO ACHE CHEMICAL INHIBITION OR MORPHOLINO KNOCKDOWN. 99
FIGURE 2.3) MALATHION EXPOSURE DISRUPTS INTESTINAL DEVELOPMENT IN BOTH *L. LAEVIS* AND *X. LAEVIS*. 101
FIGURE 2.4) ACHE SEQUENCES ARE HOMOLOGOUS BETWEEN *L. LAEVIS* AND *X. LAEVIS*. 102
FIGURE 2.5) EXPOSURE TO ACHE INHIBITORS ALTERS GUT ENDODERM STRUCTURES. 104
FIGURE 2.6) EXPOSURE TO MALATHION DISRUPTS ENDODERM STRUCTURES FROM NF 41 - 46. 105
FIGURE 2.7) ACHE KNOCKDOWN DISRUPTS ENDODERMAL STRUCTURES AT NF 41 AND NF 46. 107
FIGURE 2.8) EXPOSURE TO ACHE INHIBITORS ALTERS GUT MESODERM STRUCTURES. 109
FIGURE 2.9) EXPOSURE TO MALATHION DISRUPTS MESODERM STRUCTURES FROM NF 41 - 46. 110
FIGURE 2.10) ACHE KNOCKDOWN DISRUPTS MESODERMAL STRUCTURES AT NF 41 AND NF 46. 112
FIGURE 2.11) EXPOSURE TO ACHE INHIBITORS TRANSIENTLY ALTERS NEURAL BRANCHING IN THE ENTERIC NERVOUS SYSTEM. 114
FIGURE 2.12) ACHE ACTS CELL AUTONOMOUSLY IN THE INTESTINE. 115
FIGURE 3.1) ACHE HAS A NON-ESTERASE ROLE ESSENTIAL FOR INTESTINAL ORGANOGENESIS. 149
FIGURE 3.2) ACHE IS REQUIRED FOR ENDODERM CELL MOVEMENT, REARRANGEMENT AND POLARIZATION. 152
FIGURE 3.3) ACHE IS REQUIRED FOR MICROTUBULE ORGANIZATION. 154
FIGURE 3.4) ACHE IS NOT REQUIRED FOR CELL-CELL ADHESION OR FOR CELL SUBSTRATE ADHESION TO LAMININ, BUT IS NECESSARY FOR CELL-SUBSTRATE ADHESION TO FIBRONECTIN. 155
FIGURE 3.S1) PERTURBATION OF CHOLINERGIC SIGNALING DOES NOT AFFECT GUT MORPHOGENESIS. 157
FIGURE 3.S2) ACHE CHEMICAL INHIBITORS ALTER ENDODERM CELL POLARITY, DISRUPT MICROTUBULE ORGANIZATION, AND REDUCE ACHE EXPRESSION AT THE APICAL CELL SURFACE. 158
FIGURE 3.S3) ACHE IS REQUIRED FOR ENDODERM DIFFERENTIATION. 160

FIGURE 4.1: ACHE MEDIATES ENDODERM CELL ADHESION, POLARIZATION, AND STRUCTURAL CHANGES THROUGH FIBRONECTIN 185
FIGURE 4.2) ACHE DOES NOT ACTIVATE INTEGRIN TO FACILITATE INTEGRIN-FIBRONECTIN INTERACTIONS AND INDIRECTLY ENHANCE CELL-SUBSTRATE ADHESION. 187

CHAPTER 1 – INTRODUCTION

1) Intestinal Development

1.1) Overview of vertebrate intestinal morphogenesis

Intestinal malformations comprise some of the most common human birth defects, and include a variety of forms such as, pyloric stenosis (thickened pyloic musculature, occluding the opening to the small intestine), atresia (absence of a hollow lumen), incomplete closure, and malrotations which occur as frequently as 1 in 500 live births (de Santa Barbara et al., 2002; Luks, 2011). These congenital defects often require surgical correction in young children and may necessitate altered nutritional requirements to facilitate appropriate growth and mental development (Cosnes et al., 2011; O'Sullivan and O'Morain, 2006; Vernier-Massouille et al., 2008). However, the mechanisms underlying normal gut development are incompletely understood, and very little is known about the genetic and environmental factors contributing to the etiology of intestinal defects.

Previous studies suggest that the processes underlying intestinal development are largely conserved across vertebrate species, despite differences in the initial organization of the definitive endoderm, which gives rise to the gut. In non-amniotes (fish, frogs), the definitive endoderm forms an occluded, multi-cell layered cylinder (Chalmers and Slack, 2000; Ng et al., 2005). Radial intercalation with preferential intercalation between anterior and posterior neighbors (convergent extension) drives gut tube elongation along the anterior-posterior (A-P) axis and produces a hollow tube surrounded by a single layer of columnar

epithelium (Chalmers and Slack, 2000). In contrast, the endoderm is organized as a sheet in amniotes (chicks, mice, humans). Indentations at the anterior and posterior ends of this definitive endoderm involute, converging at the midgut to produce a hollow tube surrounded by a single layer of epithelium (Cervantes, 2013). Concomitant with involution, the endoderm cells undergo convergent-extension, causing narrowing along the mediolateral axis while driving lengthening along the A-P axis. Mediolateral intercalation may also contribute to lengthening of the intestine in amniotes. Despite these differences, the cellular movements that drive gut tube lengthening (convergent-extension, intercalation) are necessary in both non-amniotes and amniotes. Additionally, the final tissue structures are very similar at anatomical and gene expression levels, suggesting a high degree of conservation in intestinal formation among vertebrates (Chalmers and Slack, 1998, 2000; Chalmers et al., 2000). Indeed, many of the same signaling pathways are essential for gut tube morphogenesis in frogs, chicks, mice, and humans (e.g. Wnt, Hedgehog, Retinoic Acid – reviewed in section 1.3).

While all models have shed light on the mechanisms underlying intestinal development, the frog/*Xenopus* model has several distinct advantages. Unlike amniote models, *Xenopus* develop *ex vivo*, in an aquatic environment, making them amenable to a wide variety of manipulations. Large clutch sizes and rapid development allow for larger sample sizes and biological replicates than can be obtained with murine models. Additionally, intestinal elongation, canalization, and counterclockwise rotation are complete within 96 hours of egg fertilization, and the major cell movements driving intestinal

morphogenesis occur in 32 hours (Chalmers and Slack, 1998; Nieuwkoop, 1994). Furthermore, *Xenopus* tadpoles are transparent, facilitating visualization of intestinal morphogenesis in live animals. Aquatic development also provides opportunities to use small molecules to identify signaling pathways involved in organogenesis and to evaluate the contributions of exogenous chemicals (e.g. pharmecuticals, cosmetics, pesticides, nanoparticles) in gut malformation etiologies (Dush et al., 2011; Wheeler and Brandli, 2009). While zebrafish offer many of the same advantages as *Xenopus*, their intestinal tracts do not elongate or rotate to the extent of tetrapods, making *Xenopus* a more attractive model for studying gut morphogenesis (Ng et al., 2005; Wallace and Pack, 2003).

1.2) Intestinal development in Xenopus laevis

Fate mapping of the early *Xenopus* embryo (8-32 cell) is well established, and embryos are amenable to targeted microinjection through late neurula stages (NF 23/24), which facilitates cell labeling and tracking in live embryos (Moody, 1987a, b; Muller et al., 2003). Elegant double labeling experiments reveal that endoderm cells radially intercalate, driving elongation of the primitive gut tube (Chalmers and Slack, 2000). Narrowing of the intestinal diameter and the preferential arrangement of labeled endoderm cells in longitudinal tracts along the A-P axis, suggest that convergent extension also contributes to intestinal lengthening (Muller et al., 2003; Reed et al., 2009). The arrangement of the endoderm cells is also highly reminiscent of paraxial mesoderm undergoing convergent extension (Keller et al., 2000; Reed et al., 2009). Prior to intercalation, endoderm cells polarize and become radially oriented, observed first in the most basally located cells and progressing to those in the center

of the PGT (Reed et al., 2009). As the cells intercalate, generating length, the layers of cells are reduced from 4-5 cells deep to a single epithelial layer surrounding a hollow lumen (Fig. 1.1) (Ashani et al., 1992; Chalmers and Slack, 2000). As observed in other vertebrates, intestinal morphogenesis commences in the most anterior and posterior regions of the digestive tract, and converges in the midgut.

The intestine is a left-right asymmetric organ, with stereotypical counterclockwise looping. In *Xenopus*, the first symmetry-breaking event in the PGT occurs at NF 41, with a concavity on the left side of the future gastro-duodenal loop and a corresponding convexity on the right (Chalmers and Slack, 1998; Muller et al., 2003). This is followed by a second break in symmetry, which occurs in the hindgut, and results in the first loop by NF 43 (Chalmers and Slack, 1998; Muller et al., 2003). Both the left and right sides contribute equally to the mature intestine, suggesting that the asymmetric looping of this organ is due to differences in cell shape as opposed to differential cell specification or proliferation (Muller et al., 2003).

1.3) Cell signaling pathways required in gut morphogenesis

Xenopus have been useful for identifying and/or clarifying the roles of several signaling pathways in driving intestinal elongation and rotation in vertebrates. The utility of this model organism in expanding the understanding of gut morphogenesis as well as its similarity to other model organisms is highlighted below (Sections 1.3.1 – 1.3.5).

1.3.1) Shroom3: Shroom3 is a PDZ- containing protein involved in directing cytoskeletal rearrangements and cell shape changes, including apical constriction and apicobasal cell

elongation in neural tube closure (Haigo et al., 2003; Hildebrand and Soriano, 1999; Lee et al., 2007). In *Xenopus,* intestine-targeted microinjection of mRNA encoding a dominant-negative mutant form of Shroom3 (DN-Shroom3) resulted in gut tubes that failed to elongate or coil, suggesting that Shroom3 is also involved in intestinal morphogenesis (Chung et al., 2010). Cells lacking Shroom3 show reduced apical constriction or remained rounded and failed to intercalate into a single epithelial layer, suggesting that Shroom3 mediates cell shape changes required for intestinal morphogenesis and elongation (Chung et al., 2010). Similar gut epithelial defects were later found in Shroom3 knockout mice, confirming a conserved function of this protein in vertebrate gut development (Grosse et al., 2011).

1.3.2) Wnt: Wnt signaling is essential for intestinal morphogenesis as knock-out/down of Wnt signaling components result in short, malformed intestinal tracts in all species studied (Cervantes et al., 2009; Dush and Nascone-Yoder, 2013; Faas and Isaacs, 2009; Li et al., 2008; Reed et al., 2009; Zhang et al., 2013). Interestingly, while canonical Wnt signaling is necessary for cell specification and maintenance of stem cell niches, the non-canonical Wnt/planar cell polarity (PCP) pathway is primarily required for elongation of the gut tube (Li et al., 2008). Isolating the exact mechanisms by which Wnt/PCP coordinates cell movements is challenging in murine models due to redundancy of Wnt components and the necessity for tissue specific knock-outs (Matsuyama et al., 2009). In *Xenopus*, specific components of the Wnt/PCP signaling cascade have been knocked down in a tissue specific manner using oligonucleotide morpholinos and dominant negative (DN) mRNAs. This has led to a better understanding of how Wnt/PCP acts specifically within the intestine. RhoA

lies downstream of the Wnt/PCP cascade, and targeted microinjection of DN-RhoA abrogates intestinal elongation (Reed et al., 2009). Injected cells remain round in shape, unpolarized and do not intercalate, unlike uninjected cells within the same embryo (Reed et al., 2009). These cells showed aberrant myosin II organization and increased expression of the adherens junction protein, e-cadherin (Reed et al., 2009). To further elucidate the involvement of RhoA and myosin II in gut development, chemical inhibitors of Rho kinase and myosin II were administered during organogenesis. Phenocopy of intestinal malformations at gross and cellular levels confirmed that the Rho/ROCK/Myosin II portion of the Wnt/PCP cascade is required for directing cell movements through mediating actomyosin contractility (Reed et al., 2009). The ability to disrupt these signaling components chemically and genetically to target intestinal organogenesis provides a significant advantage in comprehending the mechanisms underlying gut development.

The non-canonical Wnt/PCP pathway also mediates activation of Jun N-terminal kinase (JNK). Chemical inhibition or gut tube targeted morpholino knockdown of JNK results in shortened intestines, similar to perturbation of Rho/ROCK/myosin II or other parts of the Wnt signaling pathway (Dush and Nascone-Yoder, 2013; Reed et al., 2009). However, these phenotypes differ at the cellular level, as microtubule polymerization and cell adhesion are lost in cells lacking JNK activity (Dush and Nascone-Yoder, 2013). Specific inhibition of microtubule polymerization phenocopies loss of JNK activity, and suggests that a second arm of the Wnt/PCP signaling cascade mediates cell rearrangement through promoting microtubule polymerization (Dush and Nascone-Yoder, 2013).

The use of *Xenopus* tissues in *ex vivo* assays has been essential for understanding the cell movements and regulatory pathways involved in developmental processes (Davidson et al., 2008; Davidson et al., 2004; Keller et al., 2000; Weber et al., 2012). The endoderm of the gut can be examined *ex vivo* through modification of existing techniques (Dush and Nascone-Yoder, 2013; Li et al., 2008). For instance, modification of the animal cap adhesion assay on *Xenopus* intestinal tissue was used to identify differences in cell adhesion caused by perturbation of different components of the Wnt signaling cascade. This revealed that Rho kinase is involved in reducing cell-cell adhesion, while the JNK pathway increases adhesion. It suggests that two arms of the Wnt/PCP signaling network act in opposition to facilitate intestinal cell intercalation (Dush and Nascone-Yoder, 2013).

1.3.3 Hedgehog: Hedgehog signaling is required for patterning the intestinal endoderm, mesoderm, and ectoderm in all vertebrates studied to date (Ramalho-Santos et al., 2000; Sukegawa et al., 2000). Loss of Hedgehog signaling results in shortened, malrotated gastrointestinal tracts, with disruption in the structures of all three tissue layers (Ramalho-Santos et al., 2000; Sukegawa et al., 2000). Similarly, disruption of Hedgehog signaling in *Xenopus* results in malformed, shortened gut tubes (Zhang et al., 2001). *Xenopus* have been instrumental in refining the understanding of how Hedgehog mediates communication between the tissue layers of the gut during development. Targeted microinjection of a constitutively active smoothened receptor (SmoM2) (excessive Hedgehog signaling) to the gut endoderm does not affect gut development, whereas expression in the mesoderm causes severe disruption in elongation and coiling (Zhang et al., 2001). This confirms that Hedgehog

ligands from the endoderm act through binding to receptors in the mesoderm. Of more significance, this experiment also revealed that Hedgehog signaling within the mesoderm is essential for endoderm structures to form correctly, suggesting reciprocal signaling between the layers (Zhang et al., 2001).

Xenopus have also been essential for examining downstream components of Hedgehog signaling. The transcription factor, *foxf1* is upregulated in the intestinal mesoderm in response to Hedgehog signaling (Madison et al., 2009; Mahlapuu et al., 2001a). However, knockdown of *foxf1* is lethal in mice prior to gastrointestinal elongation and looping, precluding use of the murine model for understanding the function of FoxF1 in intestinal development (Mahlapuu et al., 2001b). FoxF1 has successfully been knocked down in *Xenopus* gut tubes using targeted microinjection of morpholino oligonucleotides. Loss of FoxF1 expression disrupted the mesoderm layer and severely reduced the elongation and rotation of the intestine, suggesting that FoxF1 is essential in the gut for normal gastrointestinal morphogenesis (Tseng et al., 2004). Additionally, this phenotype was rescued by co-injection with *foxf1* mRNA, confirming the importance of this downstream factor of Hedgehog in intestinal development (Tseng et al., 2004). Subsequent to this work, *foxf2* knockouts and compound *foxf1*$^{+/-}$/*foxf2*$^{+/-}$ mice were generated, which survive to birth (Ormestad et al., 2006). Similar to the observations in *Xenopus,* the intestines of these mice were deformed and showed severe disruption in mesodermal formation, suggesting conservation of function of the *foxf* genes downstream of *hedgehog* in directing morphogenesis (Ormestad et al., 2006).

1.3.4 Retinoic Acid: Retinoic acid (RA) signaling is also necessary for intestinal morphogenesis across vertebrates. However, in mice, knockout of the enzyme involved in RA synthesis (*raldh2*) is lethal prior to intestinal looping morphogenesis (Niederreither et al., 2001). Similarly, knockout of the enzyme involved in RA degradation (*cyp26a1*) is lethal in mid-late gestation, with the intestine ending in a blind sac, making it difficult to identify the function of RA in intestinal development (Abu-Abed et al., 2001). Late stage exposure of *Xenopus* (NF 35-46) with RA or RA antagonists provides insight into this molecule's function during organogenesis, without affecting other RA sensitive processes (Lipscomb et al., 2006). Exposure to RA results in severely truncated intestines, suggesting RA signaling is essential for intestinal elongation (Lipscomb et al., 2006; Zeynali and Dixon, 1998). In milder phenotypes, excessive RA leads to malrotation of the intestine, suggesting a possible role of RA in directing the left-right asymmetric process of gastrointestinal rotation (Lipscomb et al., 2006). RA antagonists also alter the direction of gut tube rotation, further suggesting a function in asymmetric development (Lipscomb et al., 2006). RA has been shown to function in left-right axis formation and asymmetric heart development in zebrafish, mice, and chick suggesting a conserved, but largely unexplored, function of RA in left-right asymmetry (Huang et al., 2011; Vermot and Pourquie, 2005).

1.3.5 Additional Signaling Pathways Studies in Xenopus have led to the identification of additional proteins likely to be involved in intestinal morphogenesis across species. While these have not received as much attention at the molecular level as those discussed above, they provide intriguing areas for future research. Morpholino or dominant-negative knock

down of Inositol-Requiring Enzyme 1α (IRE1α) results in short, straight gut tubes (Guo et al., 2013). This short gut phenotype is recapitulated by knockdown of a downstream target of IRE1α, XBP1, suggesting this pathway is important for normal elongation (Guo et al., 2013). This pathway can also activate JNK, making it tempting to speculate that the shortened gut phenotype may be the result of altered Wnt/PCP signaling, but the mechanism(s) by which IRE1α influences gut elongation are currently unknown. Similarly, Kindlin1 has been implicated in intestinal elongation, as knockdown of this protein reduces the length and coiling of *Xenopus laevis* tadpole intestines (Rozario et al., 2014). Kindlins regulate integrin activation, which can alter cell adhesion, acto-myosin contractility and activation of Rho GTPases through signal transduction (Davidson et al., 2006; Ramos et al., 1996). This pathway might also converge with the Wnt signaling pathway, though this has yet to be elucidated. Calcineurin is a calcium/calmodulin dependent serine/threonine phosphatase which is a component of the Wnt/calcium signaling pathway. Importantly, this pathway also inhibits canonical Wnt signaling, promoting a ventral as opposed to dorsal cell fate. Exposure of tadpoles to the Calcineurin inhibitors, cyclosporine A, FK506, or FK520 resulted in edematous embryos exhibiting shortened gut tubes often displaying reversal in coil direction (Yoshida et al., 2004). Other left-right asymmetric organs, including the heart and liver, were also affect by these treatments, suggesting that clacineruin may have a conserved function in directing organ laterality (Yoshida et al., 2004). Several studies suggest that mTOR and Sirtuins have a function in gastrointestinal development. mTORs are serine/threonine kinases which form complexes with FKBP12 and Raptor. Inhibition of mTOR with rapamycin

treatment results in shorter, fatter gut tubes as compared with controls (Moriyama et al., 2011). Other organs form normally, suggesting the mTORs are specifically required in gut morphogenesis (Moriyama et al., 2011). Similarly, injection of a dominant-negative *rheb* (an upstream positive regulator of mTOR) also decreases intestinal elongation and coiling (Moriyama et al., 2011). Knockouts of these proteins in mice result in embryonic lethality (Guertin et al., 2006). Zebrafish exposed to rapamycin also have gastroinstesintal defects, suggesting this pathway may be conserved in vertebrate intestinal organogensis (Makky et al., 2007). The deacetylase proteins Sirtuins (Sirts) are also implicated in regulation of the mTOR pathway. Inhibition of Sirt-1 with the specific inhibitor, Ex-527, disrupts intestinal elongation and coiling to a greater degree than mTOR inhibition. Sirt-1 also inactivates p53. p53 activity is essential for normal development through regulation of TGF-β signaling (Takebayashi-Suzuki et al., 2003; Wallingford et al., 1997). Thus, Sirt-1 may be important for regulation of multiple pathways involved in gastrointestinal development. It seems likely that these pathways regulate cell adhesion, cytoskeletal rearrangements, and/or establishment of cell polarity, possibly downstream of other known signaling pathways, to regulate elongation of the intestine, but additional research on these and other proteins at a mechanistic level is needed.

1.4) Lessons from Toxicology

One of the greatest advantages of *Xenopus* is the ability to rapidly test a large variety of exogenous chemicals to identify adverse effects on development (Dush et al., 2011; Wheeler and Brandli, 2009). Indeed, the FETAX assay was developed in *Xenopus* to assess

teratogenicity of chemicals that could have deleterious effects on humans and wildlife (Hoke and Ankley, 2005). High-throughput chemical screening in zebrafish has successfully been initiated by government regulatory agencies, but is limited in its ability to assess perturbation of gastrointestinal organogenesis. In the future, *Xenopus* could provide a similar high-throughput model, with the ability to examine gastrointestinal defects. Many toxicological studies have already been performed that identify intestinal malformations (Table 1). However, few of these studies explore the molecular mechanisms by which toxicant exposure affects organogenesis, missing opportunities to refine the understanding of mechanisms of action of toxicants and to identify proteins/pathways essential for intestinal organogenesis. While many exogenous compounds disrupt *Xenopus* intestinal development, the effects of organophosphates (OPs) and carbamates (Table 1) are intriguing as the two chemical classes share a molecular target. Both chemical classes inhibit the esterase activity of the serine hydrolase, Acetylcholinesterase (AChE). They can also inhibit non-esterase functions of AChE through conformational changes in the protein structure. Thus, the shared mechanism of action and intestinal teratogenesis resulting from exposure to these pesticides hints at a possible role for AChE and/or the cholinergic system in intestinal development.

2) Organophosphate Pesticides

Organophosphate (OPs) pesticides are among the most highly used pesticides worldwide. Initially synthesized in the late 1800's, they were developed for use as insecticides during the 1930s and 1940s (Costa 2005). OPs have a wide application both agriculturally and domestically as insecticides for food crops, ornamental plants, livestock,

and as treatment for lice, cockroaches, and termites among others. Other OPs have been developed for use in humans. While some of these have pharmaceutical applications, others have been developed as chemical warfare agents (soman, sarin, tabun, VX). Although OPs are not as environmentally persistent as other historically used pesticides (e.g. organochlorines), their high usage puts humans and non-target wildlife at risk of exposure. Thus, the impacts of OP exposure on health and physiology are important areas of research.

2.1) OP structure

The general molecular structure of OPs is shown in Fig. 1.2. A central phosphorus atom is bound to four side chains. These include single bonds to a leaving group (X) and two additional side chains, commonly alkoxy groups (R1,R2) (Costa, 2006). The leaving group is hydrolyzed in the phosphorylation of target esterases by OPs (Section 2.2). Loss of one of the additional side groups (R1 or R2) by non-catalytic hydolysis occurs in aging, resulting in permanent phosphorylation of the esterase. Additionally, there is a double bond between the central phosphorus and either a sulfur or oxygen atom. If the double bond is formed with a sulfur atom, metabolic activation must occur before the OP is biologically active. Activation occurs through oxidative desulfuration, resulting in a double bond between the phosphate and oxygen (Fig. 1.2). The sulfated version of the OP is commonly included in pesticide formulations, necessitating biological activation to form the oxon metabolite *in vivo.*

Subclassification of OPs has been based on the identity of the atoms or side chains bound to the central phosphorus (Mileson, 1998). Phosphorothionates include three single bonds to oxygen linked side chains with a double bond to sulfur. In phosphorodithioates,

single bonds exist between the phosphorus and two oxygen linked side groups and one sulfur side chain, with the double bond between phosphorus and sulfur. A third OP subclass is the phosphoroamidothiolates, in which side groups are linked to the central phosphorus through nitrogen, oxygen, and sulfur, and the double bond exists with oxygen (Mileson, 1998). OPs of all three subclassifications bind to and inhibit the serine hydrolase, AChE, following bioactivation. Reactivity of the OPs with AChE and other biological enzymes depends upon the chemical characteristics of the side groups. For instance, side chains containing nitro, cyano, halogen, ketone, or carboxylic ester groups are more reactive, while hydroxyl and carboxylic acid groups reduce OP reactivity (Mileson, 1998).

2.2) Mechanism of Action

With few exceptions, OPs share a common mechanism of action. They inhibit AChE through phosphorylating the serine hydroxyl group present in the active site of the enzyme (Costa, 2006; Mileson, 1998; Pope et al., 2005). This reaction involves loss of the leaving group on the OP (Fig. 1.2), resulting in a very stable phosphorylated enzyme (Mileson, 1998). Such inhibition may be reversed through spontaneous hydrolysis of the phosphate ester or through administration of oximes, which are highly nucleophilic and facilitate breakage of the phosphate bond between the active site and the OP (Costa, 2006; Mileson, 1998). However, AChE inhibition becomes irreversible if the bound OP loses an alkyl group (in the process of aging), which strengthens the bond between the enzyme and OP (Mileson, 1998). If aging occurs, AChE's catalytic activity is only restored upon *de novo* protein synthesis, modification, and translocation.

Phosphorylation of the serine residue prevents enzymatic cleavage of acetylcholine (ACh) by AChE, resulting in accumulation of ACh at cholinergic synapses. The accumulation of ACh results in increased cholinergic signaling through muscarinic and nicotinic ACh receptors (AChRs). Excessive cholinergic signaling is ultimately responsible for OP toxicity in both insects and non-target organisms, including humans (Costa, 2006). Acute OP poisoning in humans is identified by the cholinergic symptoms discussed in more detail in Section 2.4.1. If treated early, OP toxicity is not lethal in humans. However, in cases where death occurs, OP lethality is due to respiratory failure due to a combination of inhibition of the respiratory centers in the brainstem, bronchoconstriction, increased bronchial secretion, and flaccid paralysis of the respiratory muscles (Costa, 2006).

OPs also impact non-esterase functions of AChE. These non-esterase functions are discussed in more detail in Section 3.3 and include functions in cell-substrate adhesion, cytoskeletal rearrangement, polarized cell movement, and cell differentiation. These functions appear to be independent of AChE's esterase activity or cholinergic signaling. Interestingly, *in vitro* exposure to OPs has been shown to affect these non-esterase functions (Campanha et al., 2014; Howard et al., 2005; Inkson et al., 2004; Pizzurro et al., 2014; Yang et al., 2008). OPs bound at the active serine in AChE's catalytic gorge modify binding of other molecules to the peripheral anionic site (PAS) (Sultatos and Kaushik, 2008). The PAS is involved in non-esterase functions of AChE, suggesting that OPs can alter non-esterase functions by phosphorylating the esterase site. In some cases, effects were observed at OP exposure levels that do not significantly inhibit AChE esterase activity. Certain OPs can bind

to a second site on AChE (Aldridge and Reiner, 1969; Kardos and Sultatos, 2000). OP binding to this allosteric site can reversibly inhibit AChE activity (unlike classic inhibition of the esterase site), suggesting that conformational changes in the protein structure occur with OP binding (Aldridge and Reiner, 1969; Kardos and Sultatos, 2000). This could also contribute to alterations of non-esterase functions.

In addition to inhibition of AChE, some OPs have other biological targets. These include inhibition of Neurotoxic esterase, Butyryl-cholinesterase, Carboxylesterase, and A-esterases (e.g. Paraoxonase 1) (Pope et al., 2005). Additional biological targets have been identified for individual OPs, but do not appear to be affected by all OP compounds (Pope et al., 2005). Some OPs can also bind directly to muscarinic and nicotinic AChRs, which can alter ACh signal transduction, in addition to the altered cholinergic signaling that results from AChE inhibition (Pope et al., 2005). Thus, OPs have the potential to modify multiple aspects of cholinergic signaling and cell behavior. However, many of these off-target effects are specific to certain OPs and are observed at concentrations higher than those required to inhibit AChE's esterase activity. The differences in toxicity and potential biological targets have led some to argue for additional subclassification of OPs, based on their non-AChE molecular targets (Costa, 2006).

2.3) Bioactivation and degredation of OPs

The biologic activity of OPs in inhibiting AChE depends on the electrophilicity of the phosphorus atom (Mileson, 1998). The phosphorus – oxygen double bond is more electrophilic than the phosphorus-sulfur double bond, and is required for the reactivity of

OPs (Mileson, 1998). Most parent OP compounds contain a double bond to sulfur. This sulfur is replaced with oxygen through oxidative desulfuration (Costa, 2006). This is mediated by cytochrome P450s (CYPs), though the specific isoforms involved are still being investigated (Costa, 2006). In humans, CYP2B6 appears to be of primary importance in activating the OP insecticides, chlorpyrifos (CPF) and parathion, to their oxon forms (Foxenberg et al., 2011; Foxenberg et al., 2007). However, additional CYP isoforms (1A2, 2C19, 3A4, 3A5, and 3A7) were also found to activate these OPs (Foxenberg et al., 2007) Some OPs are also activated by flavin-containing monooxygenase, which acts on the leaving group as opposed to the double bond (Mileson, 1998). There is still much to be learned about the activation of OPs *in vivo.*

Deactivation of OPs occurs through several mechanisms. Certain CYP450s can catalyze oxidative dealkylation and dearylation, resulting in inactive OP metabolites (Mileson, 1998). CYP2C19 is involved in dearylation of both chlorpyrifos and parathion in humans, leading to inactive metabolites (Foxenberg et al., 2007). Additional enzymes are involved in metabolizing oxon derivatives to inactive metabolites. These include the A-esterases, such as paraoxonase 1 (PON1), and carboxylesterases (Mileson, 1998). The metabolic activation and deactivation of OPs greatly influences their toxicity. For instance, the low mammalian toxicity of malathion is attributed to hydrolysis of malaoxon by carboxylesterases (Costa, 2006).

2.4) OP toxicity in humans

2.4.1) Acute Toxicity: Cholinergic dysfunction results from acute OP poisoning in humans. This presents with multiple signs and symptoms, that commonly include sweating, salivation, lacrimation, increased bronchial secretion, bronchoconstriction, bradycardia, emesis, increased gastrointestinal motility, diarrhea, abdominal pain, miosis, tremors, muscular twitching, and flaccid paralysis (Aslan et al., 2011; Costa, 2006; Mileson, 1998; Pope et al., 2005). Death can result from respiratory failure. However, treatment with oximes (e.g. pralidoxime) and atropine are usually effective in treating OP poisoning. These are used to reactivate AChE (if OP aging has not occurred) and to inhibit cholinergic signaling through muscarinic AChRs, respectively. Patients often experience long-term deficits in memory and neruophysiological functions after recovery from acute OP poisoning (Mileson, 1998).

Two to three weeks after acute OP exposure, a second set of symptoms is sometimes observed. Organophosphate-induced delayed polyneuropathy (OPIDP) is characterized by tingling of the hands and feet, sensory loss, progressive weakness, and limpness of distal skeletal muscles in the hands and feet (Costa, 2006). This syndrome appears to be linked to OP inhibition of neuropathy target esterase as opposed to inhibition of AChE and is associated with degeneration of long axons in both central and peripheral nervous systems (Costa, 2006). OPIDP results from exposure to some, but not all, OPs.

2.4.2) Chronic Toxicity

Chronic OP exposure in farmworkers, pesticide applicators, pesticide manufacturers, and their families is also of concern. OP metabolites are detected at high levels in both

agricultural workers and their family members, suggesting OPs applied in the workplace are transferred to the household, with the potential for adverse health effects (Arcury et al., 2007; Arcury, 2005). Most epidemiological evidence suggests an association between OP exposure and reduced cognitive functions in OP applicators (Munoz-Quezada et al., 2016). However, other studies failed to identify a link between OPs and altered neurobehavioral function (Starks et al., 2012). Additional work is required to determine how chronic OP exposure may affect cognition and behavior. Multiple studies also report an association between chronic OP exposure and increased risk for neurodegenerative diseases including Parkinson's disease and Alzheimer's disease (Baltazar et al., 2014; Hancock et al., 2008; Paul et al., 2016; Sanchez-Santed et al., 2016). *In vitro* studies in PC12 cells and *in vivo* studies on neonatal rats using low doses of OPs show differential gene expression in genes associated with Parkinson's disease, further indicating a link between OP exposure and disease development (Slotkin and Seidler, 2011). Additionally, associations have been made between chronic OP exposure and cancer (Jones et al., 2015; Koutros et al., 2013; Lerro et al., 2015). These studies suggest that chronic OP exposure may have adverse health effects in adult populations.

2.4.3) Developmental Toxicity: Children are also exposed to low doses of OPs *in utero* and throughout adolescence. This is evidenced by the presence of OP metabolites in cord blood at birth and in urine and superficial samples from older children (Arcury et al., 2007; Arcury, 2005; Huen et al., 2012; Whyatt et al., 2004; Wilson et al., 2010). EPA mandated phase-out of certain OPs for household use has reduced the potential for childhood exposure to OPs, and a concurrent decrease in OP metabolites in children have been reported (Whyatt et al.,

2004; Wilson et al., 2010). However, children have a lower ability to metabolize OPs to inactive forms than adults, are more likely to ingest OP residues because of high hand-to-mouth contacts, and are exposed to OPs during critical periods of development, which could place them at greater risk from OP exposure. OP levels in chord blood have been linked to lower birth weight and length (Whyatt et al., 2004). OP exposure is also associated with decreased reflex responses in newborns (Engel et al., 2007). Prenatal and postnatal OP exposure has also been linked to reduced neurobehavioral function in children (Bouchard et al., 2011; Gonzalez-Alzaga et al., 2014; Roberts et al., 2012; Rohlman et al., 2005). Some studies also provide evidence suggesting OPs can contribute to autism (Shelton et al., 2014). Cumulatively, these epidemiological studies indicate neurobehavioral sensitivity of children to low dose OP exposures.

Less research has been conducted on the contribution of OPs to human birth defects, but the few studies that have been performed suggest OP exposure may affect additional structures outside of the nervous system. A female agricultural worker experienced symptoms of acute cholinergic toxicity after entering a field sprayed with a mixture of OP and carbamate pesticides (Romero, 1989). She was four weeks pregnant at the time of exposure, which corresponds with fetal organogenesis. A full term child was born with multiple birth defects (Romero, 1989). The heart was malformed, displaying a septal defect with stenosis of the pulmonary valve and a hypertrophied right ventricle (Romero, 1989). A second case study also identified structural defects of the heart in infants exposed *in utero* to a CPF containing compound (Sherman, 1996). A more recent, hypothesis generating, study

reported an association between OP exposure during the first trimester of pregnancy and pulmonary valve stenosis in infants (Carmichael et al., 2014). CPF exposure was specifically linked with risk for atrial septal defects (Carmichael et al., 2014). While caution must be exercised in interpreting these results, similar heart defects have previously been reported in zebrafish, African clawed frog, and chick embryos exposed to OPs during development, suggesting conserved developmental pathways are disrupted by OP exposure during organogenesis (Pamanji et al., 2015a; Pamanji et al., 2015b; Snawder and Chambers, 1990; Wyttenbach and Thompson, 1985). In the case studies where OP exposure during human organogenesis was known, defects were also observed in the eyes, ears, and jaw (Romero, 1989; Sherman, 1996). Structural brain defects were observed in all five cases (Romero, 1989; Sherman, 1996). Similar defects in craniofacial structures, eye development, and brain formation have also previously been described in animal models (Wyttenbach and Thompson, 1985). These similarities suggest conservation in signaling pathways and OP toxicity during organogenesis among vertebrates.

Interestingly, OP exposure has been shown to disrupt intestinal development in *Xenopus laevis* tadpoles (Bonfanti et al., 2004; Snawder and Chambers, 1989, 1990). As discussed previously, the etiology of human intestinal defects remains largely unknown. A recent epidemiological study investigated the risk of residential agricultural pesticide exposures on several birth defects, including anorectal atresia/stenosis (Carmichael et al., 2016). While no strong associations were identified, OPs were one of only two chemical classes to show any relationship with anorectal atresia/stenosis (Carmichael et al., 2016). No

other epidemiological studies have been conducted to investigate potential links between pesticide exposure and congenital intestinal malformations. However, the phenotypic similarities between aquatic vertebrates and human case studies for other malformations, and the dearth of information on gastrointestinal defects, would make the interaction between OP exposure and intestinal malformations an interesting path to pursue further.

3) Acetylcholinesterase (AChE)

AChE is an evolutionarily conserved serine hydrolase. The catalytic triad, consisting of serine, histidine, and glutamate residues, resides at the bottom of a deep, narrow gorge (Silman and Sussman, 2005; Soreq, 2001). AChE is a rapid enzyme despite the depth of the catalytic cleft, which is attributed to the conserved amino acids lining the gorge that help position ACh (Silman and Sussman, 2005; Soreq, 2001). The neurotransmitter, ACh, is cleaved at this site into choline and acetate. This esterase activity is essential for terminating cholinergic signaling within the peripheral and central nervous systems. In addition to this esterase site, AChE has non-enzymatic, adhesive functions and shares sequence and structural homology with a number of adhesion molecules including neuroligins, gliotactin, and neurotactin (Botti et al., 1998; Lenfant et al., 2014). Several studies suggest there may be functional overlap of AChE's non-enzymatic functions with those described for neruoligins and other homologues (Darboux et al., 1996; Grifman et al., 1998; Johnson et al., 2008). Furthermore, AChE has been reported to possess aryl acylamidase activity. While no physiological function has been identified for this activity, some evidence suggests that the site is involved in cross-regulation between the cholinergic and serotonergic systems and

may also be involved in plaque formation in Alzheimer's disease (Ciro et al., 2012; Wright et al., 1993). Interestingly, the aryl acylamidase activity of AChE appears to be more sensitive than the esterase activity to inhibition by the anti-cholinesterase drugs, Huperzine and donepezil, used for treatment of Alzheimer's disease (Rajesh et al., 2009). Thus, AChE may have multiple biological functions. The structure and functions of AChE are reviewed in more detail below.

3.1 AChE structure

AChE is expressed in diverse taxa, from organisms that lack nervous systems entirely (e.g. plants, sponges) to those with complex neural circuitry (e.g. humans). Most species contain a single gene copy of AChE, though a few exceptions (e.g. *C. elegans*) have as many as four AChE genes, which may each have their own distinct functions (Massoulie, 2002). The protein variants observed in vertebrates are due to alternative splicing of the AChE pre-mRNA. In mammals, this results in three major isoforms, T (or S) (tailed/synaptic), H (or E) (hydrophobic/erythrocytic), and R (readtrhough) (Massoulie, 2002; Soreq, 2001). These three isoforms share exons 1-4 but differ in their carboxy-terminal sequences. Specifically, AChE-T contains exon 6 instead of exon 5, which is found in the other two forms. AChE-H and AChE-R forms differ only in the inclusion of a portion of intron 4 in the AChE-R isoform (Soreq, 2001). All three isoforms have catalytic activity, but their ability to associate with various proteins and subsequent subcellular localization varies based upon the carboxy terminus. AChE-T monomers dimerize through disulfide bonds between cysteine residues near the carboxy terminus. Additional tetramerization occurs through hydrophobic

interactions (Soreq, 2001). AChE-T tetramers can further interact with a hydrophobic subunit (Proline Rich Membrane Anchor - PRiMA) or a collagen subunit (ColQ). Both PRiMA and ColQ contain a proline rich attachment domain (PRAD) near their amino-termini (Massoulie, 2002; Silman and Sussman, 2005). The PRAD domain interacts with a tryptophan (W) amphiphilic tetramerization (WAT) domain that is conserved across vertebrates in the carboxy terminus of AChE-T (Massoulie, 2002; Silman and Sussman, 2005). PRiMA contains extracellular, transmembrane, and cytoplasmic domains. PRiMA's extracellular domain anchors AChE to the cell membrane (Massoulie, 2002). The proteins that may interact with PRiMA's cytoplasmic domain are still largely unknown (Massoulie, 2002). ColQ similarly anchors AChE to basal lamina. It seems likely that other proteins in the basal lamina (e.g. heparan sulfate, transglutaminases, lysyl hydroylases) may also associate with collagen-tailed AChE (Massoulie, 2002). AChE-H undergoes modification in the endoplasmic reticulum, which results in the attachment of a glycophosphatidylinositol group at the carboxy-terminus of the protein (Massoulie, 2002). This glycophosphatidylinositol group anchors AChE to the membrane of erythrocytes (Massoulie, 2002). The functional significance of these isoforms is incompletely understood. However, shifts in the splicing pattern of AChE have been observed, with increased transcription of AChE-R in response to stress, trauma, or exposure to AChE inhibiting compounds (Soreq, 2001). Additionally, AChE isoforms are expressed at different relative levels in neural and non-neural cells and tissues (Montenegro et al., 2014; Thullbery et al., 2005). This suggests the different isoforms of AChE may have different biological functions.

3.2 AChE Classical Role – Neurotransmission and acetylcholine degradation

The esterase activity that cleaves ACh, terminating cholinergic transmission in the nervous system is the best-defined function of AChE. The importance of this function is underscored by the large number of naturally occurring and human engineered compounds that inhibit activity. These have been widely used as pesticides (OPs, carbamates), warfare agents (sarin, soman, VX), and, more recently, in the treatment of mental degeneration and neuromuscular diseases (Alzheimer's, myasthenia gravis).

AChE is expressed at cholinergic synapses and muscular junctions. ACh binds to muscarinic or nicotinic AChRs to transmit electrical signals in the nervous system, alter muscle contraction, and regulate the activity of organs innervated by the parasympathetic nervous system. The subsequent degradation of ACh is essential for terminating nervous signaling, and allowing the neuron/muscle to return to a relaxed state. AChE hydrolyzes ACh at the bottom of the catalytic trough. Essential to this function are the invariant residues that comprise the catalytic triad – serine, histidine, and glutamate (Fig. 1.3). Although the amino acids surrounding these residues are not directly involved in hydrolysis, they are highly conserved across species, and mutation can reduce or eliminate the esterase activity (Behra et al., 2002; Sternfeld et al., 1998). ACh may first interact with the peripheral anionic site (PAS), which helps orient the molecule as it enters the catalytic trough. The conserved residues lining the gorge facilitate movement of the ligand to the catalytic triad, and contribute to the rapid hydrolytic reaction. Interaction between ACh and the hydroxyl group of the serine residue results in cleavage of ACh, releasing choline and forming an acetylated-

serine intermediate. Water is ubiquitous in and around the gorge, and is essential for hydrolysis of the acetate molecule, releasing it form the gorge. Displacement of acetate restores AChE to its native state and allows for degradation of additional ACh molecules. Inhibition of this activity results in accumulation of ACh, increased cholinergic signaling, and ultimately to toxicity as described in Section 2.4.

3.3 Non-classical functions of AChE

Much of the evidence suggesting that AChE has functions in addition to termination of nervous signaling, comes from *in vitro* work in a variety of cell lines. These studies highlight a wide range of cellular behaviors regulated, at least in part, by AChE. These include cell differentiation, proliferation, survival, cytoskeletal rearrangements, polarization, cell movement, and cell-substrate adhesion. The adhesive role may be of particular importance, as cell interactions with the substrate can influence all of the other behaviors linked to AChE. Even in non-synaptic contexts, AChE's esterase activity may be of importance in degrading ACh involved in paracrine signaling. For simplicity, the evidence for non-classical functions of AChE *in vitro* are reviewed by cell type below.

3.3.1 AChE in neurons: Interestingly, much of the evidence for non-classical activities comes from work in neuronal cell lines. Some of these studies were conducted in non-cholinergic neurons, which nevertheless, express AChE. AChE is also found in cholinergic neurons prior to the expression of other cholinergic proteins (e.g. ChAT, AChRs) during neurite extension. *In vivo*, the observation that AChE is expressed prior to synaptogenesis, further suggests a function in addition to the degradation of ACh in neuronal transmission. Work in neuronal

cell lines also establishes a function of AChE in cell-substrate adhesion, cytoskeletal rearrangements, and cell differentiation.

AChE can bind directly to extracellular matrix proteins (e.g. laminin I, collagen IV) through specific peptides located in or near the PAS (Johnson and Moore, 2003, 2004). Antibodies that recognize the PAS reduce neuroblastoma adhesion, but do not necessarily inhibit catalytic function (Johnson and Moore, 1999, 2004). Similarly, AChE inhibitors that bind to the PAS reduce neuroblastoma adhesion, while catalytic specific inhibitors do not (Johnson and Moore, 1999). This suggests that AChE mediates cell adhesion to substrate independently of its esterase activity. However, interactions at the PAS can influence AChE esterase activity and indicate that esterase activity is reduced when AChE is involved in cell-substrate adhesion (Johnson and Moore, 2004). In the rat R28 cell line, AChE is both bound to the cell membrane and secreted (Sperling et al., 2012). When plated on laminin-1, AChE activity in membrane bound AChE is decreased relative to cells cultured on poly-L-lysine or gelatin (Sperling et al., 2012). Similarly, a reduction in AChE activity was observed when rat primary superior cervical ganglia were cultured on laminin compared with poly-D-lysine (Howard et al., 2005). This suggests that membrane bound AChE directly interacts with ECM to mediate cell adhesion. Additionally, this suggests that the subset of AChE involved in cell-substrate adhesion might be different from that portion involved in ACh cleavage. This is further supported by the observation that unlike membrane-bound AChE, secreted AChE activity was unaffected by substrate type (Sperling et al., 2012). AChE is associated with adhesion to multiple substrates, which is unsurprising given the electrostatic nature of

the PAS. Neuronal (and other cell types) are also adherent on AChE coated coverslips, further highlighting the adhesive role of this protein (Campanha et al., 2014). However, some evidence suggests that the interaction with ECM proteins is specific to cell type, the presence of heparan sulfates, and stage of differentiaion/development. For instance, in a separate neuroblastoma cell line (N1E.115) overexpression of AChE results in increased cell adhesion to plastic, poly-L-lysine, collagen I and collagen IV while AChE knock-down reduces adhesion on these substrates (Sharma et al., 2001). However, the level of AChE expression had no influence on cell adhesion to laminin (Sharma et al., 2001). Dorsal root ganglia cells show greater neurite outgrowth on matrigel compared with Collagen I, though AChE activity is higher in cells placed on the collagen substrate (Dupree and Bigbee, 1994; Gupta and Bigbee, 1992). Evidence from other cell types (e.g. fibroblasts – discussed below 3.3.3), also suggests that AChE-substrate interactions are cell specific. In addition to physical adhesion to ECM, AChE may be involved in regulating expression of ECM proteins. For instance, exposure of astrocytes to the OPs, diazinon or diazoxon, results in decreased fibronectin secretion, although this change might also be linked to increased oxidative stress (Pizzurro et al., 2014).

AChE is also involved in neurite outgrowth. Overexpression of AChE results in increased neurite length, while reduced AChE expression is associated with significantly shorter neurites, relative to controls (Giordano et al., 2007; Sharma et al., 2001; Sperling et al., 2012; Sternfeld et al., 1998; Yang et al., 2008). Exposure to AChE inhibitors also reduces neurite outgrowth in cultured neurons (Campanha et al., 2014; Chang et al., 2006; Dupree

and Bigbee, 1994; Howard et al., 2005; Pizzurro et al., 2014; Yang et al., 2008). This neuritogenic property may be independent of esterase activity. AChE transfection of FB5 cells from the neuroblastoma cell line (N19TG2) increases neurite outgrowth (Giordano et al., 2007). This neurite outgrowth is inhibited with exposure to the PAS inhibitor, fasciculin, but not by the esterse site inhibitor, edrophonium (Giordano et al., 2007). A membrane bound, catalytically inactive form of AChE increased neurite length to the same degree as a catalytically active membrane bound version of AChE (Sternfeld et al., 1998). Catalytically active versions of AChE not tethered to the cell membrane had no influence on neurite outgrowth (Sternfeld et al., 1998). This suggests that membrane bound AChE has functions in neuritogenesis that are independent of esterase activity. As the PAS is involved in cell adhesion, and also appears to function in neuritogenesis, this data also suggests that AChE may influence neuronal development through an ECM mediated mechanism. Additional work shows that the PAS peptides involved in AChE mediated substrate adhesion also promote neurite elongation, in some cases to the same degree as full length AChE (Johnson and Moore, 2004). As these peptides lack catalytic activity, this further supports a non-esterase function of AChE in both cell adhesion and neurite extension. Futhermore, catalytically inactive AChE and the AChE homologue, neuroligin, (which lacks esterase activity and is involved in cell-substrate adhesion) rescued neuritogenesis to the same degree as enzymatically active AChE, providing further evidence of a non-esterase, adhesive role for AChE (Grifman et al., 1998). Similarly, the ECM plays an important role in mediating neurite outgrowth. Neurite length was significantly increased by either AChE overexpression

or culture on laminin, with the longest neurites were observed in AChE overexpressing cells plated on laminin (Sperling et al., 2012). The interaction of AChE with the ECM may preclude activity at the esterase site by reducing the accessibility of the gorge. For instance, CPF exposure reduces axon outgrowth on poly-D-lysine, but has no affect on neurite growth when cells are plated on laminin instead (Howard et al., 2005). This suggests that the conformation of AChE may change when interacting with ECM or substrate, and that the protein conformation is important for morphogenic properties. Similarly, the reduction in neurite extension observed due to exposure to diazinon or diazoxon can be rescued by supplementing cultures with exogenous fibronectin (Pizzurro et al., 2014). Interestingly, in AChE transfected neuroblastoma cells, culture on fibronectin or vitronectin actually decreases neurite length (Giordano et al., 2007). Neurite elongation can be restored either by co-treating the cells with the PAS inhibitor, fasciculin, or by supplementing the fibronectin with heparin sulfate proteoglycans (Giordano et al., 2007). This provides additional evidence for competition between ECM and inhibitor binding that can influence AChE's morphogenic functions and suggests that the morphogenic potential of AChE depends upon the interacting ECM proteins. At least in neurons, AChE-Laminin interactions may promote neuritogenesis, while AChE-Fibronectin interactions may inhibit outgrowth.

Neurite extension depends upon alterations in the cytoskeletal structure. Such changes in cytoskeleton can be induced through cell-substrate interactions, through activation of signal transduction pathways (Gupton and Gertler, 2010). Given AChE's role in adhesion and neurite growth, it is unsurprising that AChE is also linked to cytoskeletal changes during

neuritogenesis. In addition to the decreased neurite length reviewed above, inhibition of AChE is associated with abnormal neurofilament accumulation and phosphorylation (Chang et al., 2006; Dupree and Bigbee, 1994; Dupree et al., 1995). Furthermore, overexpression of AChE is associated with increased expression of the cytoskeletal protein, vimentin (Keller et al., 2001). Thus, AChE's morphogenic role includes regulation of cytoskeletal changes in differentiating neurons.

3.3.2 AChE in bone: Similar to neurons, AChE has non-esterase functions in bone development *in vitro*. AChE expression is detected within osteosarcoma cell lines (Saos – 2, MG63, TE85, MC-3T3, etc.) as well as in primary osteoblasts from both humans and rats (Genever et al., 1999; Grisaru et al., 1999; Inkson et al., 2004). AChE expression increases with differentiation of the bone cells both in culture and in fetal bone cross-sections. In fetal bone, AChE expression is low within the proliferating chondrocytes, and increases as the chondrocytes differentiate (Grisaru et al., 1999). Knockdown of AChE with anti-sense oligonucleotides *in vitro*, results in an increase in cell proliferation. Knockdown of the related esterase, BChE, had no effect on cells (Grisaru et al., 1999). Taken together, this suggests AChE may suppress proliferation and promote differentiation.

Additionally, AChE may facilitate cell-matrix interactions. Exposing osteoblasts to the AChE inhibitors, DFP or BW284C51, significantly reduced cell-substrate adhesion, while exposure to the BChE inhibitor, iso-OMPA, had no effect (Inkson et al., 2004). Similarly, osteosarcoma cells are adherent on AChE, but not BChE, coated coverslips, suggesting an adhesive role of AChE, independent of esterase activity (Genever et al., 1999).

AChE is expressed within the Golgi and at puncta in the cell membranes of osteosarcoma cells (Genever et al., 1999; Inkson et al., 2004). When protein transportation is inhibited in these cells, AChE activity is decreased, suggesting AChE is secreted by bone cells (Inkson et al., 2004). Furthermore, AChE is only expressed in osteoblast (matrix producing) cells in adult human bone samples (Genever et al., 1999). Mechanical stimulation triggers an osteogenic response that results in recruitment of osteoblasts and deposition of fresh matrix. Increased AChE expression is detected both within the recruited osteoblasts and in the new matrix layer (Inkson et al., 2004). Together, this suggests that secreted AChE may play an important role in cell-matrix interactions in bone tissue.

3.3.3 AChE in fibroblasts: AChE is also expressed within fibroblast cells, with AChE-T primarily expressed (Anderson et al., 2008). Despite expression of the protein, AChE enzymatic activity is very low in fibroblasts (Anderson et al., 2008). AChE is expressed near the cell surface substratum and is present at the leading edge of migrating fibroblast and astrocyte cells, suggesting a role in protrusion (Anderson et al., 2008). Further evidence supporting a role in polarized migration comes from scratch assays of confluent cells. Following wounding, fibroblasts move toward the scratch, closing the wound. Incubation of fibroblasts with either an anti-integrin antibody (positive control) or an anti-AChE antibody slows the rate of wound closure (Anderson et al., 2008). Inclusion of the anti-AChE antibody similarly impaired wound closure in 3T3 cells (Anderson et al., 2008). Overexpression of AChE-T in 3T3 cells improved wound healing, with a faster rate of closure observed compared with control cells (Anderson et al., 2008). While this work suggests a non-neuronal

role of AChE in polarized cell movement, the importance of esterase or non-esterase activities are not assessed. However, exposing 3T3 cells to ACh did not affect wound closure as would be expected if the esterase function was related to the polarized behavior of the cells (Anderson et al., 2008). Combined with the low levels of enzymatic activity found in fibroblasts, it seems likely that the role AChE plays in promoting polarized cell behavior is independent of esterase activity. Adhesion was also affected in 3T3 cells cultured with the anti-AChE antibody, suggesting that AChE may mediate polarized cell behavior through interactions with the substrate (Anderson et al., 2008). However, in a separate study, rat primary fibroblasts were not adherent on AChE coated coverslips (Genever et al., 1999). This may suggest that AChE at the cell membranes of fibroblasts is involved in adhesion to a different ECM protein, or that secreted AChE in the ECM does not facilitate adhesion in this cell type. Laminin is also unlikely to bind to AChE and contribute to AChE mediated adhesion in fibroblasts, as culture of 3T3 cells on laminin did not alter the distribution of AChE in this cell line (Anderson et al., 2008). (In contrast, the laminin binding protein α-dystroglycan was redistributed in these cells.) However, AChE can interact with a wide range of ECM proteins and may have a specific interaction in fibroblast cells that contributes to polarized movement, which has yet to be identified.

3.3.4 AChE in intestinal cells: AChE is similarly expressed in non-neuronal intestinal cell cultures. In Caco-2 cells, AChE expression increases with differentiation (Plageman et al., 2002; Xiang et al., 2008). Inhibition or knockdown of AChE reduces markers of differentiation, suggesting a role for the protein in this process (Xiang et al., 2008). AChE

expression is also correlated with cell cycle progression. Knockdown of AChE reduces the proportion of cells in the G2/M stage of the cell cycle and overexpression of AChE increases the proportion of cells in this phase (Xiang et al., 2008). AChE expression is linked with regulation of cell cycle progression in other colon cancer cell lines as well (Syed et al., 2008). Importantly, AChE only influences cell cycle progression in differentiating cells, and does not lead to an increase in cell proliferation, further supporting a role for this protein in differentiation (Syed et al., 2008; Xiang et al., 2008). There is also evidence that AChE promotes cell-substrate adhesion in intestinal cell lines (Syed et al., 2008). In HTB-38 cells, AChE overexpression significantly increased cell adhesion on fibronectin compared with controls, though no effect was observed on laminin or collagen (Syed et al., 2008). In contrast to other studies where AChE directly interacts with ECM proteins, AChE's function in cell-substrate adhesion appeared to be indirect. Incubation of these cells with an anti-AChE antibody had no affect on cell adhesion, whereas competition with integrin-fibronectin binding reduced substrate adhesion in AChE transfected cells (Syed et al., 2008). This result led to speculation that AChE positively regulates integrin expression, though AChE's relationship with integrin was not directly investigated. This study also used cell permeable and impermeable AChE inhibitors to suggest a cytosolic localization and function of AChE in intestinal cells (Syed et al., 2008). However, other studies suggest membrane localization of AChE in intestinal cell lines (Plageman et al., 2002). This discrepancy may result from specific differences between the cell lines that were used. It is also possible that both cytosolic and membrane AChE expression are required for intestinal cell behaviors.

Additionally, these studies provided indirect evidence for AChE's localization in the cell, and better information on AChE's expression pattern in intestinal cells is needed.

3.4 Role of AChE in development

<u>*3.4.1 Role of AChE in invertebrate morphogenesis:*</u> AChE is also implicated in functioning non-neuronally during invertebrate morphogenesis. For instance, AChE is expressed in the mesenchym which involutes during gastrulation in sea urchins (Drews, 1975). AChE activity increases from mid- to late gastrulation and is later expressed in the non-neural mesenchyme of the elongating spiculae (Ohta et al., 2009). Spicule elongation depends upon cytoskeletal rearrangements during sea urchin morphogenesis. However, exposure to the AChE inhibitor, eserine, results in failure of the spicules to elongate in three different sea urchin species (Ohta et al., 2009). The absence of elongation in these structures due to AChE inhibition suggests that AChE may be involved in mediating cytoskeletal rearrangements during morphogenesis, similar to the observations made *in vitro*. Similarly, in developing or regenerating octopus arms, AChE is expressed in the differentiating mesenchymal cells (Fossati et al., 2015). AChE expression initially decreases in the injured arm, while proliferation increases (Fossati, 2013). Following the proliferative stage, AChE expression is rapidly increased, corresponding with the morphogenic movements and differentiation in the regenerating arm (Fossati, 2013; Fossati et al., 2015). Eventually, AChE expression becomes restricted to nerves as the arm matures, similar to observations in chicks and amphibians (Drews, 1975; Fossati et al., 2015). Although these observations are associative, their timing and similarity

across a wide variety of taxa hints at a conserved function for AChE in cells undergoing morphogenic movements.

3.4.2 Role of AChE in vertebrate morphogenesis: Considerable evidence points to non-esterase functions of AChE *in vitro*, but the importance of these functions *in vivo* has been widely contested. The most striking evidence supporting a morphogenic role *in vivo* is associative. For instance, AChE activity first appears in cells undergoing morphogenic movements in chicks and amphibians (Drews, 1975). AChE activity persists as cells move and differentiate, and often decreases after cells have achieved their final positions (Drews, 1975). This pattern of AChE activity is observed in non-neural tissues, including the heart, gut, liver, pancreas, and limb buds (Drews, 1975). AChE activity is similarly detected in the zebrafish heart prior to innervation (Bertrand et al., 2001). The expression of AChE in non-neural organs and tissues undergoing morphogenic movements in many vertebrate species provides intriguing evidence of a non-neuronal function for the protein.

Wound healing also suggest a morphogenic role of AChE. AChE is normally absent in the epidermis of mice skin. Wounding of the skin leads to a rapid increase in AChE expression within the leading edge of the epidermis – the cells that migrate to close the wound (Anderson et al., 2008). Following wound closure, AChE expression decreases to undetectable levels in the epidermis, further suggesting a non-neural role in cell movement (Anderson et al., 2008). The similarities in AChE expression patterns in developing/regenerating tissues between vertebrates and invertebrates suggest that AChE

may have an evolutionarily conserved function in promoting cell movement and differentiation.

Several AChE mutants have been created in zebrafish, all of which have motility defects and die in early larval stages (by 144 hpf) (Behra et al., 2002; Downes and Granato, 2004). Neuromuscular defects including decreased slow muscle myosin, reduced AChR clustering at junctions, defective axon pathfinding, and increased sensory neuron death were observed in fish with a point mutation that eliminated esterase activity but presumably left the protein structure in tact (Behra et al., 2002). Muscular defects were rescued in double mutants lacking functional nicotinic AChRs and knock-down of AChE by morpholino injection phenotypically resembled the defects observed with the point mutation, leading to the conclusion that AChE's enzymatic activity is of primary importance in the formation of neuromuscular junctions in zebrafish (Behra et al., 2002). However, these results were not reproduced in two other AChE mutant lines (one with a different point mutation ablating esterase activity, and one with a premature stop codon eliminating both esterase activity and the carboxy-terminus essential for neurite outgrowth *in vitro*) (Downes and Granato, 2004). This discrepancy indicates that differences in genetic backgrounds may influence phenotypic outcomes and makes it difficult to generalize AChE's function in vertebrate development. AChR clustering was lost in these mutant lines, suggesting that AChE may be required for maintenance but not formation of neuromuscular junctions in zebrafish (Downes and Granato, 2004). Interestingly, AChE is expressed in other tissues including the developing heart (Bertrand et al., 2001; Downes and Granato, 2004). Pericardial edema was identified in

AChE mutants, which could be linked to heart malformation, though no gross abnormalities were observed (Downes and Granato, 2004). Similarly, zebrafish exposed to organophosphate or carbamate pesticides display pericardial edema, which may be related to inhibition of AChE (Lin et al., 2007; Pamanji et al., 2015a; Pamanji et al., 2015b; Schock et al., 2012). Interestingly, these exposures are also associated with abnormal heart looping, suggesting a possible role for AChE in heart morphogenesis (Pamanji et al., 2015a; Pamanji et al., 2015b; Schock et al., 2012).

Similarly, AChE-/- knockout mice show surprisingly mild phenotypes (Duysen et al., 2002; Xie et al., 2000). At birth, the nullizygous mice resemble heterozygous and wild type littermates, but die within 12 days unless supplemented with nutrient rich formula (Duysen et al., 2002; Xie et al., 2000). Grossly, internal organs appear normal, suggesting that AChE is not essential for organogenesis and/or that compensatory mechanisms are in place (Xie et al., 2000). Older AChE-/- knockout mice display a range of abnormalities including abnormal gait, posture, body tremors and seizures, and behavioral abnormalities, which could be explained by the absence of AChE's esterase activity (Duysen et al., 2002). However, other defects, such as abnormal development and maintenance of the retina and reduced body weight could be related to loss of either enzymatic or non-catalytic activities (Bytyqi et al., 2004; Duysen et al., 2002).

The absence of gross phenotypes linked to non-esterase functions in vertebrate models has led to speculation that the adhesive roles described *in vitro* are not present *in vivo*, or that redundancy of functions makes AChE's non-esterase activities non-essential *in*

vivo (Cousin et al., 2005; Johnson et al., 2008). However, internal organs were not closely examined in these models, and the defects that were observed have not been further investigated to determine how esterase or non-esterase activities may contribute to normal function. Therefore, AChE's non-neuronal role in vertebrate development remains unresolved.

3.5. AChE in Human Health and Disease: Organophosphate exposure in humans is acutely lethal at high doses, and chronic exposure has adverse health effects as discussed above (Section 2. 4). AChE inhibiting compounds have also been useful for treating human diseases including Alzheimer's disease and *myasthenia gravis*. The symptoms of both diseases result from a decrease in cholinergic signaling, which can be temporarily alleviated with AChE inhibitors, increasing the local concentration of ACh. However, AChE inhibition is associated with increased AChE transcription and altered slice variants, so may be detrimental in the long term (Soreq, 2001). Polymorphisms in humans have only recently been identified. Currently, 19 SNPs have been found within the human *ache* gene (Hasin et al., 2004; Valle et al., 2011). However, of these, only five result in a nonsynonymous substitution (Hasin et al., 2004; Valle et al., 2011). These substitutions do not occur in conserved amino acids and there is no evidence that they impact esterase activity(Hasin et al., 2004; Valle et al., 2011). Interestingly, however, there is some evidence for differences in sensitivity to OP inhibition, as well as differences in protein stability (Valle et al., 2011). There are differences in SNP frequencies among different ethnic groups, though, overall AChE SNP frequencies are low (Hasin et al., 2004; Valle et al., 2011). Though it is possible

that the nonsynonymous mutations affect other AChE functions, these studies suggest the necessity of functional AChE for survival.

AChE may contribute to several human diseases. AChE's non-esterase functions have been suggested to contribute to the formation of β-plaques in Alzheimer's disease (Ciro et al., 2012; Inestrosa et al., 2004; Small et al., 1996). Additionally, AChE is associated with several congential birth defects. *Myasthenia gravis* can result from a mutation in *colq*, which is transcribed to produce the collagen-like subunit that anchors AChE-T to the membrane (Soreq, 2001). This mutation prevents AChE from being localized to the basal laminia of the neuromuscular junction. Additionally, elevated AChE expression is diagnostic of Hirschsprung's disease (HSCR) and intestinal neuronal dysplasia (IND), both neuronal defects of the intestine (Martucciello et al., 2002; Moore and Johnson, 2005). In HSCR, aganglionosis of the colon requires surgical resection to correct. Depending upon the length of colon removed, higher caloric diets are often required for the patients to thrive. Several genes have been implicated in the etiology of HSCR, suggesting that AChE is not directly causative. However, other evidence indicates AChE may have a direct role in contributing to this congenital defect (Moore and Johnson, 2005).

4) Rationale and hypothesis

As discussed in the previous sections, AChE is a highly conserved protein essential for termination of cholinergic signaling. In addition to this esterase activity, AChE has non-esterase roles *in vitro* in cell-substrate adhesion, cell polarization, cytoskeletal rearrangements, and cell movements. Expression of AChE in non-neural tissues during

invertebrate and vertebrate morphogenesis and healing has lead many to hypothesize that AChE has conserved non-esterase functions *in vivo.* However, limitations of existing vertebrate models and difficulties in separating esterase from non-esterase linked phenotypes have precluded determination of AChE's non-neuronal functions *in vivo.* Interestingly, exposure of *Xenopus laevis* tadpoles to pesticides that inhibit AChE's esterase activity result in shortened, malrotated intestinal tracts. Binding of compounds to AChE's catalytic triad alters the protein's conformation and affects both esterase and non-esterase functions. Thus, exposure to anti-AChE compounds (e.g. organophosphates, carbamates, Alzheimer's drugs etc.) can affect both esterase and non-esterase functions of AChE. The reports of intestinal defects in *Xenopus laevis* tadpoles led us to hypothesize that AChE is required for intestinal development. We used a combination of chemical and molecular techniques to test this hypothesis with three specific aims:

1) Determine the requirement of AChE in intestinal morphogenesis
2) Determine if AChE has esterase and/or non-esterase functions in intestinal morphogenesis
3) Identify the mechanism(s) by which AChE contributes to gut morphogenesis

The results of this study have implications for a non-esterase function for AChE in vertebrate organogenesis. Our findings suggest that AChE has non-esterase functions essential for polarization, cytoskeletal reorganization, and rearrangement of the endoderm cells in the gut. These changes drive elongation of the intestine, and explain the shortened intestinal phenotypes observed with morpholino knockdown or chemical inhibition of AChE.

Additionally, we provide evidence that AChE promotes cell-substrate adhesion through a Fibronectin dependent mechanism, which likely explains AChE's ability to alter so many cellular behaviors. There are many similarities in intestinal development among vertebrates, suggesting that AChE may have a similar role in other vertebrates as well. This may be of particular relevance for human health. The etiology of human intestinal defects is still largely unknown, despite a high prevalence. Humans are also likely to be exposed to AChE inhibiting compounds as OP and carbamate pesticides are widely used. Epidemiological studies are only beginning to be conducted to consider the role agricultural pesticide exposures might play in congenital birth defects. Our work suggests that OP exposures *in utero* might contribute to human gastrointestinal defects, though this needs to be evaluated by epidemiological studies. It seems likely that AChE's non-esterase functions are conserved and that AChE is required for cell-substrate interactions, cytoskeletal changes, and polarization in the development of other organs as well. We look forward to future work that will continue to refine our understanding of AChE's roles *in vivo.*

Chemical Class	Chemicals tested	Uses	References
Azoles	Triadimefon, n-butyl isocaynate, carbendazim	Fungicide	(Lenkowski et al., 2010; Yoon et al., 2008)
Bipyridyliums	Paraquat	Herbicide	(Vismara et al., 2001; Vismara et al., 2000)
Carbamates	Carbaryl	Insecticide	(Bacchetta et al., 2008)
Carboxylic Acids	Valproic acid, pentanoic acid, butyric acid, 2-ethylhexanoic acid	Various: Plasticizer, lubricant	(Dawson, 1994)
Chlorophenoxy Acids	2,4-D	Herbicide	(Lenkowski et al., 2010)
Estrogen	17β - estradiol	Drug	(Sone et al., 2004)
Nanoparticles	CuO, ZnO, polystyrene	Various: semiconductors; drugs, skin care	(Bacchetta et al., 2014; Nations et al., 2011; Tussellino et al., 2015)
Nitroaromatic compounds	TNT, 2ADNT, 4ADNT	Explosive	(Saka, 2004)
Organochlorines	Chlorothalonil, DDT, DDD	Insecticide	(Saka, 2004; Yu et al., 2013)
Organophosphates	Malathion, Malaoxon, Parathion, Paraoxon, Dicrotophos, Monocrotophos, Chlorpyrifos, Diazinon	Insecticide	(Bonfanti et al., 2004; Modra et al., 2011; Snawder and Chambers, 1989, 1990)
Phenols	Bisphenol A, nonylphenol	Various: Plastics, resins, adjuvant	(Sone et al., 2004)
Phosphonoglycines	Glyphosate	Herbicide	(Lenkowski et al., 2010)
Triazines	Atrazine	Herbicide	(Lenkowski and McLaughlin, 2010; Lenkowski et al., 2008; Lenkowski et al., 2010)
Polymer Mixtures	Tire Debris Organic Extract	Tire product	(Mantecca et al., 2007)
Chemical Mixtures	Corexit 9500	Dispersant	(Smith et al., 2012)

Table 1.1. Anthropogeneic toxicants found to disrupt gut development in Xenopus laevis.

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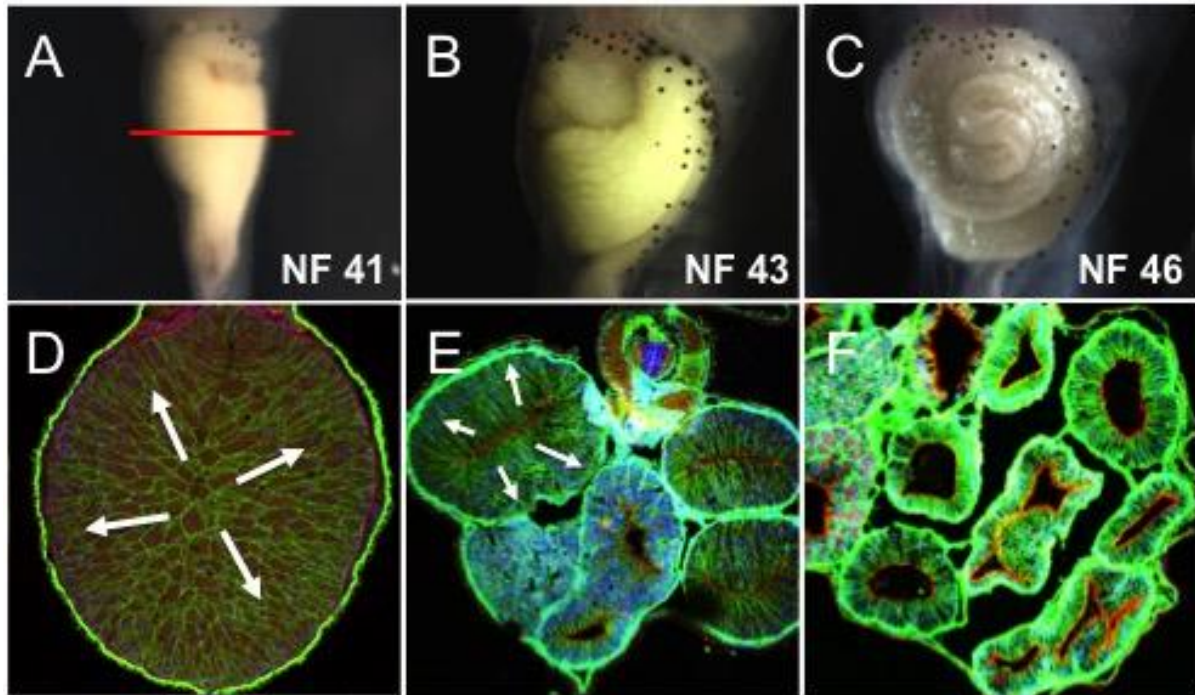

Figure 1.1) Intestinal development in *Xenopus laevis.*
The intestine is initially a short, straight primitive gut tube, with curvature first observed at NF 41 (A). The intestine rapidly elongates and coils counterclockwise within the abdominal cavity during the next 22 hours, forming a functional organ (C). This process can be visualized through the transparent skin on the ventral surface of the tadpole (A-C). The intestinal elongation observed at the gross level is due to changes occurring at the cellular level. At NF 41, a transverse section through the midgut (red line in A), reveals 4-5 layers of endoderm cells (D). These cells undergo changes in shape, polarity, and arrangement, intercalating between each other. This is evident as two endoderm cell layers at NF 43 (E) and a single cell-layered epithelium surrounding a hollow lumen by NF 46 (F).

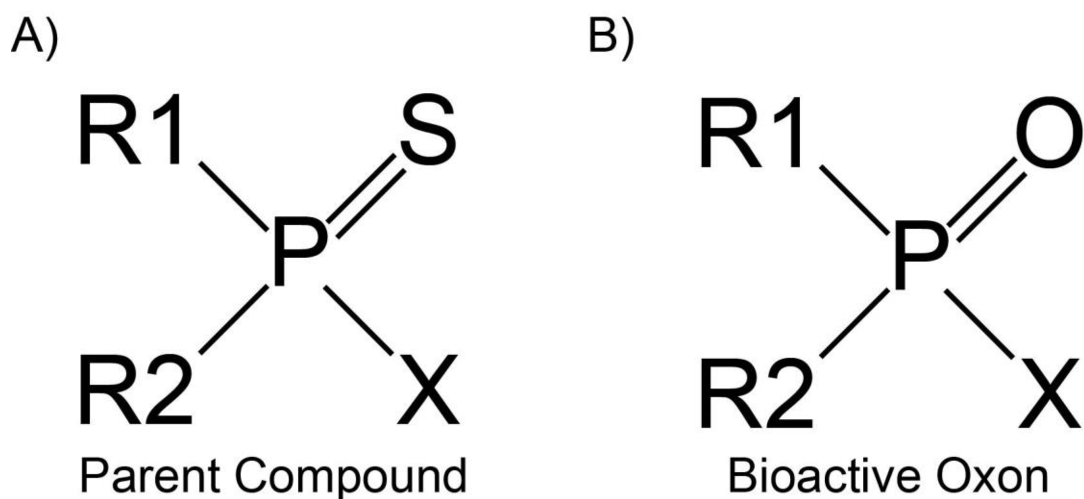

Figure 1.2) General structures of organophosphates in parental (A) and bioactive oxon (B) forms.

Metabolism of the parent compound (A) by cytochrome P450 enzymes can result in oxidative desulfuration, in which the sulfur atom is replaced by an oxygen atom (B). The oxon is highly reactive as a result of the oxygen bond and altered electrophilicity of the compound. The bioactive oxon can phosphorylate AChE.

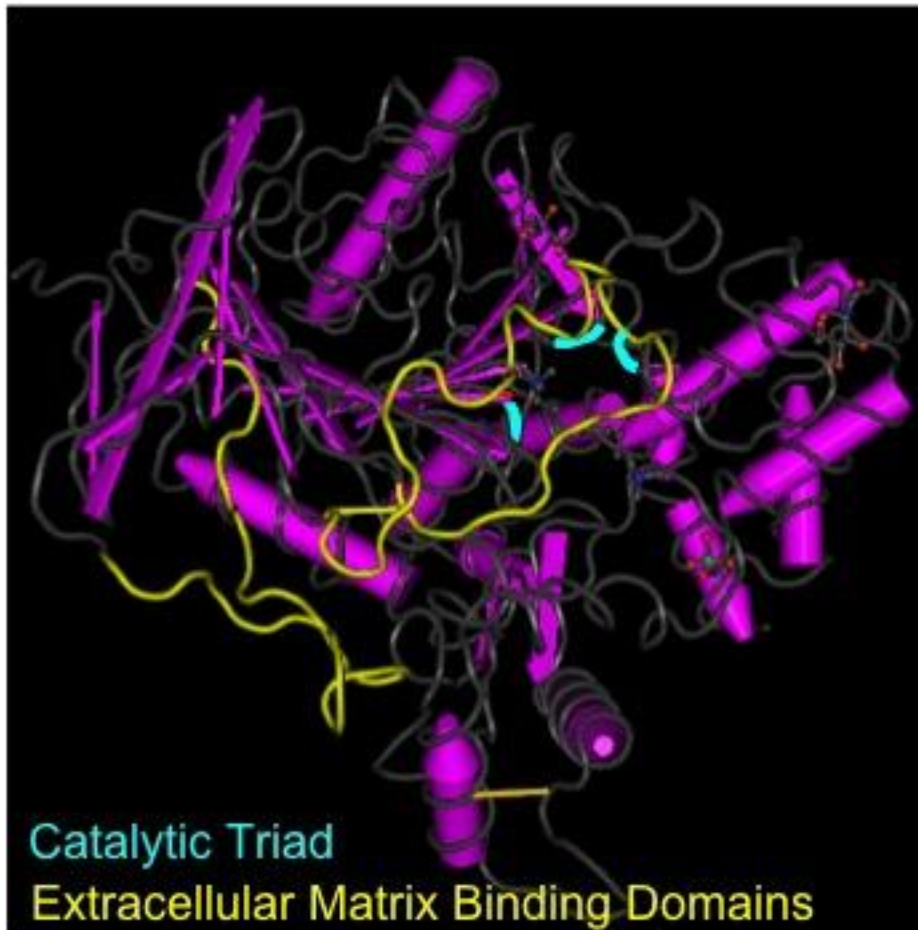

Figure 1.3) Model of AChE protein structure.
The catalytic triad is shown in turquoise. Residues involved in binding to extracellular matrix proteins (as identified by (Johnson and Moore, 2004)) are shown in yellow. The image was modified from NCBI, MMDB ID: 10053 for the mouse AChE protein.

CHAPTER 2 – CHEMICAL IHIBITION AND KNOCKDOWN OF ACETYLCHOLINESTERASE DISRUPT INTESTINAL DEVELOPMENT IN FROGS

Introduction:

Organophosphate (OP) and carbamate pesticides are among the most commonly used insecticides worldwide. These pesticide classes share a common mechanism of action through inhibition of the serine protease, acetylcholinesterase (AChE) (Costa, 2006; Mileson, 1998; Pope et al., 2005). OPs and carbamates bind to the conserved serine residue in the catalytic triad, preventing degradation of the neurotransmitter, acetylcholine (ACh) (Costa, 2006; Mileson, 1998; Pope et al., 2005), and inducing conformational changes in the protein, which can affect non-esterase functions as well (Kardos and Sultatos, 2000; Sultatos and Kaushik, 2008). These pesticides are widely applied to control insect populations. However, AChE is a highly conserved protein and exposure to OPs and carbamates can adversely affect off-target organisms, including aquatic vertebrates and humans.

OP and carbamate exposure have previously been reported to disrupt development in fish and frog species (Bonfanti et al., 2004; Pamanji et al., 2015a; Pamanji et al., 2015b; Schock et al., 2012; Snawder and Chambers, 1989, 1990). Interestingly, these pesticides disrupt organogenesis of the heart and intestine. Recent hypothesis generating studies similarly suggest an association between OP exposure and heart and intestinal birth defects in humans (Carmichael et al., 2016; Carmichael et al., 2014). Thus, a better understanding of

how these pesticides affect developmental processes is required. The shared mechanism of action among OPs and carbamates suggests that AChE may be required for morphogenesis of these organs. AChE is expressed in these organs prior to innervation (Bertrand et al., 2001; Downes and Granato, 2004; Drews, 1975) and is associated with cell migration and differentiation of non-nervous tissues (Anderson et al., 2008; Drews, 1975; Fossati, 2013; Fossati et al., 2015).

In *Xenopus laevis* tadpoles, OP exposure results in significantly shorter intestines, with fewer loops observed (Snawder and Chambers, 1989, 1990). Additionally, the diameter of the intestine is larger in OP exposed tadpoles than in solvent treated controls(Snawder and Chambers, 1990). Intestinal abnormalities have also been described in other frog species exposed to OPs during morphogenesis (Aronzon et al., 2014). Amphibian populations are declining worldwide, and pesticide exposure during sensitive windows of development might contribute to this trend. Additionally, intestinal abnormalities are among the most common of human birth defects, but their underlying etiology is largely unknown (Luks, 2011). It is likely that both genetic and environmental factors contribute to these congenital malformations. Despite the gross intestinal defects observed in frogs, the effects of OP exposure at the cellular level have not been examined. Therefore, a better understanding of how OPs affect intestinal development is required to expand our understanding of developmental and toxicological pathways.

Here we describe the effects of OP exposure on intestinal development in frog tadpoles. We show that two different OPs and a third AChE inhibitor (structurally unrelated

to the OPs) adversely affect intestinal development in a dose dependent manner in *Xenopus laevis* tadpoles. We also report similar intestinal defects in *Lepidobatrachus laevis* tadpoles exposed to the OP, malathion (MTH). Furthermore, we find that OP exposure disrupts both endodermal and mesodermal layers in the developing intestine. Similar defects are observed in the intestines of tadpoles injected with a morpholino to knockdown AChE specifically within the gut. This suggests that OPs affect intestinal morphogenesis through their interaction with AChE.

Results:

Organophosphates, Huperzine A, and AChE knockdown disrupt intestinal development

To determine the effect of organophosphate exposure on intestinal development, *Xenopus laevis* tadpoles were exposed to malathion (MTH) (0 – 20 mg/L) or chlorpyrifos-methyl (CPF-CH_3) (0 – 10 mg/L) from NF 33-46. At NF 46 tadpoles were examined for abnormalities. Intestinal defects consisted primarily of shorter gut tubes relative to solvent treated controls, but also included cases where the intestine did not coil counterclockwise (malrotated). For both OPs, we observed an increase in the percentage of embryos with intestinal defects with increasing concentrations of the pesticide (Fig. 2.1 A-H, Fig. 2.2 A,B). A slight increase in mortality was observed in the highest MTH exposure (Fig. 2.2 A'), and a large increase in mortality was found for the highest dose of CPF-CH_3 (Fig. 2.2 B'). 20 mg/L MTH and 5 mg/L CPF-CH_3 were used in subsequent experiments to evaluate the cellular effects of OP exposure, as these concentrations consistently disrupted intestinal development without causing overt mortality.

To determine if OPs affect intestinal development through inhibition of AChE, tadpoles were exposed to a structurally unrelated AChE inhibitor, Huperzine A (0-15 uM). Similar to the OPs, an increase in the percentage of tadpoles with intestinal abnormalities was found with increased Huperzine A concentrations (Fig. 2.1 I-L, Fig. 2.2 C). Huperzine A exposure did not greatly affect mortality (Fig. 2.2 C'). The similarities in intestinal phenotypes among chemically treated groups suggest that OPs affect intestinal development through inhibition of AChE. Additionally, we observed cranio-facial edema, bent spinal chords, abnormal pigmentation, hemorrhaging, and paralysis for all three chemical exposures, further suggesting a common mechanism of action. AChE activity assays were performed on whole embryos to confirm inhibition of AChE activity (data shown in Chapter 3, Fig. 3.1).

Although chemical inhibition of AChE disrupts intestinal development, many other structures are affected as well (e.g. cranio-facial edema, bent notochords). To confirm that AChE is specifically required in the intestine during morphogenesis, we knocked down AChE expression in the gut with a morpholino designed against the 5' UTR of *Xenopus laevis* AChE mRNA. *Xenopus laevis* embryos were injected at the 8-cell stage, targeting the right dorsal vegetal blastomere. This targets AChE knockdown specifically to cells of the future intestine. At NF 46, we observed a significant increase in the percentage of embryos with shortened and/or malrotated intestinal tracts, similar to those observed in chemically treated tadpoles (Fig. 2.1 M-P, Fig. 2.2 D). The percentage of tadpoles with intestinal abnormalities increased with higher concentrations of injected morpholino (Fig. 2.2 D).

Unlike chemical treatments, we did not observe defects in other structures. This suggests AChE is specifically required within the intestine during development. Morpholino injections did not increase mortality relative to control morpholino injected or uninjected controls (Fig. 2.2 D').

Malathion exposure disrupts intestinal development in Lebidobatrachus laevis

Xenopus is a useful model organism due to their experimental amenability and the ease of animal husbandry in lab (Womble et al., 2016). However, some evidence suggests that *Xenopus* are less sensitive to toxicants than other frog species (Hoke and Ankley, 2005). Their relative sensitivity compared with other frog species is still being assessed (Yu et al., 2013), but with the global decline in amphibian populations, it will be essential to have an amphibian model that can be used to assess and minimize the risk from pesticide exposures that will protect native frog species, necessitating comparison of *X. laevis* with other species. Additionally, amphibian larvae have evolved different feeding strategies (Bloom et al., 2013). Toxicants may have different effects on tadpoles with different feeding strategies due to the evolutionary divergence of signaling pathways. Similarities in toxicant effects on tadpole species may indicate conserved pathways. It is important to understand how toxicants affect development in multiple amphibian species for both the purposes of environmental regulation and to understand evolutionary processes. Recently, *Lepidobatrachus laevis,* have been introduced as a new model frog species (Amin et al., 2015). These tadpoles are much larger than *Xenopus laevis* tadpoles, grow rapidly in a laboratory environment, and appear to have similar experimental amenability to *X. laevis* (Amin et al., 2015). Additionally, *L. laevis*

are cannibalistic as tadpoles with an enlarged stomach and relatively short intestine, compared with the long, coiled intestine of the herbivorous *X. laevis* tadpoles (Amin et al., 2015; Bloom et al., 2013). However, nothing is known about the sensitivity of this frog species to pesticides. Therefore, to compare how OP exposure affects development in multiple frog species, *Lepidobatrachus laevis* and *Xenopus laevis* tadpoles were exposed to MTH at approximately the same stages of development (Gosner St 19 - 25 for *L. laevis* and NF 33-46 for *X. laevis*). Similar to observations in *X. laevis*, MTH exposure disrupted intestinal development in *L. laevis* (Fig. 2.3 A-L). The intestines were shorter in *L. laevis* tadpoles exposed to the highest dose of MTH compared with lower MTH concentrations and solvent treated controls (Fig. 2.3 A-E,K). AChE activity was assessed in whole tadpoles from each exposure group (Fig. 2.3 L). AChE activity was similar between *L. laevis* and *X. laevis*, though *L. laevis* AChE may be more sensitive to OPs than *X. laevis* AChE (Fig. 2.3 L, 1 mg/L). Additionally, the AChE sequences of the two frog species show high sequence homology (Fig. 2.4).

Chemical AChE inhibitors disrupt endoderm structure

To determine how chemical AChE inhibitors affect intestinal development, transverse cross-sections were taken through the guts of chemically treated embryos and examined immunohistochemically with a panel of antibodies chosen to examine endoderm cell shape and protein distribution. In a normal NF 46 intestine, the gut endoderm consists of a single layer of columnar epithelial cells. These cells are polarized along their apical-basal axes, and form the absorptive surface of the intestine. In solvent treated embryos, microtubules are

aligned with this axis and are enriched apically (Fig 2.5, A,A'). In contrast, microtubules are disorganized in the intestinal epithelium of tadpoles exposed to any of the chemical inhibitors (Fig. 2.5 B-D'). Endoderm cell death is also increased in these embryos, as indicated by the presence of caspase 3 positive debris in the lumen of the gut (Fig. 2.5 B-D'). Endoderm cell intercalation and polarity are also affected by AChE chemical inhibition. Integrin (a transmembrane protein involved in cell adhesion and signaling) was used to investigate endoderm cell shape (Fig. 2.5 E-G'). Compared with the columnar cell shape in DMSO treated controls (Fig. 2.5 E, E'), the endoderm of embryos exposed to chemical inhibitors appears disorganized (Fig. 2.5 F-G'). Additionally, we observe regions of the epithelium that are several cell layers thick (asterisks Fig. 2.5, Fig. 2.8), suggesting the cells do not intercalate to the degree normally observed. Polarity is also disrupted in these cells. aPKC is normally expressed at the apical surface of the epithelium (Fig. 2.5 E,E'). However, aPKC expression is reduced and shows patchy expression in the endoderm of cells exposed to AChE inhibiting chemicals (Fig. 2.5 F-G').

To determine when these cellular defects manifest, we examined the endoderm at multiple developmental stages in MTH and solvent control treated embryos (Fig. 2.6). At NF 41/42 microtubules are arranged along the apical basal endoderm cell axes (Fig. 2.6 A, A'). In MTH treated embryos, α-tubulin expression is reduced and microtubules are not as organized along the cell axes (Fig. 2.6 B,B'). As development progresses, α-tubulin becomes enriched apically and microtubules remain organized along the apical-basal cell axes (Fig. 2.6 C,C',E,E'). In MTH treated embryos, α-tubulin expression remains lower than in the

DMSO treated controls (Fig. 2.6 D,D',F,F'). Microtubules become oriented along the apical-basal axes in some of the epithelial cells, but regions with disorganized microtubules are consistently observed in both NF 43/44 and NF 46 embryos (Fig. 2.6 D,D',F,F'). At NF 41/42, endoderm cells do not display obvious differences in cell shape, and integrin expression appears similar between DMSO and MTH treated embryos (Fig. 2.6 G-H'). aPKC is only beginning to be expressed at this stage and does not appear different between the two treatments (Fig. 2.6 G-H'). By NF 43/44, endoderm cells have elongated along their apical-basal axes, and intercalated to form an epithelium 2-3 cell layers thick (Fig. 2.6 I,I'). aPKC is expressed at the apical surface of the endoderm cells lining the future lumen of the intestine (Fig. 2.6 I,I'). In MTH treated embryos, the endoderm cells are not elongated, and appear rounder in shape (Fig. 2.6 J,J'). aPKC expression appears reduced and patchy along the future luminal surface in MTH treated embryos as compared with DMSO controls (Fig. 2.6 I-J'), suggesting that polarity is disrupted. Guts from MTH treated NF 46 embryos show similar phenotypes to those of earlier stages, with reduced, patchy aPKC expression, and regions where multiple cell layers are present, as opposed to the polarized, single cell layer observed in DMSO controls (Fig. 2.6 K-L'). Expression of the cell membrane proteins, integrin (Fig. 2.6 G-L'), e-cadherin (Fig. 2.9 A-F'), and β-catenin (Fig. 2.9 G-L') do not appear to be affected by MTH exposure. Caspase 3 expression appears elevated only in NF 46 MTH treated embryos (Fig. 2.6 E-F'). This suggests that while MTH exposure induces apoptosis, cell death is likely not causative of the shortened intestinal phenotypes observed at the gross level.

AChE knockdown disrupts endoderm structure

The shared mechanism of action of the three chemicals used in this study suggests that AChE is required for endoderm morphogenesis. However, it is possible that the chemicals share an off-target mechanism. To confirm that AChE is required for gut endoderm development, we knocked down AChE specifically within the intestine by targeting microinjection of a translation blocking AChE morpholino to the dorsal, vegetal blastomeres of the eight-cell stage embryo along with GFP mRNA as a cell lineage tracer. This results in heterogeneous knockdown in the gut (i.e. cells derived from the injected blastomere express GFP and can be compared to wild-type uninjected cells in the same embryo). Immunohistochemical analysis of transverse intestinal cross-sections indicates similarities between AChE knockdown and treatment with chemical inhibitors. Similar to the MTH treatments, α-tubulin expression appears reduced, and microtubules disorganized in cells that lack AChE expression (Fig. 2.7 D-E') as compared with those from cells injected with the standard control morpholino (Fig. 2.7 A-B') instead. This difference is more evident by NF 46 where microtubles are clearly organized along the apical-basal axes of epithelial cells in control morpholino injected embryos (Fig. 2.7. G-H'), but are disorganized in the epithelium of cells in which AChE is knocked down (Fig. 2.7. J-K'). α-tubulin expression is also reduced in these cells, similar to observations in MTH treated embryos. Additionally, cell polarity is disrupted in cells that lack AChE expression. Compared with the apical expression of aPKC in control morpholino injected cells (Fig. 2.7 G, I,I'), aPKC expression is absent in AChE morpholino injected cells (Fig. 2.7 J,L,L'). Integrin expression does not

appear to be affected by AChE knockdown at NF 41 or 46 (Fig. 2.7 A,C,C',D,F,F',G, I,I',J,L,L') although we occasionally observe AChE knockdown cells that appear to express lower levels of integrin. However, endoderm cell shape is altered, with multiple layers of rounded cells observed in AChE knockdown cells (Fig. 2.7 J,L,L') compared with the single cell columnar epithelium of control morpholino injected cells (Fig. 2.7 G,I,I'). (The wt uninjected cells in the AChE morpholino injected embryos develop normally (Fig. 2.7 J,K,L)). Unlike chemically treated embryos, we do not observe an increase in apoptosis in AChE knockdown cells (data not shown). The similarities in disrupted microtubule organization, polarity, and disruption of epithelial shape suggest that OPs and Huperzine A disrupt intestinal endoderm development via inhibition of AChE.

Chemical AChE inhibitors disrupts mesoderm structure

Both the endoderm and mesoderm tissue layers contribute to intestinal lengthening and morphogenesis. To determine if AChE inhibition affects both the endoderm and mesoderm of the intestine, we investigated mesodermal protein expression immunohistochemically. Laminin is an extracellular matrix protein (ECM) normally expressed in two parallel layers in the mesoderm of NF 46 tadpoles (Fig. 2.8 A, A'). Only one laminin layer is present in tadpoles exposed to chemical AChE inhibitors (Fig. 2.8 B-D'). Smooth muscle actin (SMAC) is another mesodermal structure that normally surrounds the endoderm, and contributes to the musculature of the intestine (Fig. 2.8 E,E'). However, in chemically treated embryos, SMAC expression is reduced and patchy (Fig. 2.8 F,F'). This suggests that AChE inhibition disrupts both the mesoderm and endoderm tissue layers.

To determine when mesodermal structures are impacted by chemical exposure, we examined laminin and SMAC expression in MTH exposed tadpoles at multiple developmental stages. At NF 41/42 the two layers of laminin are already forming (Fig. 2.9 A,A'). However, only a single laminin layer is observed in tadpoles exposed to MTH (Fig. 2.9, B,B'). Laminin remains a single layer in MTH treated embryos at NF 43 and NF 46, whereas the two layers continue to form and become better defined in DMSO treated controls (Fig. 2.9 C-F'). A similar pattern is observed with SMAC expression. At NF 41/42, SMAC is expressed throughout much of the mesoderm, though it does not completely surround the endoderm at this stage (Fig. 2.9 G,G'). In contrast, SMAC expression is absent throughout most of the mesoderm in MTH treated embryos (Fig. 2.9 H,H'). By NF 43/44 SMAC is expressed throughout the mesoderm in solvent controls (Fig. 2.9. I,I'). SMAC expression is low and patchy in the MTH group (Fig. 2.9 J,J'). By NF 46, SMAC expression remains patchy in MTH treated embryos (Fig. 2.9 L,L'), in contrast to uniform expression in controls (Fig. 2.9 K,K').

AChE knockdown disrupts mesoderm structure

To determine if the mesodermal abnormalities observed in chemically treated embryos are due to AChE inhibition, we examined laminin and SMAC expression in AChE morpholino injected embryos. We occasionally observed abnormalities in these structures in embryos injected into one of the dorsal vegetal blastomeres at the eight-cell stage, but the defects were less consistently observed than in chemically treated embryos. As this might be due to limited AChE knockdown in the mesoderm, we altered our injection strategy,

injecting both of the dorsal vegetal blastomeres to increase the number of mesoderm cells lacking AChE expression. In these embryos, laminin shows similar defects to those observed in chemically treated embryos, with only one laminin layer forming, as opposed to the two parallel layers normally observed (Fig. 2.10 B,B',E,E',H,H',K,K'). This defect is apparent at both NF 41 and 46 (Fig. 2.10). We also observe defects in the distribution of the ECM protein, fibronectin, at the basement membrane of NF 46 embryos lacking AChE expression (Fig. 2.10 I,I',L,L'). Interestingly, this protein is expressed throughout the endoderm at NF 41, and appears to be asymmetrically localized toward the basal cell surface (Fig. 2.10 C,C'). In AChE knockdown, this asymmetric pattern appears to be lost, and fibronectin expression is observed around the cell membrane, which may suggest that AChE is required for the asymmetrical distribution of fibronectin (Fig. 2.10 F,F'). However, this observation is preliminary and requires further investigation. The similarities in disrupted mesoderm structures between AChE knockdown and inhibitor treated embryos suggests that the mesodermal abnormalities observed with OP or Huperzine A exposure are also due to AChE inhibition.

MTH exposure disrupts the enteric nervous system structure

Intestinal motility is coordinated by the enteric nervous system, which arises from neural crest cells that migrate to and populate the gut. Enteric neuronal abnormalities contribute to intestinal malformations in children and are associated with increased expression of AChE (Martucciello et al., 2002; Moore and Johnson, 2005). Innervation of the *X. laevis* intestine occurs at NF 41 (Holmberg et al., 2001), corresponding with the tissue

changes in the mesodermal and endodermal cell layers that drive intestinal elongation (Chalmers and Slack, 2000). Although coordinated peristaltic contractions are not observed until later (NF 43), ACh is the primary excitatory neurotransmitter of the intestine, and inhibition of AChE could affect enteric neural development (Holmberg et al., 2001; Olsson and Holmgren, 2011; Sundqvist and Holmgren, 2006). To determine if AChE inhibition affects neural development, whole intestines were dissected from NF 41, 43, and 46 embryos exposed to MTH or DMSO and immunohistochemically stained for acetylated tubulin to identify neurons. Branching neurons are evident in NF 41 DMSO treated embryos (Fig. 2.11 A), Neurons are also present in MTH treated embryos, suggesting that AChE inhibition does not affect the migration of neural crest cells to the intestine (Fig. 2.11 D). However, the neurons do not branch to the same extent as in DMSO controls, suggesting neurite outgrowth may be inhibited. By NF 44, neuron tracts parallel the curvature of the gut (Fig. 2.11 B). Although the intestines are shorter in MTH treated embryos at NF 44, the neurons show a similar pattern to those in DMSO controls (Fig. 2.11 E). Similar observations are made in NF 46 embryos, with neuronal patterning similar despite reduced length in MTH exposed embryos (Fig. 2.11 C,F). This suggests that AChE inhibition does not alter neuronal patterning in the gut, and implies that AChE is primarily required for processes in the endoderm and/or mesoderm to drive intestinal elongation.

AChE acts cell autonomously and is essential in the endoderm for intestinal morphogenesis

An advantage of *X. laevis* is that proteins can be heterogeneously knocked down within a tissue by targeting microinjection of knockdown reagents from the 8-32 cell stages (Moody,

1987a, b). The vegetal cells of the eight-cell embryo contribute to the future intestine. By microinjecting the right dorsal vegetal blastomere at the eight-cell stage, we knockdown AChE in the intestine in a subset of the intestinal cells, allowing us to compare cells that have AChE knocked down to wild-type cells through co-injection of a cell lineage marker (e.g. GFP mRNA). To determine if AChE acts cell autonomously or non-autonomously within the intestine, we compared the structure of cells lacking AChE expression to that of neighboring wild type cells that do express AChE. AChE knockdown in endoderm cells results in cells with rounded cell shapes that fail to form a single columnar epithelial layer (Fig. 2.12 A-C). However, uninjected wild type cells form a normal single cell columnar layer (Fig. 2.12 A,A'). Even when surrounded by AChE knockdown cells, the wild type cells assume a columnar shape and express both AChE (B) and aPKC (C) at their apical surfaces. This difference between injected and uninjected endoderm cells within a single embryo suggests that AChE acts cell autonomously within the intestine. Similarly, knockdown of AChE in the endoderm does not affect mesoderm development (Fig. 2.12 D-E'). Endoderm columnar structure is disrupted by AChE knockdown within the endoderm (Fig. 2.12 D-E). However, the parallel laminin layers form normally in the overlying mesoderm of AChE knockdown endoderm (Fig. 2.12 E,E'), suggesting that AChE knockdown in the endoderm has no effect on mesodermal development. Knockdown of AChE in the mesoderm also has no impact on endodermal development (Fig. 2.12 F-G'). When AChE is knocked down in the mesoderm, laminin organization is disrupted (Fig. 2.12 F-G'), with only a single laminin layer observed in regions where AChE is knocked down (Fig. 2.12 G'). However, the underlying endoderm

forms a normal single cell layered columnar epithelium (Fig. 2.12 F',G), suggesting that knockdown of AChE in the mesoderm has no impact on endodermal development. Taken together, this suggests that AChE has cell autonomous functions within both the endoderm and mesoderm of the developing intestine. It also suggests that AChE has distinct functions in the development of each tissue layer.

Discussion:

The results of this study indicate that AChE is required for intestinal morphogenesis and that OPs and other AChE inhibitors disrupt organogenesis through inhibition of AChE, as opposed to off-target mechanisms. Additionally, we describe the cellular effects of AChE inhibition and knockdown at multiple developmental stages. Both endoderm and mesoderm structures are disrupted, suggesting AChE may have multiple functions in promoting intestinal development. Interestingly, neural branching of the enteric nervous system appears largely unaffected by chemical treatments. This indicates that AChE has non-neuronal functions during intestinal morphogenesis. Importantly, knockdown of AChE in the endoderm is sufficient to disrupt intestinal morphogenesis, and indicates a developmental function of AChE in this non-neuronal tissue. This work improves our understanding of OP mediated teratogenesis in a non-target vertebrate species and indicates an important role for AChE in organogenesis.

OP exposure has previously been reported to disrupt intestinal development in *X. laevis* tadpoles, but the teratogenic mechanism(s) have not been described (Snawder and Chambers, 1989, 1990). Here, we similarly find that exposure to two different OPs (MTH

and CPF-CH_3) disrupt intestinal development, with shortened and/or malrotated intestines observed. Exposure to a structurally unrelated AChE inhibitor, Huperzine A, similarly disrupts intestinal development, suggesting that AChE inhibition is the cause of intestinal malformation. Knockdown of AChE within the gut results in similar intestinal phenotypes to those observed with chemical inhibition. Together, this suggests that AChE is required for intestinal organogenesis.

To determine if AChE is essential for intestinal development in other species, we exposed *L. laevis* tadpoles to MTH. We found that MTH exposure also disrupted intestinal lengthening in *L. laevis. L. laevis* are distantly related to *X. laevis,* but their feeding strategies and gastrointestinal morphologies differ greatly (Amin et al., 2015; Bloom et al., 2013). *L. laevis* are carnivorous tadpoles with large stomachs and relatively short intestines to digest high protein meals (Amin et al., 2015; Bloom et al., 2013). By comparison, *X. laevis*, are herbivores, with smaller stomachs and long, coiled intestines to digest nutrient poor algae (Bloom et al., 2013). Thus, the similarities in shortened intestinal phenotypes between these two frog species suggest that AChE has a conserved function in intestinal elongation. OP exposure has been reported to disrupt intestinal development in other frog species as well, further indicating a conserved role for AChE in intestinal development, at least among amphibian species (Aronzon et al., 2014). AChE is expressed within the developing intestine of multiple species during organogenesis, indicating that AChE may have a conserved function within vertebrates (Drews, 1975). While intestinal defects were not identified in the AChE knockout mouse, these mice show signs of malabsorption, which could be linked to

epithelial defects in the intestine (Duysen et al., 2002; Xie et al., 2000). Hence, AChE's role in intestinal development in other vertebrate species should be investigated.

Interestingly, *L. laevis* appeared to be less sensitive to OP teratogenesis than *X. laevis*, though AChE activity appeared slightly more prone to inhibition. This was surprising as *X. laevis* are often less sensitive to environmental pollutants than other frog species (Hoke and Ankley, 2005). This discrepancy might be explained by the difference in the degree of intestinal lengthening between the two species. Intestinal elongation is dramatic in *X. laevis*, lengthening by approximately 3.5x from NF 41 – 45 (within~22 hours) (Chalmers and Slack, 2000). Intestinal lengthening is not as extensive in *L. laevis*, and therefore may be less sensitive to chemical perturbations. Additionally, *L. laevis* have an excess of intestinal endoderm cells, which normally undergo apoptosis and do not contribute to the intestine *(unpublished observation from N.N.Y lab)*, unlike *X. laevis*, where all endoderm cells contribute to intestinal lengthening (Chalmers and Slack, 2000). This could also explain the greater sensitivity of *X. laevis* to MTH exposure. AChE activity was similar between *X. laevis* and *L. laevis* tadpoles (except in tadpoles exposed to 1 mg/L MTH, in which *L. laevis* show greater AChE inhibition). AChE predicted protein sequences are also highly conserved between these amphibian species. This is unsurprising as AChE is conserved across invertebrate and vertebrate species. However, AChE sequences from the frogs are more similar to each other than either is to mammalian AChE.

The OP concentrations used in this study are higher than those found environmentally. However, native frog species are often more sensitive to environmental

contaminants than laboratory models (Hoke and Ankley, 2005). Although OP environmental persistence is low, organogenesis occurs rapidly in frogs (often in a matter of days) and exposure to OPs or carbamate pesticides during development could adversely affect morphogenesis. Additionally, multiple OPs and carbamates are applied within agricultural regions, potentially exposing native tadpoles to mixtures of AChE inhibitors, which would be expected to have an additive effect through a shared mechanism of action (Relyea, 2009). Pesticides are suspected of contributing to the worldwide decline of amphibian populations, so understanding how pesticide exposure affects tadpole development is of paramount importance, and our study indicates that organogenesis can be disrupted by OP exposure.

To determine how AChE inhibition alters intestinal morphogenesis, we examined structures in the endoderm, mesoderm, and enteric nervous system of the gut. Similar defects were identified in both mesodermal and endodermal structures for all AChE chemical inhibitors, suggesting that AChE is required for development of both tissue layers. Malformation of both tissues is apparent as early as NF 41 and persists through NF 46. Similar defects were identified in the endoderm and mesoderm in AChE knockdown cells, suggesting that interaction of OPs or Huperzine A with AChE is responsible for the observed defects. Interestingly, the enteric nervous system was affected by MTH exposure at NF 41, but appeared to recover by NF 44-46. *In vitro*, AChE is involved in neurite outgrowth in both cholinergic and non-cholinergic nerve cells (Giordano et al., 2007; Howard et al., 2005; Johnson and Moore, 2004; Sharma et al., 2001; Sternfeld et al., 1998; Yang et al., 2008). At NF 41, MTH exposure appears to reduce neurite extension and branching, consistent with the

results of *in vitro* studies. In later stages of development, intestines from MTH treated embryos are visibly shorter than those from DMSO treated sibling controls. However, the neuronal patterning appears similar between DMSO and MTH treated embryos. Although it is possible that closer examination would reveal subtle differences in enteric nervous structures, our data suggest that AChE is not essential for branching of the enteric nervous system. This indicates that the shortened intestinal phenotypes are due to disruption of the endoderm and/or mesoderm as opposed to abnormalities in neuronal patterning. Based on the defects observed at NF 41 (but not at later developmental stages), this evidence may also suggest that redundant mechanisms are in place *in vivo* to facilitate neural patterning.

At the cellular level, endoderm defects from AChE inhibition or knockdown are characterized by rounded cell shape, disorganized microtubules, aberrant polarity, and disrupted intercalation. These defects are more obvious in cells that lack AChE than in those that were exposed to AChE inhibitors during organogenesis. For instance, a single cell layer can form in the intestines of embryos exposed to AChE inhibitors, but multiple cell layers are always observed in the cells that lack AChE protein expression entirely. aPKC expression is patchy and weak in cells from OP exposure, but completely absent in AChE morpholino injected cells. This apparent discrepancy is likely due to the complete absence of AChE expression in the knockdown cells, but presence in the inhibited cells. Although the protein structure and function is disrupted by chemical inhibitors (Kardos and Sultatos, 2000; Sultatos and Kaushik, 2008), AChE is still expressed within the endoderm. Hence, the protein may have a reduced, but not absent function in the inhibited cell population. This

would explain why the endoderm defect is more severe with AChE knockdown than inhibition. Still, regions of the AChE inhibited intestines are strikingly similar to complete absence of AChE. Portions of NF 46 intestines from each of the AChE inhibitors display multiple endodermal cell layers, disorganized microtubules, absent aPKC, and rounded cell shapes. Additionally, in regions of AChE inhibitor treated embryos where a single cell epithelium does form, the epithelium shows reduced expression of aPKC and α-tubulin relative to DMSO treated siblings. Thus, these similarities suggest a shared mechanism of action among AChE inhibitors and AChE knockdown. This suggests that OPs disrupt endodermal intestinal formation through inhibition of AChE, as opposed to an off-target mechanism.

Additionally, this data suggests that AChE is required within the non-neuronal cells of the intestine for morphogenesis to occur. This non-classical role for AChE is intriguing as AChE is an evolutionarily conserved protein, which pre-dates emergence of the nervous system (Karczmar, 2010). In addition to AChE's esterase function in the degradation of ACh, evidence suggests that AChE also has non-esterase functions. These non-esterase functions have been linked to polarized cell movement, microtubule rearrangement, and cell-substrate adhesion (Anderson et al., 2008; Howard et al., 2005; Johnson and Moore, 1999; Keller et al., 2001; Pizzurro et al., 2014; Sharma et al., 2001; Sperling et al., 2012; Yang et al., 2008). AChE shares sequence and structural homology with electrotactins, which are essential for cell-substrate adhesion (Botti et al., 1998; Johnson and Moore, 2004). Although the data presented here do not rule out the possibility of AChE esterase activity in the endoderm,

several lines of evidence suggest that AChE may have a non-esterase function within this tissue. Similar to evidence presented in other, non-neuronal cell lines, we find that AChE is required for the endoderm cells to polarize, intercalate, and organize microtubules. These characteristics are orchestrated by non-esterase AChE functions in other non-neural cells and may similarly be the result of non-esterase functions in the intestine (Anderson et al., 2008; Keller et al., 2001). Secondly, the reduced severity of endodermal defects in AChE inhibited tadpoles as compared with AChE knockdown may point to a non-esterase function. In rat primary superior cervical ganglia cultures, OP exposure reduces neurite outgrowth on poly-D-lysine, but has no affect in cells cultured on laminin (Howard et al., 2005). This suggests that OPs and substrates may compete in interacting with AChE and altering cell morphology. This is interesting as OPs and laminin interact with different protein motifs on AChE. However, binding of OPs to AChE results in conformational changes in AChE's peripheral anionic site (PAS) (Kardos and Sultatos, 2000; Sultatos and Kaushik, 2008). The PAS is involved in cell-substrate adhesion, and regions of the PAS bind directly to ECM proteins, including laminin and collagen (Johnson and Moore, 2003, 2004). Similarly, cells plated on laminin display reduced AChE activity as compared with cells plated on gelatin, poly-L-lysine, or poly-D-lysine (Howard et al., 2005; Sperling et al., 2012). This suggests that AChE's esterase and non-esterase functions may be mutually exclusive. In our study, high concentrations of OPs were used to disrupt intestinal morphogenesis. This may have been necessary for OPs to compete with AChE interactions with substrate. OPs alter AChE's protein structure, affecting both esterase function and non-esterase interactions (Kardos and

Sultatos, 2000; Sultatos and Kaushik, 2008). Thus, the possibility that OPs alter non-esterase functions during organogenesis should be considered.

We also found that AChE inhibiton or knockdown disrupted mesodermal intestinal structures. Laminin normally forms two parallel layers around the endoderm layer of the intestine. However in AChE inhibited embryos, we often observed only a single layer. The intestinal musculature was also impacted by inhibitor treatments, as SMAC expression appeared reduced and patchy in the mesoderm as compared with DMSO treated controls. Similarly, AChE knockdown in the mesoderm affected laminin deposition at both NF 41 and 46. AChE knockdown also alters fibronectin structure in the mesoderm of NF 46 embryos. This suggests that AChE is required in both the endoderm and the mesoderm for intestinal morphogenesis. AChE has been shown to bind to laminin *in vitro*, suggesting that AChE may have a direct role in organizing laminin within the mesoderm (Johnson and Moore, 2003, 2004). Additionally, AChE's interaction with laminin is independent of its esterase activity (Johnson and Moore, 2004), further supporting a non-esterase function of AChE within the intestine. Interestingly, laminin and SMAC structure were only sporadically altered when one vegetal blastomere was microinjected with AChE morpholino. Mesodermal defects were more frequently observed when two blastomeres were microinjected at the eight-cell stage, increasing the number of mesodermal cells that lack AChE expression. This data suggests that AChE may have a role in the formation of mesodermal structures, but that the mesoderm may be more resilient to loss of AChE than the endoderm.

Both the endoderm and mesoderm of the intestine play essential roles in the development of the gut, and AChE appears to function in the development of both layers. However, it is worth noting that AChE knockdown in the endoderm disrupts intestinal elongation, cell shape, polarity, microtubule arrangement, and endoderm cell movement without affecting mesodermal structures. This suggests that AChE plays a critical endodermal role, necessary for intestinal morphogenesis, and that its role in the mesoderm is separate from its endodermal function. This may also suggest that the intestinal defects observed in OP treated embryos are primarily due to disruption in endoderm maturation, with mesodermal abnormalities playing a secondary role. This data also indicates that AChE acts cell autonomously within the intestine. This is further supported by the observations that AChE knockdown in the mesoderm does not affect the structure of the underlying endoderm and, that the uninjected endoderm cells acquire normal columnar shape, polarity, and arrangement in embryos where AChE is heterogeneously knocked down in the intestine.

Our research reveals a previously unappreciated function for AChE during intestinal development in a vertebrate model. Although OP or carbamate exposure has previously been reported to disrupt intestinal development in frogs (Bacchetta et al., 2008; Bonfanti et al., 2004; Snawder and Chambers, 1989, 1990), the cellular impacts of these exposures have not been described. Our data indicate that both the endoderm and mesoderm layers of the intestine are disrupted by exposure to AChE inhibitors. AChE knockdown within the intestine suggests that endodermal defects may be primarily responsible for intestinal malformation, with mesodermal abnormalities as a secondary effect. Our lab has previously

reported that perturbation of the Wnt/PCP signaling pathway disrupts endoderm cell shape, polarity, and cytoskeletal dynamics, thereby preventing elongation of the intestine (Dush and Nascone-Yoder, 2013; Reed et al., 2009). This study similarly identifies the importance of these cellular processes in driving intestinal development, while identifying a novel role for AChE in directing these processes.

Congenital intestinal malformations are common in humans, but their underlying etiologies remain largely unknown. Both genetic and environmental factors likely contribute to these maladies. The role AChE may play in the development of the human intestine is unknown, although overexpression is associated with several intestinal defects including Hirschsprung's disease and intestinal neuronal dysplasia (Martucciello et al., 2002; Moore and Johnson, 2005). AChE polymorphism has only recently been identified in humans, and the impacts of these polymorphisms on health and development are currently unknown (Hasin et al., 2004; Valle et al., 2011). Additionally, wide usage of OP and carbamate pesticides worldwide, as well as the development of anti-AChE drugs for treatment of diseases (e.g. Alzheimer's, *myasthenia gravis*, glaucoma) places humans at risk for exposure to AChE inhibitors. Human AChE is more susceptible to inhibition than *X. laevis*, AChE – thus fetal development may be at higher risk from *in utero* OP exposure (Shapira et al., 1998). Future studies should be conducted to determine if AChE's function in organogenesis is conserved within vertebrates and to determine if OP exposure is associated with intestinal birth defects.

Materials and Methods:

Embryos and chemical exposures

Xenopus laevis tadpoles were obtained by *in vitro* fertilization, dejellied in 2% cysteine solution (pH 7.9), and reared between 14 - 23°C in 0.1X Marc's Modified Ringers (MMR) solution (Sive et al., 2000). Embryos were staged according to Nieuwkoop and Faber (Nieuwkoop, 1994). Embryos were exposed to a range of AChE inhibitor concentrations (1-50 mg/L malathion, 1-10 mg/L chlorpyrifos-methyl, or 0.1 - 15 μM Huperzine A) or to an equal volume of the DMSO solvent from NF stage 33 through stages 41-46. Embryos were anesthetized in 0.05% MS222 for gross morphological analysis and preserved in 4% PFA.

Lepidobatrachus laevis were obtained by natural pair matings and placed in 10% Holtfreter's solution. Tadpoles were exposed to MTH (0.1 – 50 mg/L) from Gosner stage 18/19 - 25 at 23°C or to an equal volume of DMSO solvent. Embryos were anesthetized in 0.05% MS222 for gross morphological analysis.

Morpholino Knockdown

Morpholino oligonucleotides (GeneTools, Inc) were designed to bind to the 5' UTR region near the translation start site of *Xenopus laevis* AChE mRNA (5'-CATGGCTGCTCCTCTGTGGGATTAC-3') or to a splice mutant of human β-globulin (5'-CCTCTTACCTCAGTTACAATTTATA-3') as a standard control. A pCS2 vector containing a coding region for cMyc tagged GFP was lineralized with *Not1*, capped mRNA synthesized with the mMessage Machine kit (Ambion), and mRNA purified by lithium chloride extraction. The synthesized GFP mRNA was co-injected with either the AChE or control

morpholinos at 8-cell stages. The right dorsal vegetal blastomere was targeted with 7.6 ng morpholino in 8 cell stage embryos to target knockdown to the midgut. Embryos were raised to NF stages 41-46, anesthetized in MS222, evaluated for morphological analysis, and fixed for immunohistochemistry. Both dorsal vegetal blastomeres were targeted with 7.6 ng morpholino in 8 cell stage embryos to increase the proportion of cells with reduced expression of the AChE protein in the mesoderm.

AChE Activity Assays

Whole NF stage 46 *X. laevis* and Gosner stage XX. *L. laevis* from MTH exposures where anesthetized in 0.05% and frozen at -80°C. Embryos were homogenized in 1% Triton 0.05M Tris-HCL (pH 7.4) buffer at a 1:10 (w:v) ratio and centrifuged for 5 minutes at 15,000g at room temperature. Protein concentrations of the supernatant were determined with the Pierce BCA protein assay (ThermoScientific). Protein preparations were used in the Ellman AChE activity assay, modified for use in a 96 well plate (Ellman et al., 1961). Briefly, 20 µg of protein from each sample was added to 1% Triton 0.05M Tris-HCL (pH 7.4) buffer to a final volume of 300 µL. 10 µL of 0.33mM f,f'-dithio-bis (2-nitrobenzoic acid) (DTNB) were added to each well and the plate incubated at room temperature for 10 minutes. 2 µL of 0.075 M acetylthiocholine iodide was added to each well and the absorbance read at 412 nm for 15 minutes. All samples were read in triplicate and 300µL Tris buffer with DTNB and acetylthiocholine idodide served as a blank. Activity was calculated as nmol acetylthiocholine iodide hydrolyzed per minute per µg protein.

Comparison of AChE sequences

In a separate study in our lab, mRNA was isolated from the intestine of *L. laevis* tadpoles at Gosner stage 19 and the transcriptome was sequenced on an Illumina NextSeq sequencer. An mRNA sequence homologous to AChE was identified in the transcriptome and predicted to be *L. laevis* AChE mRNA. The predicted protein sequence was generated using ExPASy (Gasteiger et al., 2003) and compared with the AChE protein sequences obtained from the NCBI database: *X. laevis* (NCBI NP_001121332.1), mouse (NCBI NP_001276939.1), and human (NCBI NP_001289551.1). Clustal Omega freeware was used to align AChE protein sequences from the four species (Sievers et al., 2011).

Immunohistochemistry

Stage 41 to 46 embryos from chemical exposure and 8-cell morpholino microinjections were anesthetized in 0.05% MS222 and fixed in 4% PFA at 4°C overnight. Embryos were rinsed in DENTs fixative (80% methanol/20% DMSO) and stored overnight at -20°C. Embryos were rehydrated in 100 mM NaCl, 100 mM Tris pH 7.6 and transferred to sucrose/gelatin (15% sucrose/15% cold-water fish geletin) overnight. Embryos were embedded in OCT (Tissue-Tek) and 10µm serial sections transferred onto charged slides (Fisher Superfrost Plus). Sections dried overnight at room temperature. Slides were dipped in acetone for one minute and rinsed in 1X PBS. Antigen retrieval was performed by washing slides in 1% SDS in PBS for 2.5-5 minutes followed by three washes in 1X PBS. Slides were blocked for 30 minutes at room temperature in TNB buffer (0.1 M Tris pH 7.4, 0.15 M NaCl, 0.05% Tween 20, 5% lamb serum, 0.5% casein-based blocking reagent, NEN). Primary antibodies were

applied to slides in TNB buffer overnight at 4°C for the following proteins: E-cadherin (DSHB, 5D3; 1:200), β-catenin (SCBT, H-102; 1:100), aPKC (Santa Cruz, sc216; 1:200), α-tubulin (Sigma, T9026; 1:1000), active caspase 3 (Cell Signaling Technology 9661; 1:300), cMyc (Santa Cruz 9E10, 1:1000), AChE (Abcam, ab 97298; 1:200) GFP (1:500), Fibronectin (*Kind gift from D.W. Simone*, 1:1000), Laminin (Sigma, L9393; 1:200) , smooth muscle actin (Sigma, A5228; 1:1000). Slides were washed twice in PBST and incubated for 3 hours at room temperature in TNB buffer containing Alexa 488-conjugated goat anti-mouse IgG (Invitrogen, A11029; 1:2000) and Alexa 555 conjugated goat anti-rabbit IgG (Invitrogen, A11035; 1:2000). Slides were washed twice in PBST, followed by two washes in PBS. Nuclei were stained with ToPro3 (Invitrogen, 1:1000) in 1X PBS for 15 - 30 minutes at room temperature. Autofluorescence was quenched in Eriochrome Black (0.2% w/v in PBS, 30 seconds - 1 minute). Quenched slides were rinsed in 1X PBS, mounted in Prolong Gold, cured in the dark overnight at room temperature, and sealed with nail polish. Fluorescence was visualized on a Leica SPEII confocal microscope, with 10X, 20X, and/or 40X dry objectives.

Whole intestines were dissected from MTH and DMSO exposed tadpoles at NF 41, 44, and 46 for whole mount immunohistochemistry. Intestines were fixed in MEMFA and processed for immunohistochemistry as published (Lee et al., 2008). Intestines were incubated overnight at 4°C in WMBS containing the following primary antibodies: acetylcated tubulin (Sigma Aldrich, T-7451, 1:500), laminin (Sigma, L9393; 1:100), AChE (abCam 97299; 1:200). Intestines were washed five times in TBST and incubated in

overnight at 4°C in WMBS containing the following secondary antibodies: Alexa 488-conjugated goat anti-mouse IgG (Invitrogen, A11029; 1:2000) and Alexa 555 conjugated goat anti-rabbit IgG (Invitrogen, A11035; 1:2000). Following TBST washes, intestines were imaged on a Nikon AZ100-C2 macroconfocal microscope.

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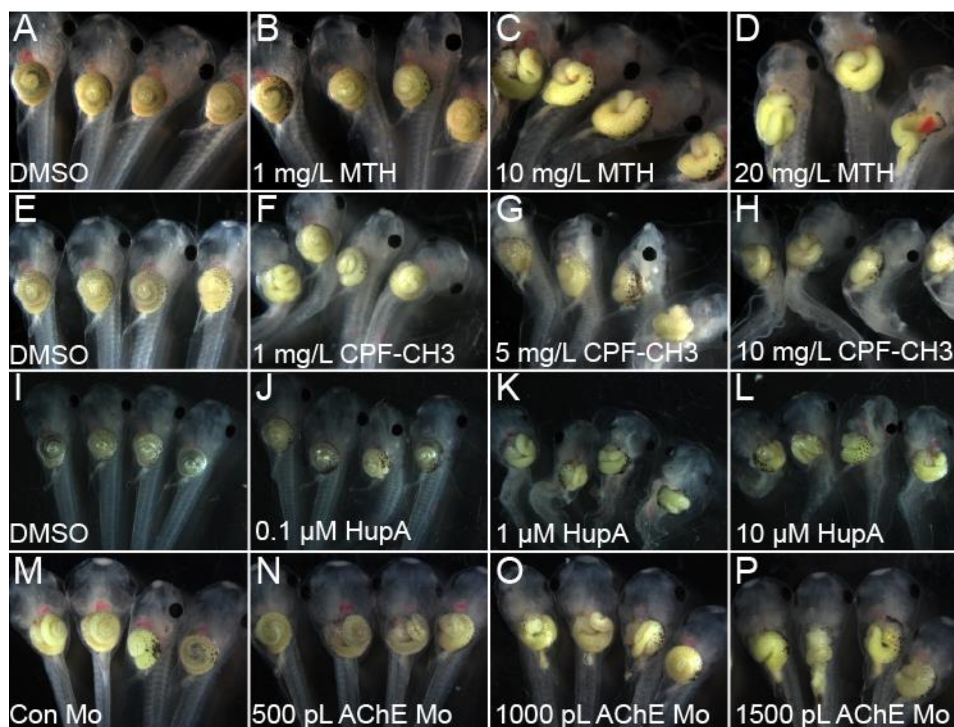


Figure 2.1) AChE inhibition or knockdown result in intestinal shortening and malrotation in a dose dependent manner.

A-D) Compared with the counterclockwise rotation and elongation in (A) solvent treated embryos, tadpoles exposed to increasing concentrations of (B-D) malathion display shortened and malrotated intestines.

E-H) Intestinal defects in length and coiling are observed with increasing concentrations of (F-H) chlorpyrifos-methyl.

I-L) Exposure to increasing concentrations of (J-L) Huperzine A, similarly disrupts intestinal development as compared with solvent controls (I)

M-P) Intestines elongate and coil normally in (M) control morpholino injected embryos. Intestine targeted morpholino AChE knockdown disrupts intestinal elongation and rotation, with increased severity observed with injection of higher concentrations of morpholino.

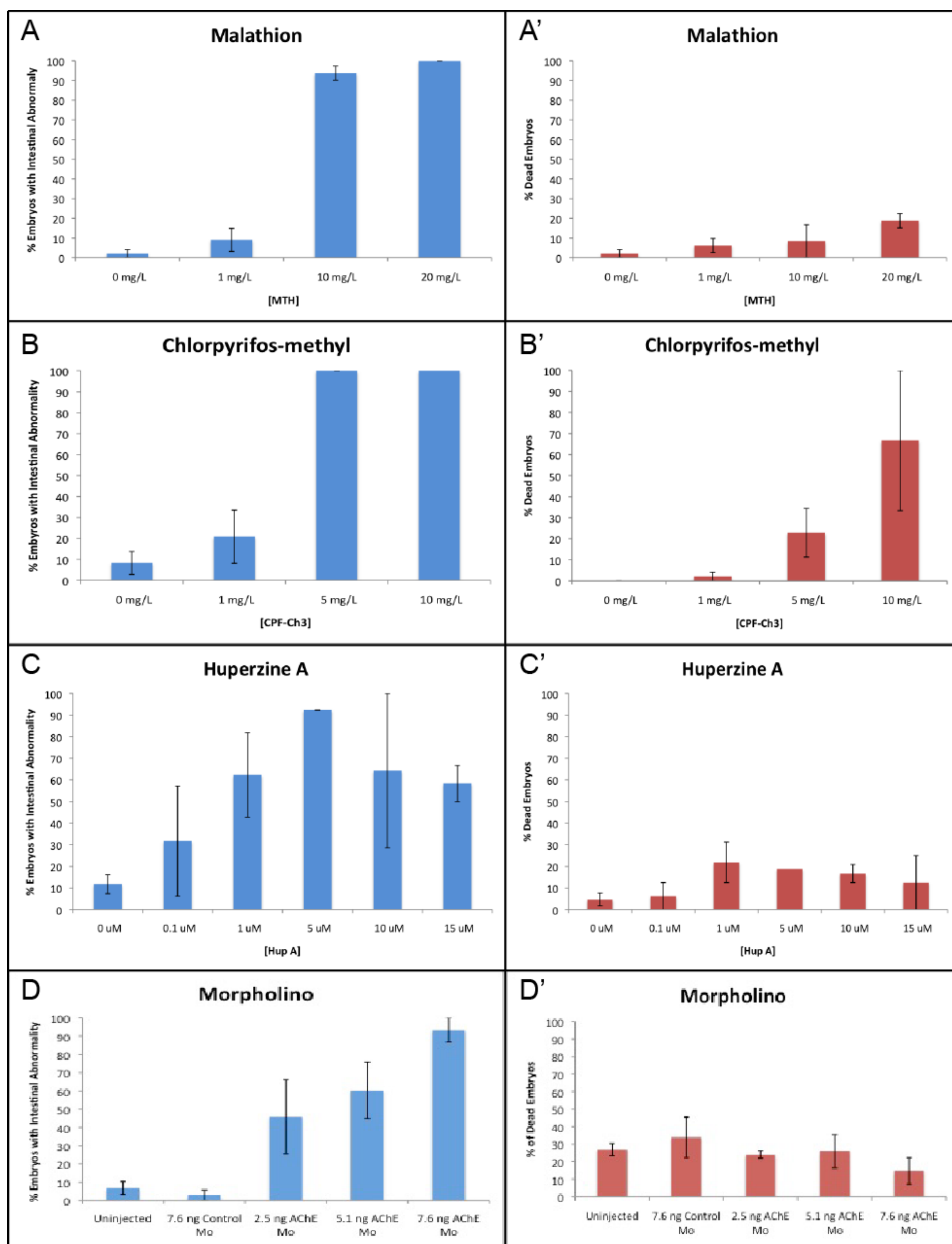
Figure 2.2) Quantification of intestinal malformation and embryo mortality in response to AChE chemical inhibition or morpholino knockdown.

A) Exposure to increasing concentrations of the OP, malathion, results in an increased percentage of tadpoles with abnormal intestines. A') malathion is not highly lethal at concentrations that disrupt intestinal morphogenesis.

B) Exposure to increasing concentrations of the OP, chlorpyrifos-methl, results in an increased percentage of tadpoles with abnormal intestines. B') Chlorpyrifos-methyl exposure also results in increased embryonic death.

C) Exposure to increasing concentrations of the AChE inhibitor, Huperzine A results in an increased percentage of tadpoles with abnormal intestines. C') Huperzine A is not toxic at concentrations that disrupt intestinal development.

D) Microinjection of higher concentrations of AChE morpholino to knockdown AChE within the intestine, results in an increased percentage of tadpoles displaying shortened/malrotated intestinal tracts. D') Morpholino knockdown at the 8-cell stage is not lethal.


A
Malathion
% Embryos with Intestinal Abnormaly
0 mg/L
1 mg/L
10 mg/L
20 mg/L
[MTH]
A'
Malathion
% Dead Embryos
0 mg/L
1 mg/L
10 mg/L
20 mg/L
[MTH]
B
Chlorpyrifos-methyl
% Embryos with Intestinal Abnormality
0 mg/L
1 mg/L
5 mg/L
10 mg/L
[CPF-Ch3]
B'
Chlorpyrifos-methyl
% Dead Embryos
0 mg/L
1 mg/L
5 mg/L
10 mg/L
[CPF-Ch3]
C
Huperzine A
% Embryos with Intestinal Abnormality
0 uM
0.1 uM
1 uM
5 uM
10 uM
15 uM
[Hup A]
C'
Huperzine A
% Dead Embryos
0 uM
0.1 uM
1 uM
5 uM
10 uM
15 uM
[Hup A]
D
Morpholino
% Embryos with Intestinal Abnormality
Uninjected
7.6 ng Control Mo
2.5 ng AChE Mo
5.1 ng AChE Mo
7.6 ng AChE Mo
D'
Morpholino
% of Dead Embryos
Uninjected
7.6 ng Control Mo
2.5 ng AChE Mo
5.1 ng AChE Mo
7.6 ng AChE Mo

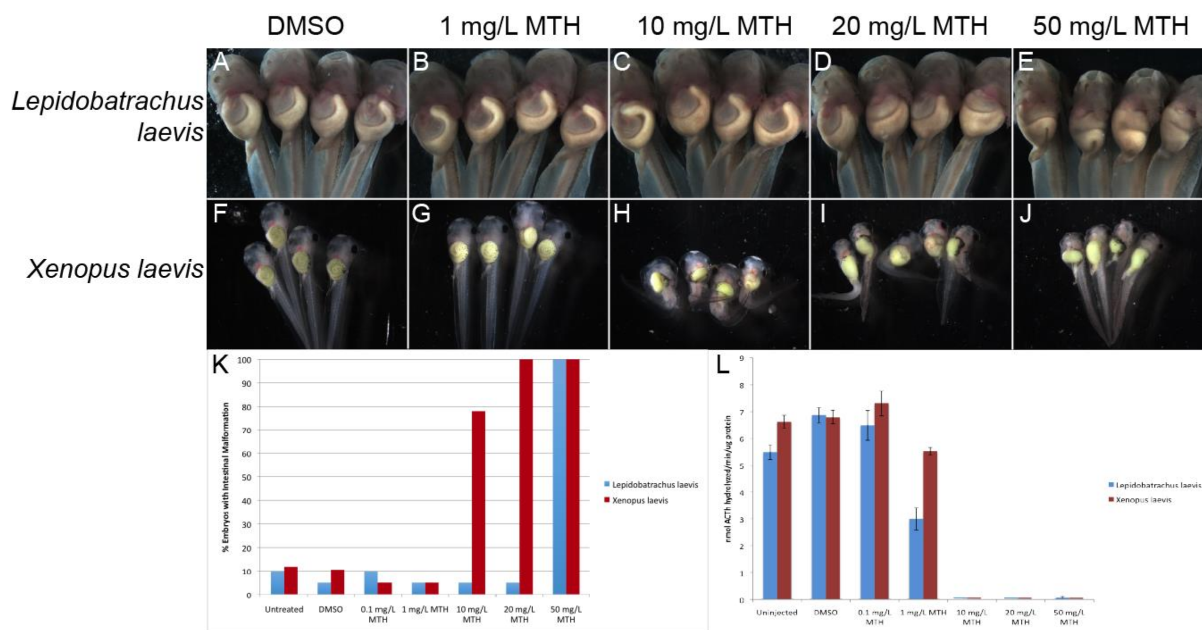


Figure 2.3) Malathion exposure disrupts intestinal development in both *L. laevis* and *X. laevis*.

A-E) Exposure to increasing concentrations of malathion result in shortened intestinal tracts in *L. laevis.*

F-J) Exposure to increasing concentrations of malathion result in shortened and malrotated intestinal tracts in *X. laevis*.

K) Quantification of the percentage of embryos with intestinal malformations for both *L. laevis* (blue) and *X. laevis* (red) exposed to increasing concentrations of malathion.

L) Quantification of AChE esterase activity in *L. laevis* (blue) and *X. laevis* (red) tadpoles exposed to increasing concentrations of malathion. Malathion exposure inhibits AChE activity in both species

Figure 2.4) AChE sequences are homologous between *L. laevis* and *X. laevis*. Clustal Omega alignment of the predicted protein sequences for L. laevis, X. laevis, Mus muscularis, and Homo sapiens reveals high sequence homology among the vertebrate AChE proteins. Human and mouse AChE's share approximately 90% amino acid identity. L. laevis shares 65% identity with both human and mouse AChE sequences, with X. laevis slightly less similar to mammalian AChE, sharing 62% homology. The two frog sequences are very similar, with 87% sequence identity between the two. Symbols are standard for Clustal alignment (* = fully conserved residue, : - conservation between groups of strongly similar properties, . = conservation between groups of weakly similar properties)

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CLUSTAL O(1.2.2) multiple sequence alignment


Mus                       PPVGSRRFMPPEPKRPWSGVLDATTFQNVCYQYVDTLYPGFEGTEMWNPNRELSEDCLYL      60
Homo                      PPMGPRRFLPPEPKQPWSGVVDATTFQSVCYQYVDTLYPGFEGTEMWNPNRELSEDCLYL      60
Lepidobatrachus           PPVDKLRFRRTEPKKPWSDVWDATSYPNACYQYFDTLYPGFSGMEMWNPNRPMSEDCLYL      60
Xenopus                   ----------SEPKKPWTDVWDATAYPDACYQYFDTLYPGFPGMEMWNPNRPMSEDCLYL      50
                                     ***:**: * ***:: ..****.******* * ******* :*******

Mus                       NVWTPYPRPASPTPVLIWIYGGGFYSGAASLDVYDGRFLAQVEGAVLVSMNYRVGTFGFL      120
Homo                      NVWTPYPRPTSPTPVLVWIYGGGFYSGASSLDVYDGRFLVQAERTVLVSMNYRVGAFGFL      120
Lepidobatrachus           NIWVPSPRPKNA-TVMVWIYGGGFYSGSSSLDVYDGRYLCHTENVIVVSMNYRVGAFGFL      119
Xenopus                   NIWVPHPRPSNA-TVMVWIYGGGFYSGSSSLDVYDGRYLCHAENVIVVSMNYRVGAFGFL      109
                          *:*.* *** .   *::**********::********:* :.* .::********:****

Mus                       AL-PGSREAPGNVGLLDQRLALQWVQENIAAFGGDPMSVTLFGESAGAASVGMHILSLPS      179
Homo                      AL-PGSREAPGNVGLLDQRLALQWVQENVAAFGGDPTSVTLFGESAGAASVGMHLLSPPS      179
Lepidobatrachus           TLTPGSADAPGNVGLFDQRLALQWIQDNIVFFGGDPRTVTIFGESAGGASVGMHVLSPGS      179
Xenopus                   TLTPGSVDAPGNVGLFDQRLALQWVQDNIAFFGGDPRTVMIFGESAGAVSVGMHVISPGS      169
                          :* *** :*******:********:*:*:. ***** :* :******..*****::*  *

Mus                       RSLFHRAVLQSGTPNGPWATVSAGEARRRATLLARLVGCPPGGAGGNDTELIACLRTRPA      239
Homo                      RGLFHRAVLQSGAPNGPWATVGMGEARRRATQLAHLVGCPPGGTGGNDTELVACLRTRPA      239
Lepidobatrachus           HQLFSKAVLQSGSPNTPWATVTPQESRRRAELLGKLLDCKQ----GNDTDLLNCLRTKPP      235
Xenopus                   HHLFSKAALQSGTPNTPWATITPQEARRRTEMMGKKLDCRM----GNDTELLNCLRAKQP      225
                          : ** :*.****:** ****:   *:***:  :.: : *      ****:*: ***::

Mus                       QDLVDHEWHVLPQESIFRFSFVPVVDGDFLSDTPEALINTGDFQDLQVLVGVVKDEGSYF      299
Homo                      QVLVNHEWHVLPQESVFRFSFVPVVDGDFLSDTPEALINAGDFHGLQVLVGVVKDEGSYF      299
Lepidobatrachus           QKLIDHEFSVLPAPSVFRFAFVPVPDGDFFPDAPETLMNMGRFKPCPLIVGVNQNEGSYF      295
Xenopus                   QELIDHEFSVLPAPSVFRFAFVPVPDGDFFPEPPEVLMNMGRFKPCPLLMGVNQNEGSYF      285
                          * *::**: ***  *:***:**** ****: : **.*:* * *:   :::** ::*****

Mus                       LVYGVPGFSKDNESLISRAQFLAGVRIGVPQASDLAAEAVVLHYTDWLHPEDPTHLRDAM      359
Homo                      LVYGAPGFSKDNESLISRAEFLAGVRVGVPQVSDLAAEAVVLHYTDWLHPEDPARLREAL      359
Lepidobatrachus           LLYGAPGFSKNNESLITREEFLGGVRMSVPHANDIALEAVVMQYTDWADEHAPIKNREAM      355
Xenopus                   LLYGAPGFSKNNESLINREEFLGGVKMSVPHANDIALEAVVMQYTDWADEHAGIKNREAM      345
                          *:**.*****:*****.* :**.**::.**:..*:* ****::**** . .   : *:*:

Mus                       SAVVGDHNVVCPVAQLAGRLAAQGARVYAYIFEHRASTLTWPLWMGVPHGYEIEFIFGLP      419
Homo                      SDVVGDHNVVCPVAQLAGRLAAQGARVYAYVFEHRASTLSWPLWMGVPHGYEIEFIFGIP      419
Lepidobatrachus           DQLVGDHNVICPMTHFAGKVSEFGNRVYAYYFDHRASNLAWPQWMGVPHGYEIEFVFGLP      415
Xenopus                   DQLVGDHNVICPLTYFAGKASETGNRVYTYYFDHRASNLAWPQWMGVPHGYEIEFVFGLP      405
                          . :******:**:: :**: :  * ***:* *:****.*:** ************:**:*

Mus                       LDPSLNYTTEERIFAQRLMKYWTNFARTGDPNDPRDSKSPQWPPYTTAAQQYVSLNLKPL      479
Homo                      LDPSRNYTAEEKIFAQRLMRYWANFARTGDPNEPRDPKAPQWPPYTAGAQQYVSLDLRPL      479
Lepidobatrachus           LEPKLNYTKQEADLSRRMMRYWANFARTGDPNENATGRQTKWPVYTATEQRYLALNNKPV      475
Xenopus                   LVANLNYNPQEEALSRKMMRYWANFARTGDPNEGNDARQQRWPLYTASEQRYIALNNRPQ      465
                          *  . **. :*  :::::*:**:*********:    :  :** **:  *:*::*: :*

Mus                       EVRRGLRAQTCAFWNRFLPKLLSATDTLDEAERQWKAEFHRWSSYMVHWKNQFDHYSKQE      539
Homo                      EVRRGLRAQACAFWNRFLPKLLSATDTLDEAERQWKAEFHRWSSYMVHWKNQFDHYSKQD      539
Lepidobatrachus           QNLQGIRVQTCTFWNRFLPKLLNITDNIDEAERQWKVEFHRWSAYMMRWKNQFDHYSKQE      535
Xenopus                   QDLQGIRVQTCMFWNRFLPKLLNITDNVDEAERQWKEEFHRWSTYMMRWKNQFDHYSKQE      525
                          :  :*:*.*:* **********. **.:******** ******:**::***********:

Mus                       RCSDL      544
Homo                      RCSDL      544
Lepidobatrachus           RCSEL      540
Xenopus                   RCSEL      530
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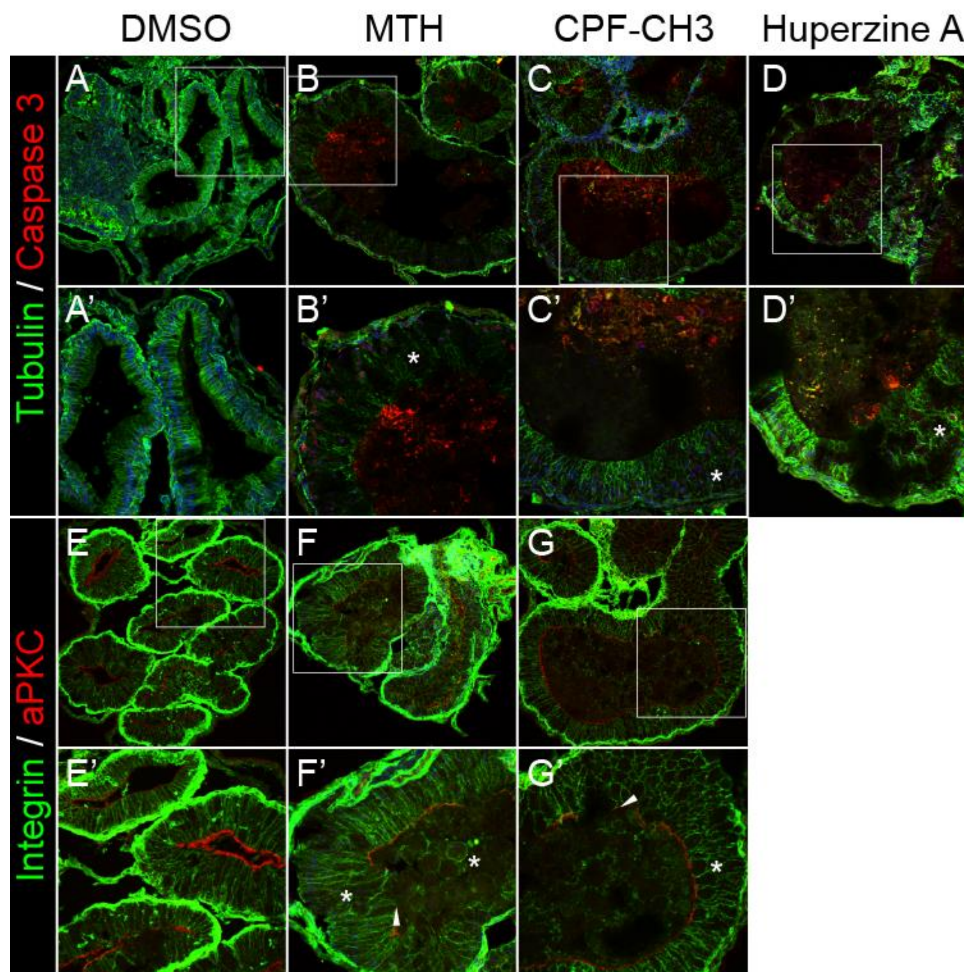
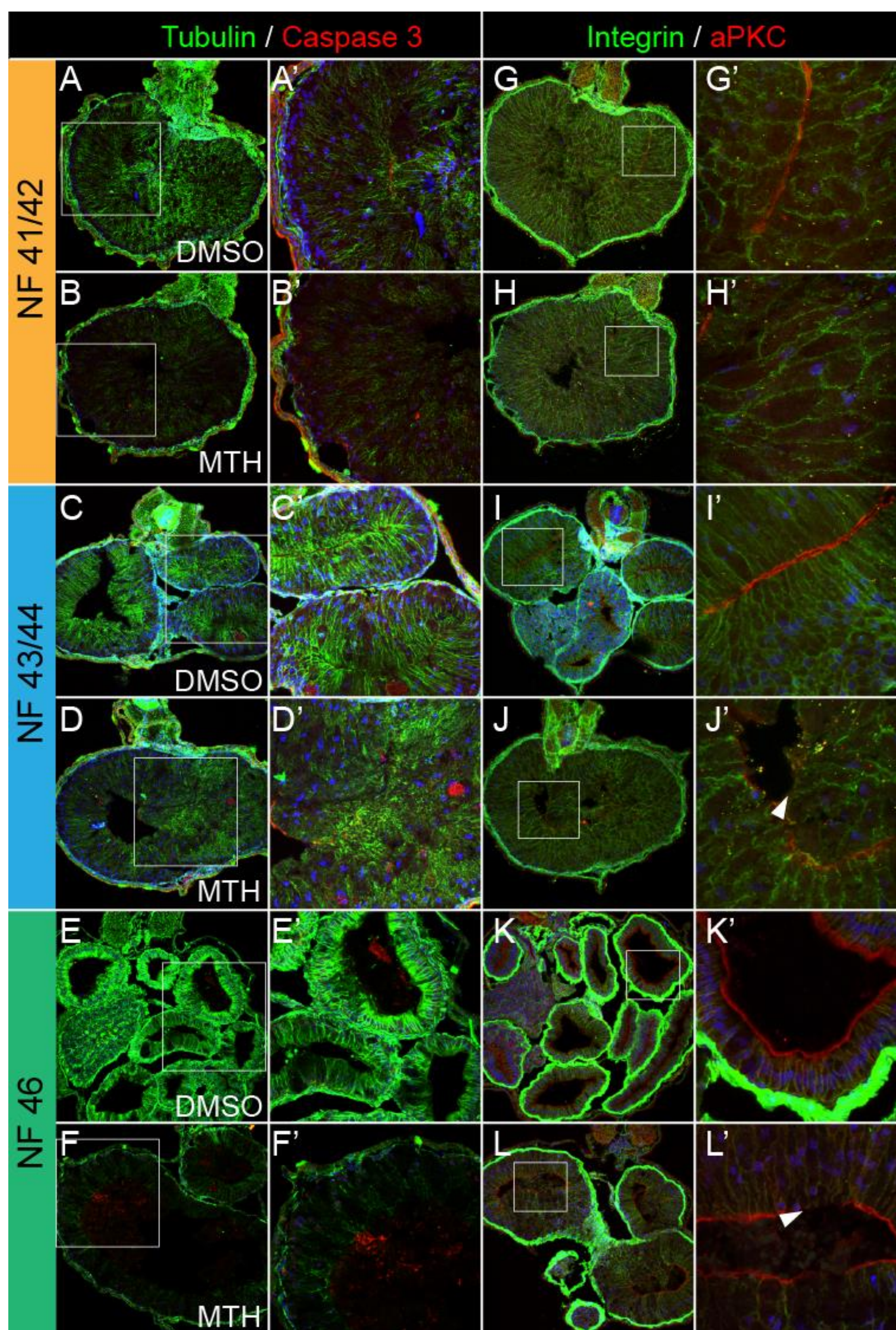


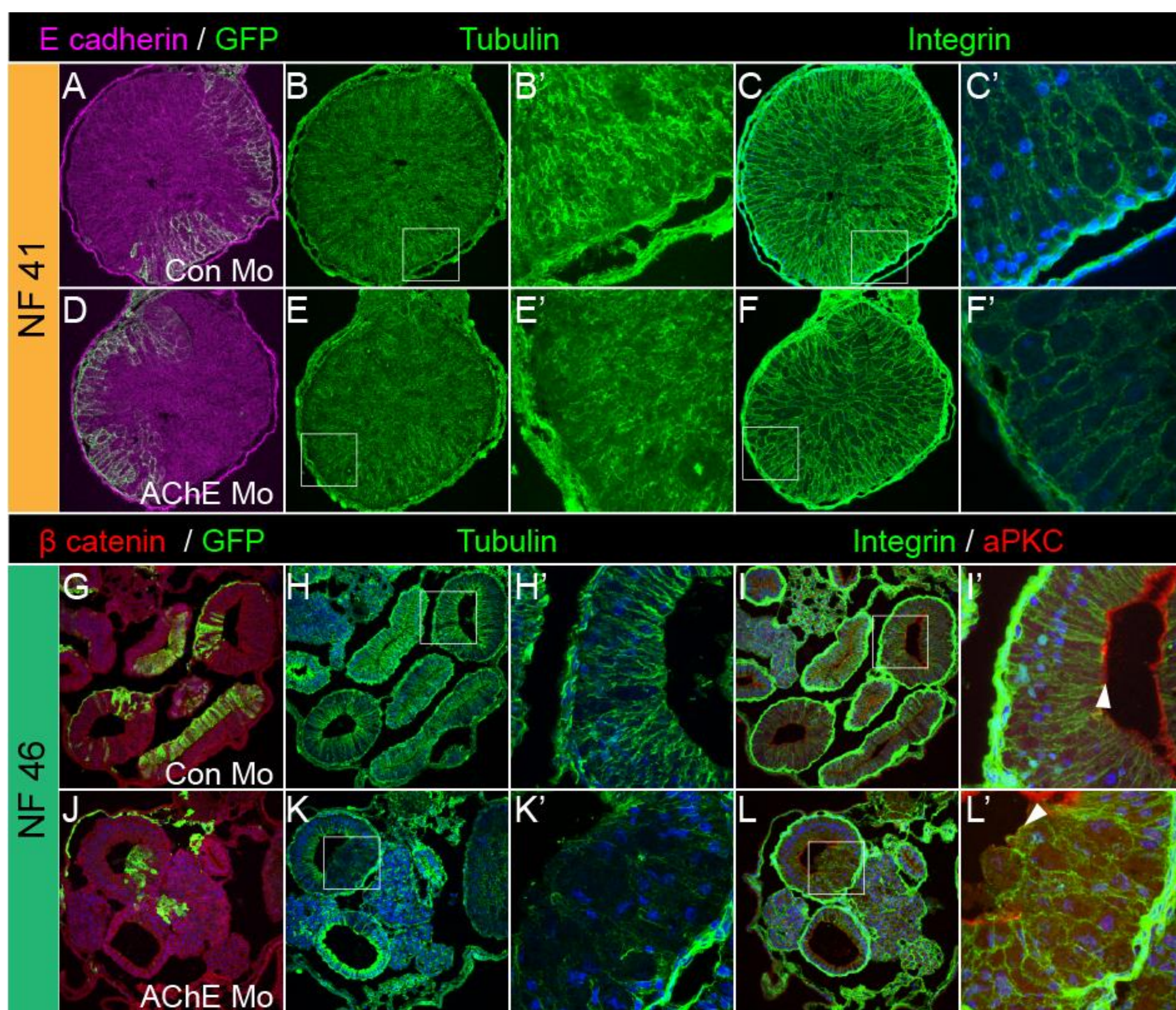
Figure 2.5) Exposure to AChE inhibitors alters gut endoderm structures.
A-D') Compared with the regular expression of α-tubulin (green) on microtubules in DMSO treated controls (A,A') exposure of tadpoles to any (B-D') AChE inhibitor disrupts microtubule structure (asterisks B'-D') and reduces α-tubulin expression AChE inhibitor exposure also increases apoptosis (B-D', red). (A'-D') Higher magnification images of boxed regions in (A-D).
E-G') Compared with the single layer of columnar epithelium in (E,E') DMSO treated embryos (visualized by integrin expression (green), multiple layers of rounded cells are observed in the intestines from tadpoles exposed to organophosphates (F-G', asterisks in F' and G'). Compared with the uniform expression of the apical marker, aPKC (red) in (E,E') DMSO treated embryos, aPKC expression is weak and patchy in organophosphate exposed tadpoles (F-G', arrowheads, F' and G'). (E'-G') Higher magnification images of boxed regions in (E-G).

Figure 2.6) Exposure to malathion disrupts endoderm structures from NF 41 - 46.
A-B') At NF 41/42 microtubules (green – detected by α-tubulin expression) are already organized along the future apical-basal axes of endoderm cells of (A, A') DMSO treated controls. In contrast, α-tubulin (green) expression is reduced in (B,B') MTH treated embryos. Apoptosis (red) is low in intestines of both (A,A') DMSO and (B,B') MTH treated embryos at this stage. (A', B') higher magnification images of the boxed regions in (A,B).
C-D') By NF 43/44, α-tubulin (green) expression increases and becomes apically enriched in the intestines of (C,C') DMSO treated tadpoles. α-tubulin expression remains lower in (D,D') MTH treated embryos relative to (C,C') DMSO controls. Additionally, microtubules are disorganized in (D,D') MTH treated embryos compared with their alignment along the apical-basal endoderm cell axes in (C,C') DMSO controls. Apoptosis of endoderm cells appears similar between (C,C') DMSO and (D,D') MTH treated tadpoles. (C',D') higher magnification images of the boxed regions in (C,D).
E-F') At NF 46 microtubules (green) are arranged along the apical-basal axes of the single epithelial layer, with α-tubulin expression enriched apically in (E,E') control embryos. As observed in earlier stages, α-tubulin is expressed at a lower level in (F,F') MTH treated embryos, and microtubules are less organized along the apical-basal axes. In contrast to earlier stages, apoptosis is increased in (F,F') MTH exposed tadpoles as compared with (E,E') DMSO treated embryos. (E',F') higher magnification images of boxed regions in (E,F).
G-H') Endoderm cell shape (visualized by the expression of the cell membrane protein, integrin (green)) is not obviously different between (G,G') DMSO and (H,H') MTH treated embryos at NF 41/42). aPKC (red) is only beginning to be expressed in the endoderm at this stage. (G', H') higher magnification images of the boxed regions in (G,H).
I-J') Compared with the columnar shape of the intestinal endoderm cells in NF 43/44 (I,I') DMSO treated tadpoles, cells are rounder and less organized in (J,J') MTH exposed embryos. aPKC (red) is expressed at the apical endoderm surface, lining the future lumen in (I,I') DMSO treated embryos. In contrast, aPKC expression is reduced and patchy in (J,J') MTH exposed embryos (arrowhead in J'). (I', J') higher magnification images of boxed regions in (I,J).
(K-L') The columnar, polarized single epithelial layer observed in the intestines of (K,K') DMSO treated embryos, is disrupted in (L,L') MTH treated embryos with patchy aPKC expression (red, arrowhead in L') and regions of multiple cell layers (e.g. L region below the box). (K', L') Higher magnification images of boxed regions in (K,L)



Tubulin / Caspase 3
Integrin / aPKC
NF 41/42
NF 43/44
NF 46
A
A'
G
G'
DMSO
B
B'
H
H'
MTH
C
C'
I
I'
DMSO
D
D'
J
J'
MTH
E
E'
K
K'
DMSO
F
F'
L
L'
MTH

Figure 2.7) AChE knockdown disrupts endodermal structures at NF 41 and NF 46.
A-F') Endoderm structure and protein expression in NF 41 embryos injected with (A-C') control or (D-F') AChE morpholinos. (A,D) Morpholinos were co-injected with GFP mRNA to identify (green) injected cells in the heterogeneous intestines (cells that do not contain GFP are wildtype, and express AChE). E-cadherin (magenta) was used to outline cell shape. Serial sections from these embryos were obtained to investigate endoderm protein expression and cell structure (B,C,E,F). Compared with the strong expression of α-tubulin (green) in control injected NF 41 embryos (B,B'), expression of α-tubulin appears reduced in cells that lack AChE expression (E,E') Although microtubules are beginning to become aligned along the apical-basal endoderm cell axes at this stage (B,B'), they appear less organized along this axis in cells that do not express AChE (E,E'). Endoderm cell shape (integrin expression, green) does not appear different between (C,C') control and (F,F') AChE morpholino injected embryos at NF 41. (B',C',E',F') higher magnification images of boxed regions in (B,C,E,F) respectively.
G-L') Endoderm structure and protein expression in NF 46 embryos injected with (G-I') control or (J-L') AChE morpholino. (G,J) Morpholinos were co-injected with GFP mRNA to identify (green) injected cells in the heterogeneous intestines (cells that do not contain GFP are wildtype, and express AChE). β-catenin (red) was used to outline endoderm cell shape. Serial sections from these embryos were obtained to investigate endoderm protein expression and cell structure in (H,I) control and (K,L) AChE knockdown embryos. In cells containing (H,H') control morpholino, microtubules (green) orient along the apical-basal axis of the single layer of epithelium, with α-tubulin expression enriched apically. In contrast, microtubules are randomly organized and α-tubulin expression is reduced in endoderm cells that lack AChE expression (K,K'). The columnar cell shape of epithelial cells (integrin, green) in (I,I') is lost with AChE knockdown, where multiple layers of rounded endoderm cells are present instead (L,L'). aPKC expression at the apical surface of the mature epithelium (I,I', arrowhead in I') is absent in cells lacking AChE expression (L,L', arrowhead in L'). (H',I',K',L') higher magnification images of the boxed regions in (H,I,K,L) respectively.


E cadherin / GFP
Tubulin
Integrin
NF 41
A
Con Mo
B
B'
C
C'
D
AChE Mo
E
E'
F
F'
β catenin / GFP
Tubulin
Integrin / aPKC
NF 46
G
Con Mo
H
H'
I
I'
J
AChE Mo
K
K'
L
L'

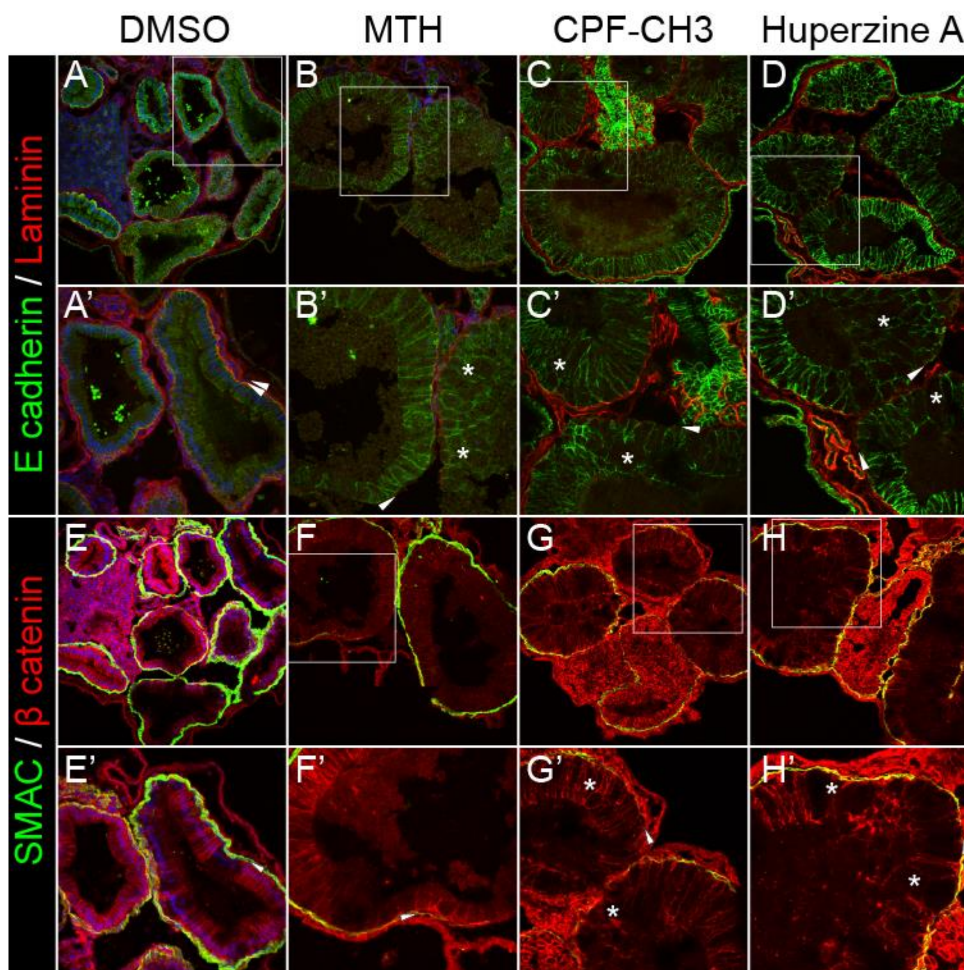


Figure 2.8) Exposure to AChE inhibitors alters gut mesoderm structures.
A-D') E-cadherin (green) and laminin (red) expression in (A,A') DMSO treated and (B-D') AChE inhibitor treated embryos. Compared with the expression of two parallel laminin (red) layers around the gut tube in (A,A') DMSO controls, exposure of tadpoles to any (B-D') AChE inhibitor disrupts laminin structure (arrowheads A'-D'), with only a single, reduced layer of laminin present. (A'-D') Higher magnification images of boxed regions in (A-D). E-G') β-catenin (red) and smooth muscle actin (SMAC, green) expression in intestines from (E,E') DMSO and (F-H') AChE inhibitor treated tadpoles. Compared with the single layer SMAC (green) expressed in the mesoderm of (E,E') DMSO treated embryos, SMAC expression is reduced and patchy (arrowheads E'-H') in intestines from tadpoles exposed to any of the (F-H') AChE inhibiting chemicals. (E'-H') higher magnification images of the boxed regions in (E-H).

Figure 2.9) Exposure to malathion disrupts mesoderm structures from NF 41 - 46.
A-B’) At NF 41/42 laminin (red) is already becoming organized into two parallel structures in the mesoderm, surrounding the endoderm of the intestine (arrowheads in A’). In contrast, laminin expression is reduced in (B,B’) MTH treated embryos and is present in only a single layer (arrowhead in B’) at the basement membrane of the gut. (A’, B’) higher magnification images of the boxed regions in (A,B).
C-D’) By NF 43/44, laminin (red) expression is increased and the parallel layers are well defined in the intestines of (C,C’, arrowhead in C’) DMSO treated controls. Laminin is still expressed in a single layer in NF 43/44 tadpoles exposed to (D,D’ arrowhead in D’) MTH, at lower levels than in controls. (C’,D’) higher magnification images of boxed regions in (C,D).
E-F’) Laminin expression increases in the parallel layers surrounding the gut in (E,E’) DMSO treated embryos (arrowheads E’). In contrast, only a single laminin layer is present in the (F,F’) MTH treated embryos and laminin expression remains lower than in controls (arrowhead in F’). (E’,F’) higher magnification images of boxed regions in (E,F).
G-H’) The smooth muscle layer (SMAC, green) nearly encircles the intestine by NF 41/42, with few gaps in expression observed in (G,G’) DMSO treated embryos. In contrast, muscle development is absent or delayed in (H,H’) MTH treated embryos at his stage, as indicated by absence of SMAC surrounding the gut. (G’,H’) higher magnification images of the boxed regions in (G,H).
I-J’) Compared with the uniform expression of SMAC within the intestine of NF 43/44 embryos exposed to (I,I’) DMSO, tadpoles exposed to (J,J’) MTH display patchy, reduced expression of this muscle marker. (I’, J’) higher magnification images of boxed regions in (I,J).
K-L’) SMAC expression in the mesoderm increases, ubiquitously surrounding the epithelium in (K,K’) NF 46 control tadpoles. Although SMAC expression around the intestine increases in (L,L’) MTH exposed embryos relative to earlier developmental stages (H,H’,J,J’), SMAC expression is still reduced and patchy compared with (K,K’) solvent treated siblings. (K’, L’) Higher magnification images of boxed regions in (K,L).

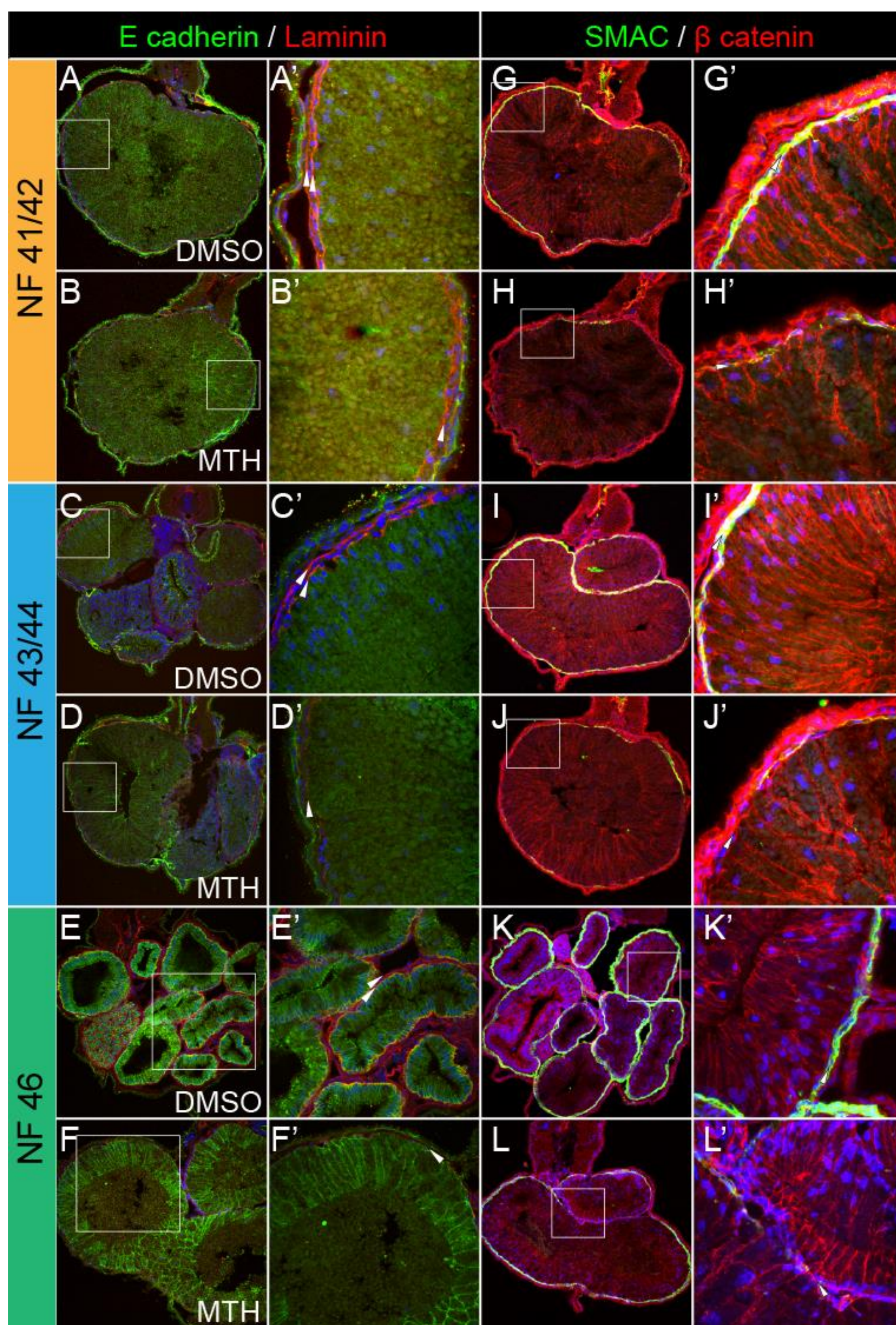
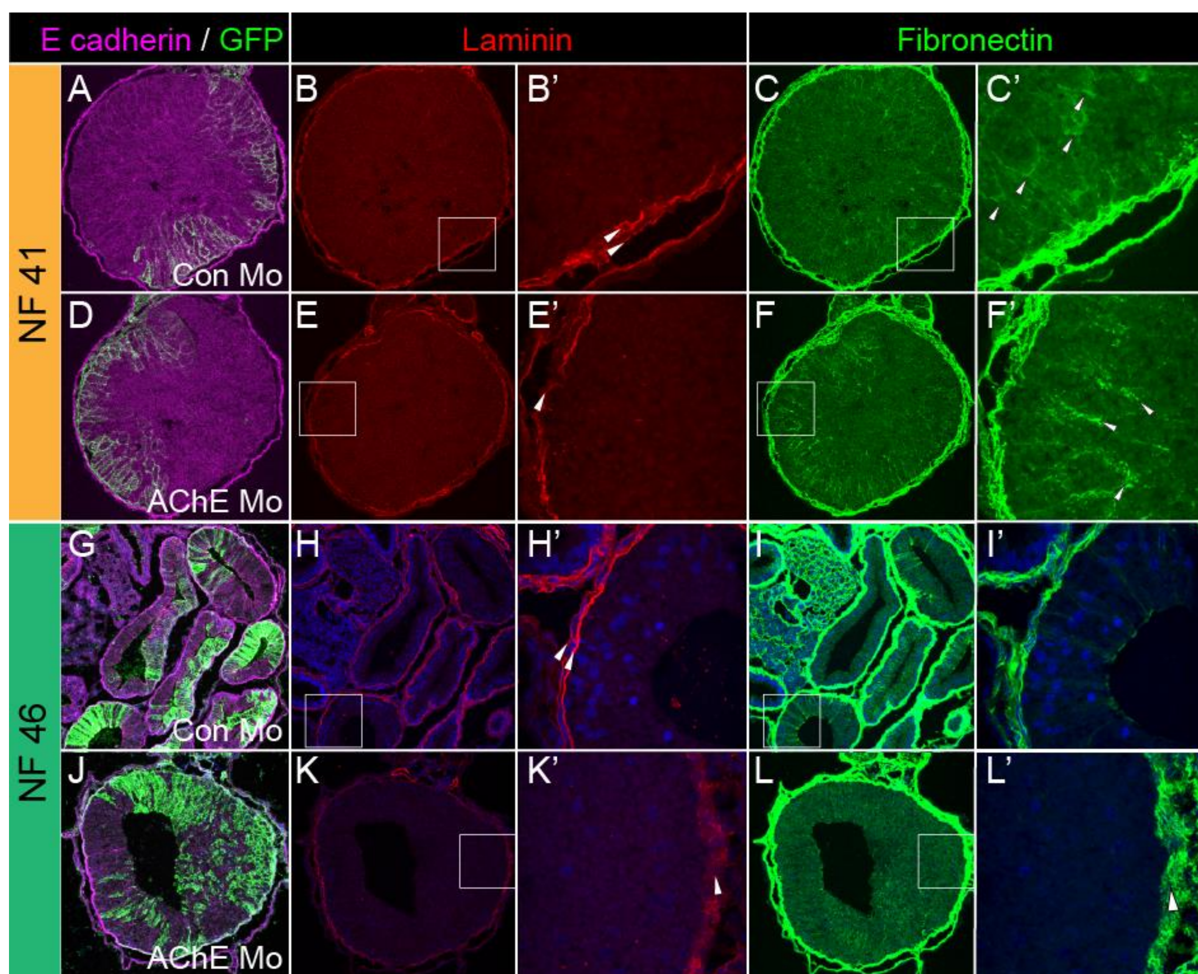

E cadherin / Laminin
SMAC / β catenin
NF 41/42
NF 43/44
NF 46
A
A'
G
G'
DMSO
B
B'
H
H'
MTH
C
C'
I
I'
DMSO
D
D'
J
J'
MTH
E
E'
K
K'
DMSO
F
F'
L
L'
MTH

Figure 2.10) AChE knockdown disrupts mesodermal structures at NF 41 and NF 46. A-F') Expression and organization of ECM proteins in NF 41 embryos injected with (A-C') control or (D-F') AChE morpholinos. (A,D) Morpholinos were co-injected with GFP mRNA to identify (green) injected cells in the heterogeneous intestines (cells that do not contain GFP are wildtype, and express AChE). Serial sections from these embryos were obtained to investigate ECM protein expression and structure (B,C,E,F). Laminin (red) is becoming organized into two parallel layers around the basement membrane of the gut in (B,B') control embryos (arrowheads A'). In contrast, only a single laminin layer is present when AChE is knocked down in the mesoderm (E,E', arrowhead in E'). Fibronectin (green) is expressed in both the endoderm and mesoderm at NF 41 (C,C'), and shows asymmetric expression biased toward the basal surface of endoderm cells (arrowheads C'). Interestingly, endodermal expression of fibronectin appears altered with loss of AChE expression (F,F'). Fibronectin appears to be distributed at lateral and apical cell surfaces (arrowheads F') in AChE knockdown cells. (B',C',E',F') higher magnification images of boxed regions in (B,C,E,F) respectively.

G-L') ECM structure and protein expression in NF 46 embryos injected with (G-I') control or (J-L') AChE morpholino. (G,J) Morpholinos were co-injected with GFP mRNA to identify (green) injected cells in the heterogeneous intestines (cells that do not contain GFP are wildtype, and express AChE). Serial sections from these embryos were obtained to investigate endoderm protein expression and cell structure in (H,I) control and (K,L) AChE knockdown embryos. In (H,H') control morpholino injected embryos, laminin (red) is expressed in two parallel layers in the intestinal endoderm (arrowheads H'). In contrast, laminin distribution appears disorganized, with no parallel structures identifiable in mesoderm lacking (K,K') AChE expression (arrowhead in K'). Fibronectin (green) expression is limited to the mesoderm in (I,I') control morpholino injected intestines. In (L,L') AChE morpholino injected mesoderm, this organization appears broad and scattered, similar to laminin disorganization (arrowhead L') in these embryos, as opposed to the single fibronectin layer in controls (I,I'). (H',I',K',L') higher magnification images of the boxed regions in (H,I,K,L) respectively.


E cadherin / GFP
Laminin
Fibronectin
NF 41
NF 46
A
B
B'
C
C'
D
E
E'
F
F'
G
H
H'
I
I'
J
K
K'
L
L'
Con Mo
AChE Mo
Con Mo
AChE Mo

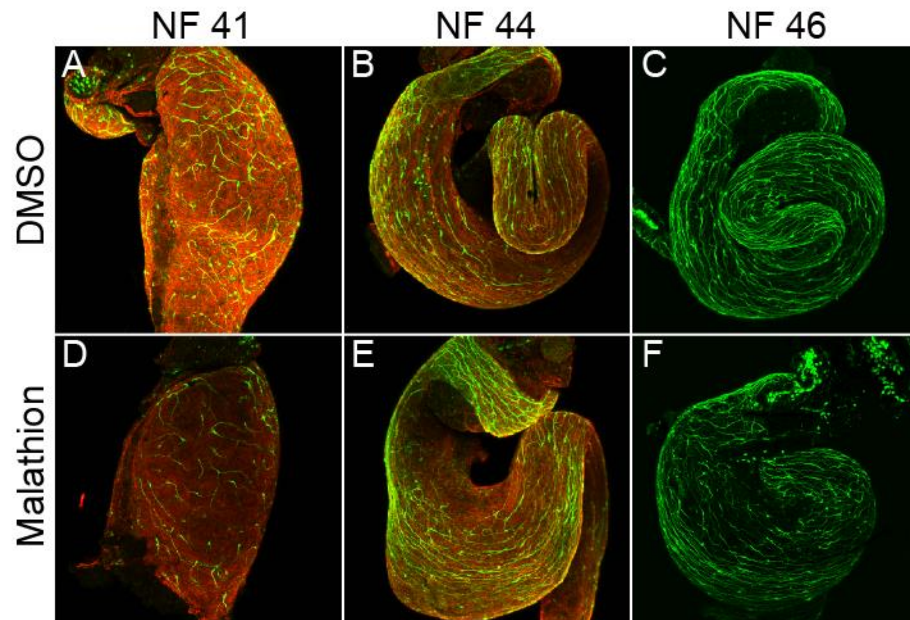
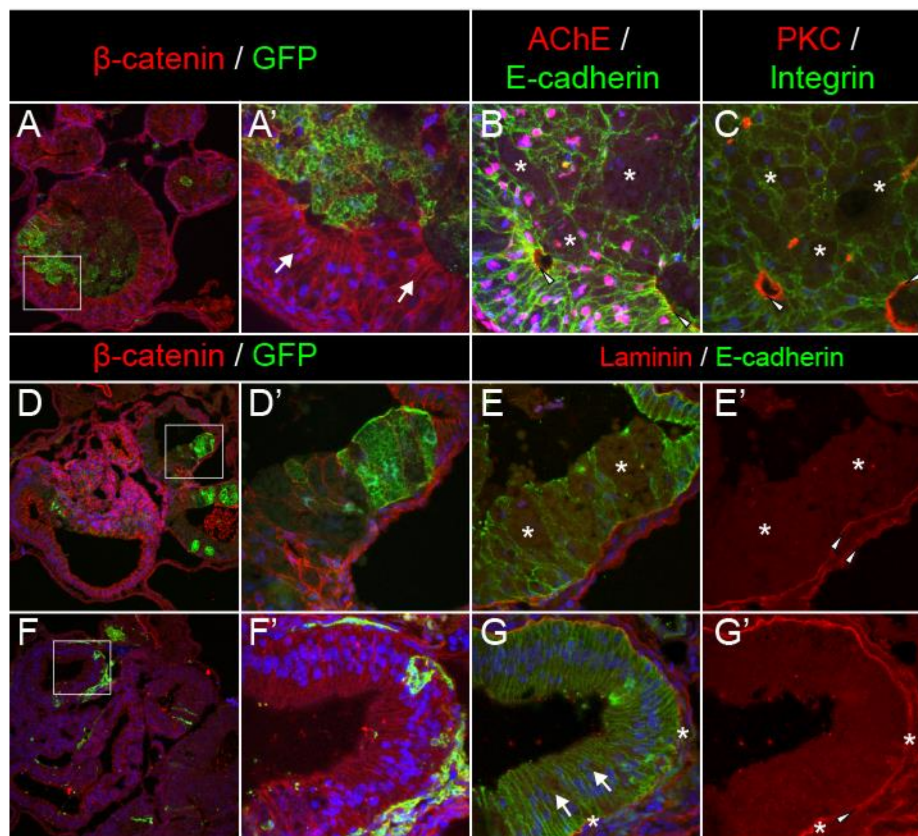


Figure 2.11) Exposure to AChE inhibitors transiently alters neural branching in the enteric nervous system.

Neurons are detected by acetylated tubulin (green) expression in intestines from (A-C) DMSO and MTH (D-F) treated tadpoles at NF 41 (A,D), NF 44 (B,E), and NF 46 (C,F). Expression of laminin (red) is shown as a counterstain in (A,B,D,E) outlining the shape of the intestines. Compared with extensive neural branching in (A) control embryos at NF 41, neurons in (D) MTH treated groups show fewer, shorter extensions. In (B) NF 44 and (C) NF 46 embryos exposed to DMSO, neuron tracts are parallel to the curvature of the intestine. Although intestines from (E,F) MTH treated tadpoles are visibly shorter than (B,C) DMSO counterparts, neuronal structures appear similar among the groups, as they are oriented parallel to the curvature of the intestine.

Figure 2.12) AChE acts cell autonomously in the intestine.
A-C) Serial sections from an embryo co-injected with AChE morpholino and GFP mRNA at the eight-cell stage, targeting knockdown to the future intestine. Injected cells are identified by (A,A') GFP expression (green). AChE knockdown in the endoderm affects cell shape in injected cells (asterisks B,C), but does not alter development in uninjected wild type cells (arrows A'). Uninjected cells form a single, columnar epithelium (arrows A') and show normal apical expression of (B) AChE (red, arrowheads) and (C) PKC (red, arrowheads) even when surrounded by endoderm cells that do not express AChE (green cells A', asterisks B,C). (A') higher magnification image of boxed region in (A). (B,C) High magnification images of serial sections near the region shown in (A').
D-E') Serial sections from an embryo where AChE is knocked down in the endoderm, but not in the mesoderm of the intestine. (D,D') GFP positive cells (green) identify cells in which AChE has been knocked down. (D') AChE is knocked down in the endoderm, but expressed in the mesoderm. (E,E') AChE knockdown in the endoderm results in large round endoderm cells instead of the normal columnar epithelia (asterisks E (e-cadherin – green, outlines cell membranes),E') but does not affect laminin (red) organization in the mesoderm (arrowheads E'). (D') High magnification image of the boxed region in (D). (E,E') high magnification image from serial section of same region shown in (D').
F-G') Serial sections from an embryo where AChE is knocked down in the mesoderm, but not in the endoderm of the intestine. (F,F') GFP positive cells (green) identify cells in which AChE has been knocked down. (F') AChE is knocked down in the mesoderm, but expressed in the endoderm. (G,G') AChE knockdown in the mesoderm (asterisks) disrupts laminin (red, arrowheads) structure in the mesoderm, as only a single layer is observed instead of the two parallel layers that normally form in the intestine. The underlying endoderm, which expresses AChE forms normally as a single cell layered columnar epithelium is present (arrows G, e-cadherin (green) outlines endoderm cell membranes). (F') High magnification image of the boxed region in (F). (G,G') high magnification image from serial section of same region shown in (F')


β-catenin / GFP
AChE / E-cadherin
PKC / Integrin
A
A'
B
C
β-catenin / GFP
Laminin / E-cadherin
D
D'
E
E'
F
F'
G
G'

CHAPTER 3 – ACETYLCHOLINESTERASE PLAYS A NON-NEURONAL, NON-ESTERASE ROLE DURUING ORGANOGENESIS

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In Preparation for Submission to *Developmental Cell*

Introduction:

Acetylcholinesterase (AChE) is a highly conserved serine esterase, well studied for its enzymatic cleavage of the neurotransmitter, acetylcholine (ACh), in the nervous systems of invertebrates and vertebrates. The importance of this protein in termination of nervous signaling is underscored by the wide number of natural and man-made toxicants that inhibit AChE's esterase activity (Costa, 2006; Mileson, 1998; Pope et al., 2005). AChE inhibition elicits toxicity because of increased ACh concentrations and excessive cholinergic signaling (Costa, 2006; Mileson, 1998; Pope et al., 2005). Inhibition of AChE has also proven beneficial for the treatment of Alzheimer's disease and *Myasthenia gravis*, in which reduced cholinergic signaling is responsible for many of the associated symptoms (Greig et al., 2013; Pope et al., 2005).

Interestingly, AChE is expressed in non-neuronal tissues and in organisms that lack nervous systems (e.g. plants, fungi, sponges) (Karczmar, 2010). AChE also shares sequence and structural homology with electrotactins, non-enzymatic proteins that function in cell-substrate adhesion (Botti et al., 1998; Gilbert and Auld, 2005; Soreq, 2001). *In vitro,* AChE

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has been shown to promote cell-substrate adhesion in a wide variety of cell types, leading to speculation that AChE has an ancient, conserved role in cell-substrate adhesion, independent of its esterase activity (Anderson et al., 2008; Genever et al., 1999; Inkson et al., 2004; Johnson and Moore, 1999, 2004; Sharma et al., 2001). Indeed, many of these studies suggest that esterase activity is dispensable for AChE's adhesive properties, and peptide motifs outside of the enzymatic gorge bind directly to extracellular matrix (ECM) proteins (Johnson and Moore, 2004). Intriguingly, AChE shows dynamic expression patterns during organogenesis in sea urchins, amphibians, and chicks (Drews, 1975). AChE expression is associated with cell morphogenic movements and differentiation of non-neuronal tissues in these embryos (Drews, 1975). AChE expression is similarly associated with cell movements during wound healing and tissue regeneration, in which developmental programs may be redeployed (Anderson et al., 2008; Fossati, 2013; Fossati et al., 2015). Furthermore, non-esterase functions of AChE have been attributed to changes in cytoskeletal structure and polarity (Anderson et al., 2008; Genever et al., 1999; Inkson et al., 2004). Cell-substrate interactions are well known to mediate changes in these cell behaviors, which are critical for directed cell movements during morphogenesis (Davidson et al., 2006; Marsden and DeSimone, 2001, 2003; Matsui et al., 2007; Ramos et al., 1996; Trinh and Stainier, 2004; Weber et al., 2012). Hence, it has been hypothesized that AChE has non-neuronal, non-esterase functions during development (Paraoanu et al., 2006; Vogel-Hopker et al., 2012).

Consistent with a role of AChE in organogenesis, exposure of zebrafish, frog, or chick embryos to AChE inhibiting chemicals (organophosphate (OP) and carbamate

pesticides) results in both neural and non-neural structural defects. Abnormalities in size and looping were observed in both the heart and the intestine of exposed zebrafish, frog, and chick embryos (Pamanji et al., 2015a; Pamanji et al., 2015b; Schock et al., 2012; Snawder and Chambers, 1989, 1990), and AChE expression is found prior to innervation in both organs (Bertrand et al., 2001; Downes and Granato, 2004; Drews, 1975). Case studies in humans have reported similar heart defects in infants exposed to organophosphates *in utero* (Romero, 1989; Sherman, 1996). These similarities may suggest a conserved role for AChE in vertebrate organogenesis. However, chemical exposures are blunt tools that affect both esterase activity and non-esterase functions, by causing conformational changes in the AChE protein structure (Kardos and Sultatos, 2000; Sultatos and Kaushik, 2008). Chemicals may also have off-target effects, limiting their utility for interpreting developmental pathways. AChE mutant zebrafish die in early larval stages, have severe motility defects, and display abnormalities in development/maintenance of neuromuscular junctions (Behra et al., 2002; Downes and Granato, 2004). Pericardial edema may suggest underlying cardiac defects, but organs appear grossly normal (Downes and Granato, 2004). Surprisingly, AChE$^{-/-}$ mice survive to birth, but die shortly thereafter unless supplemented with high caloric diets and extra care (Duysen et al., 2002; Xie et al., 2000). They display a number of neural, behavioral, and optical abnormalities and never grow as large as wild type littermates (Duysen et al., 2002). However, like zebrafish, the internal organs appear to develop normally (Xie et al., 2000). The relatively minor defects in AChE mutant models have led to speculation that the non-esterase functions observed *in vitro* are redundant or that

compensatory mechanisms are engaged in the absence of AChE *in vivo* (Cousin et al., 2005; Johnson et al., 2008*)*. This conclusion seems premature, particularly because organ structure has not been examined at the cellular level, and the defects that have been described in knockouts are not mechanistically resolved. Esterase or non-esterase functions of AChE could contribute to the observed defects, such as the need for a higher caloric intake but reduced body size in $AChE^{-/-}$ mice (Duysen et al., 2002). The ubiquity of AChE expression and unanswered questions about its non-neural functions led us to examine AChE's role during organogenesis.

Here we show that AChE expression in non-neuronal, gut endoderm cells is required for intestinal elongation. We also demonstrate that endodermal AChE is necessary for cell polarity, cytoskeletal reorganization, and the cellular rearrangements that drive intestinal elongation. AChE's esterase activity is not essential for direction of these cellular changes, indicating non-esterase functions are necessary *in vivo.* Our evidence suggests that AChE is not required for cadherin mediated cell-cell adhesion but is involved in cell-substrate adhesion. Interestingly, AChE is required for cell-substrate adhesion on fibronectin, but not laminin. Cell interactions with fibronectin are essential for directing cytoseletal rearrangements and establishing polarity in migrating cells. Fibronectin is also essential for intestinal development. In combination with our results, it seems likely that AChE directs cellular changes during organogenesis via a fibronectin-mediated mechanism.

Results:

Acetylcholinesterase is required for intestinal elongation

Xenopus laevis tadpoles were exposed to the organophosphate pesticides malathion or chlorpyrifos-methyl, or to the AChE inhibitor, huperzine A, during intestinal development (NF 33 – NF 46). Exposure to any of these AChE inhibitors results in short, malrotated intestines as compared with DMSO treated sibling controls, suggesting that AChE is required for intetestinal development (Fig. 3.1 A-E). Edema, bent notochords, and hemorrhaging were also noted for all three chemicals. Inhibition of AChE activity by these compounds was confirmed by detecting whole body AChE activity with the Ellman assay (Ellman et al., 1961)(Fig. 3.1 F).

To determine where AChE is expressed, whole intestines were dissected from NF 41 embryos, when the intestine is still occluded, just prior to midgut elongation (Chalmers and Slack, 1998; Muller et al., 2003). Foreguts were removed and cDNA was prepared from mid-hind guts containing all tissue layers or from isolated mid-hind gut endoderm. PCR confirms that gut endoderm was successfully isolated away from the mesodermal layer as the endoderm specific marker IFABP can be detected, but the mesoderm specific marker, FoxF1, is absent in endoderm preparations (Fig. 3.1, G). Importantly, AChE is expressed within the non-neuronal endoderm cell population, suggesting a non-classical role for AChE in development. Endogenous AChE protein is expressed at endoderm cell membranes and is also present in the cytoplasm at NF 41 (Fig. 3.1, H,H'). By NF 46 when the intestine has elongated and rotated counterclockwise, AChE's endodermal expression is localized to the

apical epithelial membrane, with reduced expression along lateral cell membranes (Fig. 3.1, J,J'). Injection of GFP tagged AChE at the 8 cell stage, targeting the future midgut yields a similar protein expression (Fig. 3.1, I,I',K,K'). Although AChE mRNA and protein are expressed in the gut endoderm, enzymatic activity within the intestine could not be detected using the Ellman enzymatic assay (Data not shown). Previous studies similarly report low AChE activity in non-neural cells that express AChE protein, which may point to non-esterase functions in these cells (Anderson et al., 2008; Thullbery et al., 2005).

While malathion, chlorpyrifos-methyl, and Huperzine A share a common mechanism of action, the potential for off-target effects exists (Costa, 2006; Pope et al., 2005). Therefore, to confirm that the observed intestinal defects were due to chemical interactions with AChE, a morpholino was designed against the 5' UTR of *Xenopus laevis* AChE mRNA. The morpholino was injected into the right dorsal vegetal blastomere at the 8-cell stage, to target knockdown of AChE to the midgut of the intestine. Morpholino knockdown of AChE similarly resulted in shortened, malrotated gut tubes compared with standard control morpholino injected siblings (Fig. 3.1 L,M,P). Co-injection of 8-cell embryos with AChE morpholino and wild type (wt) AChE mRNA rescues gut elongation and rotation, indicating that AChE is required for intestinal development (Fig. 3.1 N,P).

AChE has both enzymatic and non-enzymatic roles *in vitro*. Chemical inhibition of the esterase site causes structural changes that affect both of these functions (Kardos and Sultatos, 2000; Sultatos and Kaushik, 2008). To determine the relative contributions of such functions during gut morphogenesis, we co-injected 8-cell embryos with the AChE

morpholino and a mutated (mut) AChE mRNA that lacks enzymatic activity when translated. This enzymatically null mutant rescued intestinal elongation and rotation to the same degree as wt AChE mRNA (Fig. 3.1 O,P). This strongly suggests that non-esterase functions of AChE are essential for gut morphogenesis. Non-esterase functions are further suggested by the absence of any effect on gut development in embryos exposed the ACh receptor (AChR) agonist, carbachol (Fig. 3.S1). Carbachol exposure should mimic the excessive cholinergic signaling that results from AChE inhibition, and is lethal at higher doses. Similarly, antagonism of AChRs has no effect on intestinal development, and does not rescue the phenotypes elicited by exposure to AChE inhibitors (Fig. 3.S1). This further indicates that AChE has non-esterase functions during organogenesis.

The biological activity of the injected reagents was confirmed by injecting 1-cell embryos with morpholinos or mRNA for total AChE knockdown or overexpression. Embryos were harvested at NF 35 and the Ellman activity assay performed on whole embryos (Fig. 3.1 Q). Injection of the AChE morpholino resulted in a significant decrease in AChE activity relative to controls (Fig. 3.1 Q). Injection of wt AChE mRNA resulted in a significant increase in AChE activity, while injection of mut AChE mRNA did not alter activity from endogenous levels (Fig. 3.1 Q).

Acetylcholinesterase is required for ***gut endoderm*** *cell shape, arrangement, and polarization*

Intestinal endoderm cells radially intercalate between neighboring cells, driving elongation of the gut tube as cell layers are reduced from 4-5 cells thick to a single cell epithelium surrounding a hollow lumen (Chalmers and Slack, 2000; Reed et al., 2009). This process

requires cells to change shape and polarize as they elongate along their apical-basal axes and narrow mediolaterally. To determine the effects of AChE knockdown at a cellular level, we sectioned both control and AChE morpholino-injected embryos, and stained these sections with a panel of antibodies chosen to reflect cell shape and polarity. In control morpholino injected cells and in uninjected cells, the mature epithelium consists of a single layer of columnar epithelial cells (Fig 3.2, A,E,I). These cells are elongated along their apical-basal axes and polarized, as indicated by the expression of proteins, such as aPKC, at only the apical surface (Fig 3.2, E,I (red)). In contrast, cells that lack AChE do not form a single cell layer, revealing defects in cell rearrangement (Fig 3.2, B,F,J). These cells are also rounder in shape, failing to form the columnar epithelium characteristic of a mature intestine (Fig. 3.2 B,F,J). This difference in cell shape is quantified by a significant reduction in the Length:Width ratio of the endoderm cells that do not express AChE as compared with controls (Fig. 3.2 M). In addition to the defects in rearrangement and shape, endoderm cells do not polarize without AChE as indicated by the absence of aPKC at the apical surface (Fig. 3.2, F,J (red)).

These defects are rescued by co-injection of the AChE morpholino with either wt AChE mRNA or mut AChE mRNA (Fig. 3.2 C,D,G,H,K,L,M). This suggests that AChE is required for gut endoderm cells to change shape, polarize, and intercalate between their neighbors to form a mature epithelium. Importantly, the catalytically inactive mut AChE was able to restore cell shape, polarity, and movement to cells lacking endogenous AChE (Fig. 3.2 D,H,L,M). This suggests that AChE has a non-esterase function in directing intestinal

development *in vivo*. Exposure to AChE inhibiting compounds results in similar defects in cell shape and polarity (Fig. 3S2), though somewhat less severe than in AChE morpholino injected embryos. This suggests that these compounds disrupt non-esterase functions of AChE *in vivo.*

To determine if altered differentiation, proliferation, or cell death contribute to the cellular phenotypes associated with loss of AChE, intestinal cross-sections were examined for pH3 (proliferation), caspase 3 (apoptosis), and intestinal fatty acid binding protein (IFABP, differentiation). IFABP is expressed in the mature epithelium of the proximal two-thirds of the small intestine (Chalmers and Slack, 1998). AChE expression was required for cell differentiation, but did not appear to have a role in proliferation or apoptosis (Fig. 3.S3).

Acetylcholinesterase is required for cytoskeletal rearrangement in ***gut endoderm*** *cells*

AChE is required for cells to change shape and become elongated along their apical-basal axes (Fig. 3.2 M). Prior work suggests that cell shape changes and polarization depend upon rearrangement of the cytoskeleton (Weber et al., 2012). Therefore, it is unsurprising that perturbing AChE expression also affects cytoskeletal organization. In control morpholino injected endoderm cells, microtubules are arranged along the apical-basal axis, and are enriched apically (Fig 3.3 A,E,I). In contrast, cells that lack AChE expression display disorganized microtubules that do not align with any cell axis and show no evidence of apical enrichment (Fig. 3.3 B,F,J).

Microtubule organization was restored by co-injection of the AChE morpholino with either wt AChE mRNA or mut AChE mRNA, suggesting that the enzymatic activity of

AChE is non-essential for cytoskeletal rearrangement (Fig. 3.3 C,D,G,H,K,L). Similar defects in cytoskeletal structure are observed in tadpoles exposed to AChE inhibitors during intestinal development (Fig. 3.S2). This suggests that AChE's non-esterase functions are required for the organization of cytoskeletal structure *in vivo*.

*Acetylcholinesterase is required for **gut endoderm** cell adhesion on fibronectin but not laminin*

Cell-cell and cell-substrate adhesion play crucial roles in directing cell shapes, polarity, cytoskeletal rearrangements, and cell movements due to differences in physical force and activation of signaling cascades (Davidson et al., 2006; Marsden and DeSimone, 2003; Weber et al., 2012). Furthermore, changes in cell-cell adhesion have been linked to loss of cell polarity and altered endoderm shape in the intestine (Dush and Nascone-Yoder, 2013; Reed et al., 2009). To determine if AChE is required for cell-cell adhesion, we isolated intestinal endoderm cells from NF 41.5 embryos injected into both dorsal vegetal blastomeres at the 8-cell stage with either standard control morpholino or AChE morpholino, increasing the number of injected cells in the intestine. Intestines were dissected from morpholino injected embryos, foreguts removed, and endoderm cells dissociated with Calcium Magnesium Free Media (CMFM). Morpholino injected cells (GFP positive) were separated from uninjected cells from the same embryo. CMFM was replaced with MBS containing calcium ions and cell reaggregation monitored for 30 minutes. Cells lacking AChE reaggregated to the same degree as wild type cells or control morpholino cells, suggesting AChE is not required for cell-cell adhesion (Fig. 3.4, A,B). Similarly, exposure to

AChE inhibiting chemicals, AChR agonists, or AChE antagonists had no effect on cell-cell reaggregation (data not shown), suggesting AChE is not required for cell-cell adhesion.

AChE has previously been shown to interact with ECM proteins and to facilitate cell-substrate adhesion through these same peptide motifs independently of its esterase activity (Johnson and Moore, 2004). Endoderm cells were isolated from morpholino injected embryos as described above. Following dissociation in CMFM, endoderm cells were plated on laminin or fibronectin substrates. Endoderm cells lacking AChE were significantly less adherent on fibronectin compared with cells injected with the standard control morpholino (Fig. 3.4 C). There was no significant difference in adhesion when cells were plated on laminin (Fig. 3.4 D). This suggests that AChE promotes cell-substrate adhesion by a fibronectin dependent mechanism in the intestine. Overall, gut endoderm cell adhesion is lower on laminin than fibronectin (Fig. 3.4 C,D), which likely reflects differences in the location of laminin and fibronectin in the developing gut. At NF 41.5 laminin is located only in the basement membrane (Fig. 3.4 F,F'). By comparison, fibronectin is expressed throughout the many cell layers of the gut endoderm at this stage (Fig. 3.4 E,E'). Therefore, all of the endoderm cells interact with fibronectin, but only the most basal endoderm cells interact with laminin at this stage. Similar to fibronectin, AChE is expressed throughout the intestinal endoderm at NF 41.5 (Fig. 3.1 H-I'), which might suggest a specific interaction between these proteins.

Discussion:

Our work presents direct evidence for an essential, non-esterase function of AChE during organogenesis. Such a role has long been predicted, but has not been substantiated by experimental evidence until now. Consistent with *in vitro* observations, we find that AChE is required for cell polarization, cytoskeletal organization, and rearrangement of intestinal cells, independently of esterase activity. Additionally, we find that AChE is required for cell adhesion to fibronectin, but not laminin. Cell-substrate interactions are crucial for directing polarization, cytoskeletal changes, and movement during morphogenesis (Brafman et al., 2013; Davidson et al., 2006; Marsden and DeSimone, 2003; Rozario and DeSimone, 2010; Weber et al., 2012). Thus, we suggest that AChE promotes intestinal development through a fibronectin-mediated mechanism.

AChE is required for organogenesis

The observation that AChE expression corresponds with migrating and differentiating cells during organogenesis was made over 40 years ago (Drews, 1975). This association was seen in multiple vertebrate species as well as in invertebrate sea urchins, and suggested an ancient, conserved role of AChE during development (Drews, 1975). Exposure to the AChE inhibiting organophosphate and carbamate pesticides similarly disrupts diverse developmental processes including spicule elongation in sea urchins (Ohta et al., 2009), heart development in zebrafish, frogs, and chicks (Pamanji et al., 2015a; Pamanji et al., 2015b; Schock et al., 2012; Snawder and Chambers, 1990; Wyttenbach and Thompson, 1985), and intestinal development in frogs (Bacchetta et al., 2008; Bonfanti et al., 2004; Snawder and

Chambers, 1989, 1990). The shared mechanism of action of these compounds also suggests a role for AChE in the development of non-neural tissues. However, experimental evidence to directly test the role of AChE in organogenesis has been lacking.

We found that exposure to AChE inhibiting chemicals disrupted intestinal development in *Xenopus laevis* tadpoles (Fig. 3.1 A-F), as has been previously reported (Bonfanti et al., 2004; Snawder and Chambers, 1989, 1990). These intestinal defects are recapitulated when AChE is knocked down within the gut endoderm (Fig. 3.1 L,M). Intestinal development is restored with co-injection of exogenous wt AChE mRNA (Fig. 3.1 N, P), providing direct experimental evidence that AChE is required for organogenesis in a vertebrate model. Importantly, intestinal development is also rescued by co-injection of mut AChE mRNA (Fig. 3.1 O,P). Rescue by this catalytically null mutant provides evidence of a non-esterase function *in vivo*. AChE has been predicted to have non-esterase functions during organogenesis, but this function has not previously been demonstrated. The absence of teratogenesis in tadpoles exposed to AChR agonists or antagonists similarly suggests a non-esterase function (Fig. 3.S1). These reagents act similarly to ACh, and would be expected to elicit an intestinal phenotype if AChE's esterase activity was of primary importance during development. Furthermore, AChE activity is low in the intestine despite mRNA and protein expression throughout the non-neuronal endoderm (Fig. 3.1 G-K'). Low esterase activity has previously been reported in non-neuronal cells, and may point towards non-esterase functions of this ancient protein (Anderson et al., 2008; Thullbery et al., 2005).

Our data suggest AChE plays an essential role in intestinal morphogenesis. Interestingly, $AChE^{-/-}$ mice require high caloric diets to survive, but remain smaller than wildtype littermates throughout life (Duysen et al., 2002). While this metabolic/growth deficiency might be related to loss of esterase activity, the authors suggest it may result from poor nutrient absorption, unrelated to esterase function (Duysen et al., 2002). This could indicate structural defects in the intestine, which limit nutrient absorption. Although intestinal defects were not identified in post-natal mice, subtle differences in epithelial polarity or cytoskeletal structure may have been overlooked (Xie et al., 2000). Intestinal malformations are common in humans, but the underlying etiologies are largely unknown. Human AChE is more susceptible to inhibition by organophosphates than *Xenopus* AChE, and may put infants at greater risk during development (Shapira et al., 1998). For instance, *in utero* and early adolescent exposure to organophosphates is associated with decreased cognitive functions in children (Bouchard et al., 2011; Gonzalez-Alzaga et al., 2014; Roberts et al., 2012). Similarly, exposure to AChE inhibitors *in utero*, may impact organogenesis, as congenital heart defects are associated with organophosphate exposure and organophosphates were one of the few compounds to show any association with anorectal stenosis/atresia (Carmichael et al., 2016; Carmichael et al., 2014; Romero, 1989; Sherman, 1996). AChE expression is associated with several congenital intestinal defects (e.g. Hirschsrung's disease, intestinal neuronal dysplasia), but its importance in has not been assessed (Friedrich et al., 1994; Martucciello et al., 2002; Moore and Johnson, 2005). Our results suggest that both

esterase and non-esterase functions of AChE should be evaluated in the context of human birth defects.

AChE has non-esterase functions in vivo

The intestinal endoderm is initially organized as multiple layers of unpolarized cells. As organogenesis proceeds, these cells become polarized along their apical-basal axes, and radially intercalate, producing a single cell layered columnar epithelium and simultaneously driving elongation of the tract (Chalmers and Slack, 1998, 2000; Reed et al., 2009). This process depends upon changes in cell polarity, cytoskeletal dynamics, and adhesion (Dush and Nascone-Yoder, 2013; Reed et al., 2009). AChE has a non-esterase function essential for these changes to occur. Knockdown of AChE results in multiple layers of round, unpolarized cells with disorganized cytoskeletal structures (Fig. 3.2, 3.3). Additionally, cells that lack AChE expression do not differentiate, though proliferation and cell death do not appear to be affected (Fig. 3.S3). These cellular defects are rescued by either wt or mut AChE mRNA, restoring the polarized, columnar epithelium characteristic of the mature intestine (Fig. 3.2,3.3, 3.S3). To our knowledge, this provides the first *in vivo* experimental evidence of a non-esterase AChE function in directing polarized cell behavior, cytoskeletal alterations, and differentiation. Similar defects in epithelial polarity and cytoskeleton are seen in tadpoles exposed to AChE inhibitors (Fig. 3.S2), suggesting that non-esterase functions are altered due to inhibition of the active site. Conformational changes occur due to organophosphate binding to the catalytic triad, and explain the ability of active site inhibitors to disrupt non-esterase functions *in vivo* (Kardos and Sultatos, 2000; Sultatos and Kaushik, 2008).

Non-esterase functions of AChE in polarity, cytoskeletal organization, and differentiation have previously been described *in vitro*. For example, AChE is expressed at the leading edge of fibroblasts and facilitates cell migration, indicating a role for AChE in polarized cell behavior (Anderson et al., 2008). Anti-body sequestration of AChE disrupts wound healing, whereas excess ACh has no effect on fibroblast movement, suggesting that AChE's role in polarized migration is independent of its esterase activity (Anderson et al., 2008). Our data also support a non-esterase role for AChE in cell polarization and movement. The similarity in AChE's non-esterase role in these two non-neural cell populations suggest a conserved function that has received little attention relative to AChE's well established role in the nervous system.

Cell polarization often depends upon cytoskeletal rearrangements (Gupton and Gertler, 2010; Weber et al., 2012). Extensive cytoskeletal rearrangements are involved in neurite outgrowth. AChE expression is associated with changes in cytoskeletal protein expression and localization in neurons (Chang et al., 2006; Dupree and Bigbee, 1994; Dupree et al., 1995; Keller et al., 2001). Additional studies suggest that AChE expression is required for neurite outgrowth, suggesting a role for AChE in directing cytoskeletal dynamics (Giordano et al., 2007; Sharma et al., 2001; Sperling et al., 2012; Sternfeld et al., 1998; Yang et al., 2008). Significantly, AChE peptide motifs that share homology with adhesive proteins, but do not contain the esterase site, can promote neuritogenesis to the same degree as full length AChE (Johnson and Moore, 2004). This indicates that AChE has non-esterase functions *in vitro* that can induce changes in cell structure and behavior. Our work suggests a

similar role for AChE *in vivo* (Fig. 3.3). Exposure of sea urchin larvae to OPs, inhibits spicule elongation, a process which also depends upon changes in cytoskeletal structure (Ohta et al., 2009). Hence, our data validates previous observations made *in vitro* and supports an evolutionary function of the protein in influencing multiple cell behaviors.

AChE is essential for cell-substrate adhesion on fibronectin

The importance of cell adhesion in driving cell polarization and directed protrusion, cytoskeletal rearrangements, differentiation, and movement during morphogenesis cannot be understated (Brafman et al., 2013; Davidson et al., 2006; Marsden and DeSimone, 2003; Matsui et al., 2007; Muhamed et al., 2016; Trinh and Stainier, 2004; Weber et al., 2012). *In vitro*, AChE has a well-described role in promoting cell adhesion to the ECM. AChE shares sequence homology with electrotactins, which have an adhesive function, but do not have esterase activity. Furthermore, AChE can bind specifically to collagen and laminin through non-esterase protein motifs (Johnson and Moore, 2004). AChE-ECM interactions are electrostatic, and AChE expression has been shown to correlate with the expression and adhesion of other ECM proteins, including fibronectin (Giordano et al., 2007; Syed et al., 2008). Consistent with a role in cell-substrate adhesion, we found that AChE is required for endoderm cell-substrate adhesion on fibronectin in *ex vivo* assays (Fig. 3.4). Interestingly, AChE is not required for cell-substrate adhesion on laminin or for cell-cell adhesion (Fig. 3.4). Fibronectin, but not laminin, is essential for directing the differentiation of human embryonic stem cell lines toward definitive endoderm – which gives rise to the intestine (Brafman et al., 2013). Furthermore, fibronectin knockdown disrupts intestinal development

in *Xenopus laevis*, suggesting the ECM protein is essential for organogenesis (Davidson et al., 2006; Marsden and DeSimone, 2001). Fibronectin interactions with other cell surface proteins (e.g. integrin) are essential for cell polarity, cytoskeletal dynamics, and cell migration during morphogenesis. For instance, fibronectin-mediated interactions are required for mesoderm cells undergoing convergent-extension (Davidson et al., 2006; Marsden and DeSimone, 2001, 2003) and for heartfield midline convergence (Matsui et al., 2007; Trinh and Stainier, 2004). The decrease in cell-substrate adhesion on fibronectin as a result of AChE knockdown, and the importance of fibronectin for cytoskeletal dynamics, cell polarity, and movement in other developmental contexts, suggests that AChE directs organogenesis by a fibronectin-dependent mechanism.

Based on AChE's non-esterase, adhesive role, *in vitro,* it seems likely that AChE at the cell membrane directly interacts with fibronectin in the endoderm to drive intestinal morphogenesis. The specific mechanics between these two proteins deserves more attention, but is beyond the scope of the current study. However, several observations in this study support a non-esterase AChE-fibronectin interaction, and should be mentioned. In contrast to keratinocytes where ACh acts as a signaling molecule to regulate cell-cell adhesion (Nguyen et al., 2003; Nguyen et al., 2000), we found no evidence for a role of ACh or AChE in cell-cell adhesion (Fig. 3.4). This suggests that AChE is not required for degradation of ACh in these cells and further supports a non-esterase function. Secondly, AChE esterase activity was very low in the developing intestine, despite protein and mRNA expression (Fig. 3.1). Prior studies report a decrease in AChE esterase activity in cells adherent on laminin, and

their data suggest competition exists between the esterase and adhesive properties of this protein (Howard et al., 2005; Johnson and Moore, 2004; Sperling et al., 2012). Hence, an adhesive interaction between AChE and fibronectin could explain the low enzymatic activity. Last, and most importantly, AChE and fibronectin are both expressed throughout the endoderm, whereas laminin expression is limited to the basement membrane. Thus, interactions between AChE and fibronectin are more likely to be required for directing cell polarization and movement than AChE-laminin interactions, and explain the differences in endoderm adhesion on the two substrates.

Materials and Methods:

Embryo Culture

Xenopus laevis tadpoles were obtained by *in vitro* fertilization, dejellied in 2% cysteine solution (pH 7.9), and reared between 14 - 23°C in 0.1X Marc's Modified Ringers (MMR) solution (Sive et al., 2000). Embryos were staged according to Nieuwkoop and Faber (Nieuwkoop, 1994). Following chemical treatment or microinjection, tadpoles were raised to NF 41-46 and anethetized in 0.05% MS222 for gross morphological analysis, dissections, and fixation.

Chemical exposures

Embryos were then exposed to the AChE inhibitors (20 mg/L malathion, 5 mg/L chlorpyrifos-methyl, or 10 µM Huperzine A) or to an equal volume of the DMSO solvent from NF stage 33 through stages 41-46. Optimal chemical doses were selected based on

dose-response curves to identify concentrations that disrupted intestinal development without causing lethality.

Morpholino Knockdown

Morpholino oligonucleotides (GeneTools, Inc) were designed to bind to the 5' UTR region near the translation start site of *Xenopus laevis* AChE mRNA (5'-CATGGCTGCTCCTCTGTGGGATTAC-3') or to a splice mutant of human β-globulin (5'-CCTCTTACCTCAGTTACAATTTATA-3') as a standard control. A pCS2 vector containing a coding region for cMyc tagged GFP was lineralized with *Not1*, capped mRNA synthesized with the mMessage Machine kit (Ambion), and purified by lithium chloride extraction. GFP mRNA was co-injected with morpholinos at 1- cell (40-80 ng/injection – whole body knockdown) and 8-cell (7.6 ng/injected blastomere – midgut targeted knockdown) stages.

AChE mRNA Injections

Total RNA was isolated from 10 NF 41 tadpole intestines using TRIzol (Ambion) and RT-PCR used to generate cDNA. *Xenopus laevis* AChE was amplified with PfuUltra II polymerase (Agilent) using the following forward and reverse primers: (F: 5' GCCGGATTCATGGCACTTGTACCC 3', R: 5' ATGTGGAACCCCCATCCACTGTGGCCAAG 3'). wt AChE DNA was inserted into the pCS2 vector and transformed into competent E. coli cells. The conserved serine (Ser224 in *X. laevis*) in the esterase catalytic triad was mutated to an alanine with PCR using the following forward and reverse primers (F: 5' GGGGAAGCTGCTGGTGCCGTCTCTGTGGG 3', R: 5' ACCAGCAGCTTCCCCAAAAATCATGACAGTCCTGGGATC 3'). mut AChE was

inserted into the pCS2 vector (NEBuilder HiFi DNA assembly kit) and mutation confirmed by sequencing. wt and mut AChE mRNA was synthesized as already described for GFP. wt or mut AChE mRNA were injected into 1 cell embryos (1200 pg/injection), or co-injected with morpholino into 8 cell embryos (800-1000 pg/blastomere). GFP tagged AChE was similarly inserted into the pCS2 vector (NEBuilder HiFi DNA assembly kit). The sequence was confirmed, mRNA synthesized as described, and 8 cell embryos injected (600-1000 pg/blastomere), targeting the dorsal, vegetal blastomeres.

AChE Activity Assays

Stage 33 to 46 embryos from chemical exposure and 1-cell morpholino and AChE mRNA microinjections were anesthetized in 0.05% MS222 and frozen at -80°C in groups of four. Embryos were homogenized in 1% Triton 0.05M Tris-HCL (pH 7.4) buffer at a 1:10 (w:v) ratio and centrifuged for 5 minutes at 15,000g at room temperature. Protein concentrations were determined with the Pierce BCA protein assay (ThermoScientific). Protein preparations were used in the Ellman AChE activity assay, modified for use in a 96 well plate (Ellman et al., 1961). Briefly, 20 µg of protein was added to 1% Triton 0.05M Tris-HCL (pH 7.4) buffer (total volume = 300 µL). 10 µL of 0.33mM f,f'-dithio-bis (2-nitrobenzoic acid) (DTNB) was added per well and the plate incubated at room temperature for 10 minutes. 2 µL of 0.075 M acetylthiocholine iodide was added per well and the absorbance read at 412 nm for 15 minutes. All samples were read in triplicate and 300µL Tris buffer with DTNB and acetylthiocholine idodide served as a blank. Activity was calculated as nmol acetylthiocholine iodide hydrolyzed per minute per µg protein.

Immunohistochemistry

Section immunohistochemistry was performed on transverse intestinal sections from NF 41 and 46 embryos as previously described (Dush and Nascone-Yoder, 2013). Primary antibodies in blocking buffer were applied to the slides at 4°C overnight at the following dilutions: E-cadherin (DSHB, 5D3; 1:200), β-catenin (SCBT, H-102; 1:100), aPKC (Santa Cruz, sc216; 1:200), α-tubulin (Sigma, T9026; 1:1000), active caspase 3 (Cell Signaling Technology 9661; 1:300), cMyc (Santa Cruz 9E10, 1:1000), AChE (Abcam, ab 97298; 1:200) GFP (1:500), pH3 (Millipore, 06-570; 1:500), IFABP (Kind gift 1:1000), Fibronectin (Kind gift from D.W. DeSimone, 1:1000), laminin (Sigma, L9393; 1:200). Secondary antibodies, Alexa 488-conjugated goat anti-mouse IgG (Invitrogen, A11029; 1:2000), Alexa 546-conjugated goat anit-rabbit IgG (Invitrogen, A11035; 1:2000), Alexa 488- conjugated goat anti-rabbit (Invitrogen, ;1:2000), and/or Alexa 546-congated donkey anti-mouse (Invitrogen, ;1:2000), were diluted in blocking buffer and applied to slides for 3 hours at room temperature. Fluorescence was visualized on a Leica SPEII confocal microscope, with 10X and/or 40X dry objectives.

Ex vivo cell assays

Intestines of 1 to 4 tadpoles were dissected from morpholino injected NF 41-42 tadpoles. Foreguts were removed and the intestines splayed open along the anterior-posterior axis. Intestines were inverted so that endoderm cells faced the media. Mid-hind gut explants were transferred to 9 mL calcium magnesium free media (CMFM) on 1% agarose coated well, and dissociated for 30 minutes at room temperature (Sive et al., 2000). Eyelash knives were used

to further dissociate cells, and the underlying mesoderm core removed by pipet. Cells were then used to assess cell-cell or cell substrate adhesion.

Cell-Cell adhesion

Dissociated cells were moved to the center of the 1% agarose coated dishes. CMFM was removed until endoderm cells began to roll along the agarose bottom. The media was replaced with 1XMBS containing calcium and magnesium ions. Cells were allowed to reaggregate for 30 minutes at RT.

Cell-Substrate adhesion assay

Plates were coated with 50 µg/ml laminin at 4°C overnight, washed with sterile PBS, and blocked with 0.2% BSA for 1 hour at 37°C. Plates were washed a with 1X PBS and 4 mL of cell media added (50% 1X MBS, 49% Leibovitz solution, 1% FBS, 50 µg/mL Gentamysin) (Sternfeld et al., 1998). Plates were coated with 50 µg/ml fibronectin for 30 minutes at room temperature, rinsed three times with 1X PBS, and 4 mL of cell media added. Dissociated cells were transferred to either laminin or fibronectin coated plates and allowed to adhere for 60-70 minutes at room temperature. Five 12X images of each plate were taken with a Zeiss Lumar microscope to determine the number of cells plated. 2 mL of cell media were removed from each plate and cells washed three times with 1X MBS. Cells were fixed in formaldehyde-gluteraldehyde (FG) overnight at 4°C. FG was removed, plates washed three times with PBST, and filled with 4 mL PBST. Five 12X images of each plate were aquired after fixation to determine the number of adherent cells on each substrate. Adobe Photoshop CS6 was used to automatically align the five images for each plate, to show the entire field of

plated or adherent cells. FIJI was used to automatically threshold images and quantify cell number. The percentage of adherent cells was determined by dividing the number of adherent cells by the number of plated cells and multiplying by 100 for each plate.

Statistics

Analysis of variance (ANOVA) was used to evaluate differences in means among three or more treatments. Student's t-test was used to evaluate the difference in means between two treatment groups. Differences were considered significant for p-value ≤ 0.05. StatCrunch statistical software was used for analysis. Error bars represent the standard error of mean in all graphs.

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Figure 3.1) AChE has a non-esterase role essential for intestinal organogenesis.
A-D) Gross phenotypes of NF 46 tadpoles exposed to AChE inhibiting chemicals during organogenesis (NF 32-46). Normal intestinal elongation and counterclockwise rotation is observed in tadpoles exposed to the solvent control, DMSO (A). Exposure to AChE inhibitors, (B) malathion, (C) chlorpyrifos-methyl, or (D) Huperzine A results in shortened, malrotated intestinal tracts. Scale bars = 1000 µm.
E) Quantification of the percentage of tadpoles with intestinal abnormalities in chemical exposures. (Percentages of embryos with intestinal abnormalities were averaged from at least 5 experimental replicates. Error bars represent the Standard Error of the Mean (S.E.M) among the separate experiments. n = the number of experimental replicates. Different letters indicate significant differences, $p<0.05$, ANOVA.)
F) Malathion, chlorpyrifos-methyl, or HuperzineA significantly inhibited whole body AChE esterase activity. (Bars show the average activity for at least 9 biological replicates ± S.E.M. Different letters indicate significant differences, $p<0.05$, ANOVA)
G) RT-PCR reveals expression of AChE mRNA in the non-neuronal endoderm cells of the intestine. Endoderm was successfully isolated from the mesodermal layer of the intestine as indicated by the expression of endoderm specific IFABP mRNA, but the absence of mesoderm specific FoxF1 mRNA in the sample.
H-K') Transverse cross-sections through the mid-guts of (H-I') NF 41 and (J-K') NF 46 embryos to determine AChE protein localization. Endogenous AChE (red) is expressed at endoderm cell membranes (arrows) and in the cytoplasm at (H,H') NF 41 and becomes localized to the apical epithelial surface by (J,J', arrowhead) NF 46, with reduced expression at the cell membranes (arrows). (H,J) E-cadherin (green) used to identify cell membranes. Injected exogenous GFP-tagged AChE (green) is similarly expressed at endoderm cell membranes (arrows) and in the cytoplasm of (I,I') NF 41 embryos. AChE is expressed at the apical epithelial surface (arrowhead) though membrane expression (arrows) and cytosolic AChE expression are also noted in (K,K') NF 46 embryos heterogeneously expressing GFP tagged AChE. (I,K) α-tubulin (magenta) was used to outline cell cytoskeletal structure. Scale bars = 25 µm.
L-O) Gross phenotypes of NF 46 embryos injected a the 8-cell stage with standard control morpholino, AChE morpholino (knockdown), or AChE morpholino with either exogenous wt AChE mRNA or mutant AChE mRNA, targeting the future midgut. Compared with embryos injected with the (L) standard control morpholino, embryos injected with the (M) AChE Mo display shortened, malrotated intestines. Intestinal malformations are rescued with co-injection of either (N) wt AChE mRNA or (O) mutant AChE mRNA, that lacks catalytic activity when translated. Scale bars = 1000 µm.
P) Quantification of the percentage of tadpoles with intestinal abnormalities in midgut targeted injected embryos. (Percentages of embryos with intestinal abnormalities were averaged from at least 3 experimental replicates. Error bars represent the Standard Error of the Mean (S.E.M) among the separate experiments. n = the number of experimental replicates. Letters indicate significant differences between groups, $p<0.05$, ANOVA.)

Q) Quantification of AChE activity in whole embryos injected at the 1 cell stage with morpholinos or mRNA. AChE morpholino injection inhibits AChE activity. Exogenous wt AChE mRNA injection increases AChE activity while injection of mutant AChE has no effect on endogenous activity. Control morpholino or GFP mRNA injection has no effect on AChE activity. (Bars show the average activity for at least 9 biological replicates ± S.E.M. Different letters indicate significant differences, $p<0.05$, ANOVA)

Figure 1

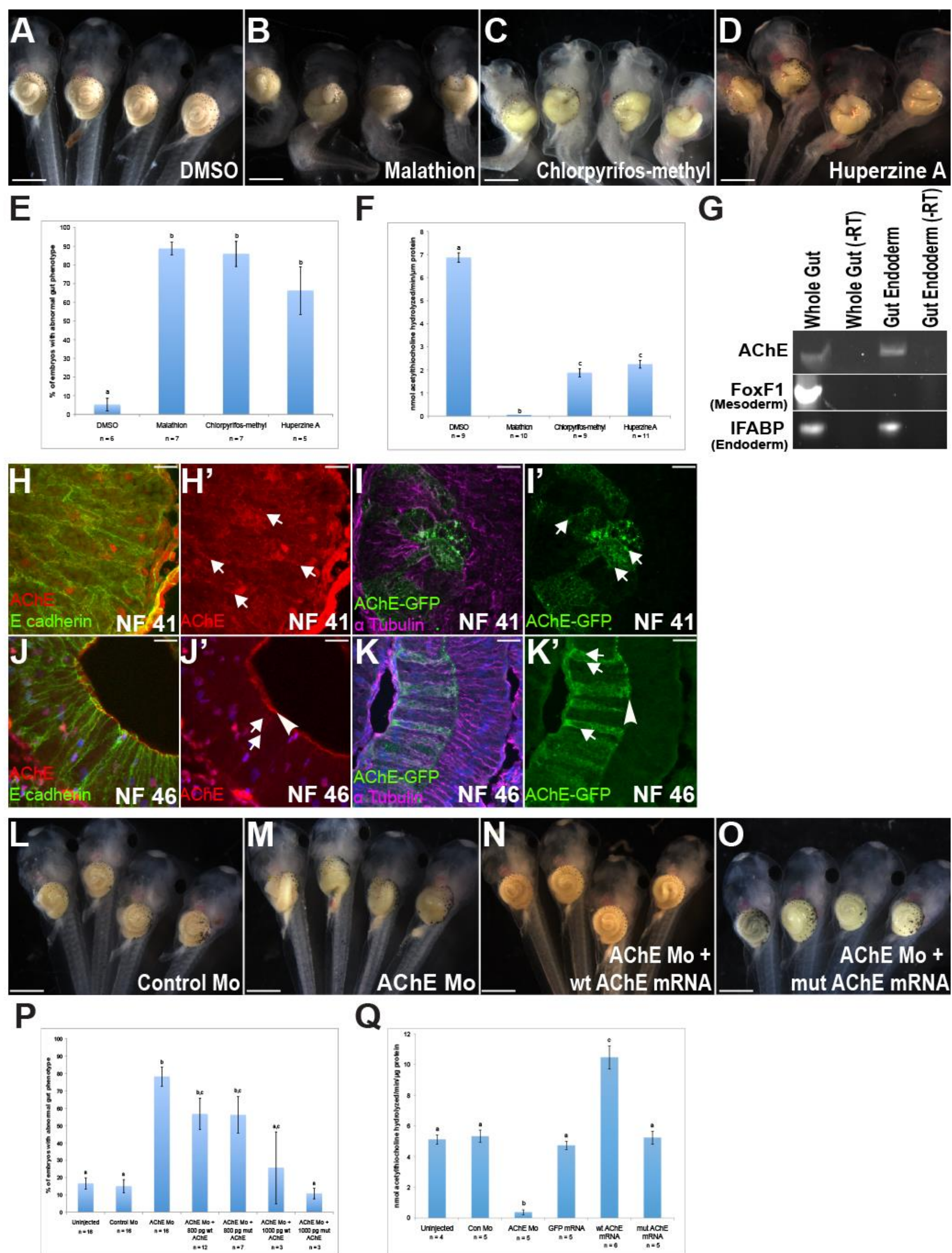

Figure 3.2) AChE is required for endoderm cell movement, rearrangement and polarization.

A-D) Transverse cross-sections through the midguts of NF 46 embryos injected at the 8-cell stage with (A) control morpholino, (B) AChE morpholino, (C) AChE morpholino + wt AChE mRNA, or (D) AChE morpholino + mut AChE mRNA. All morpholinos were co-injected with GFP to identify injected cells (green). β-catenin (red) outlines cell membranes of injected (green) and uninjected intestinal cells. Scale bars = 100 μm.

E-H) Serial sections from the same embryos in A-D immunostained for integrin (green – cell membranes) and PKC (red – apical epithelial surface). Scale bars = 100 μm.

I-L) Higher magnification images of boxed regions in E-H. (I) A single columnar epithelium composed of polarized cells is formed in control morpholino injected intestines. (J) AChE morpholino injected cells are rounder, fail to move to form a single epithelial layer, and lack expression of the apical marker, aPKC, suggesting they are not polarized. These endodermal defects are rescued by co-injection with (K) wt AChE mRNA or (L) mut AChE mRNA. Scale bars = 25 μm.

M) Quantification of cell shape (Length:Width) in injected and uninjected endoderm cells. (* indicates significant difference compared with other injected cells, $p<0.05$, ANOVA. Bar indicates significant difference between injected and uninjected cells, $p<0.05$, t-test.)

Figure 2

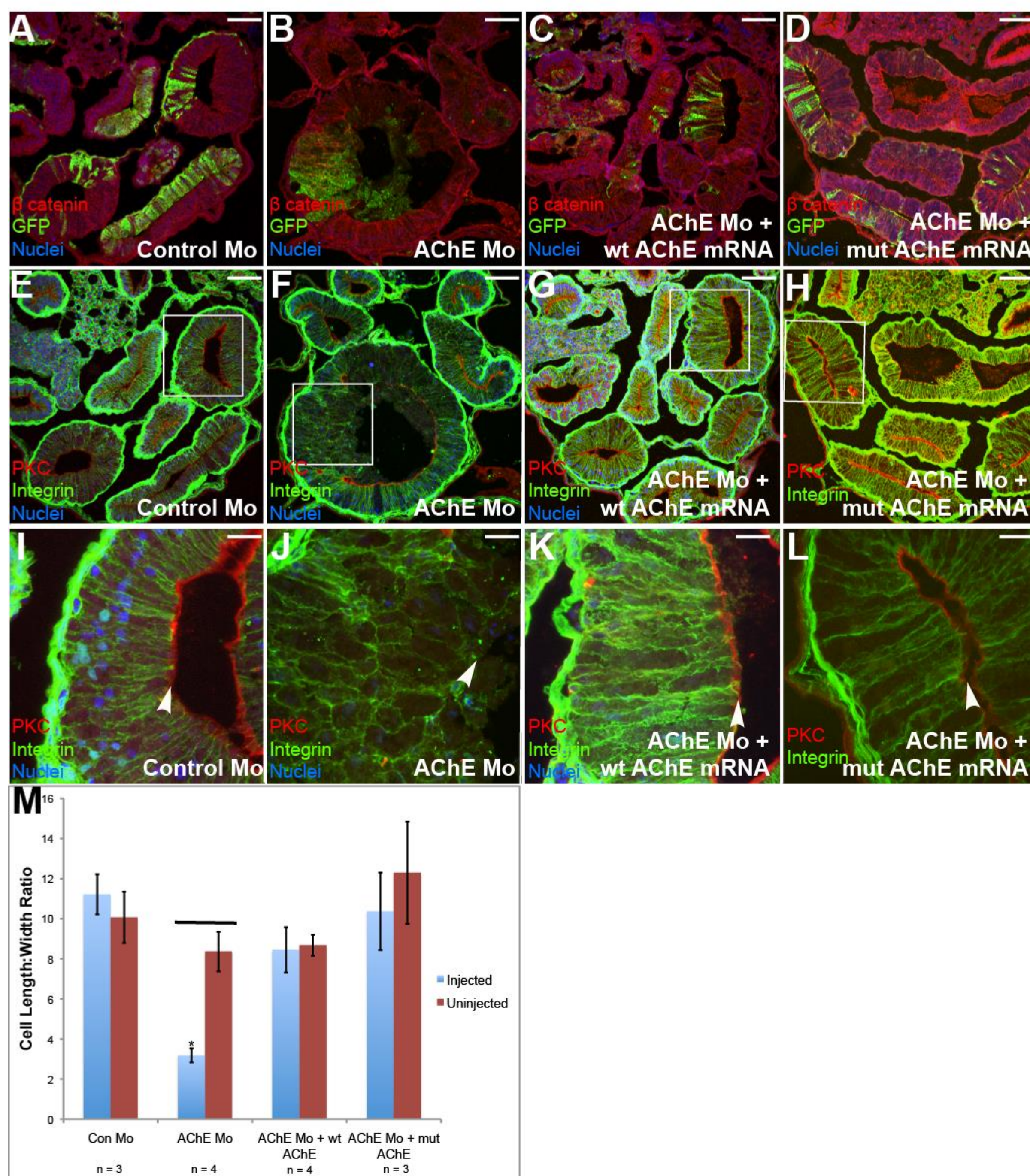

Figure 3

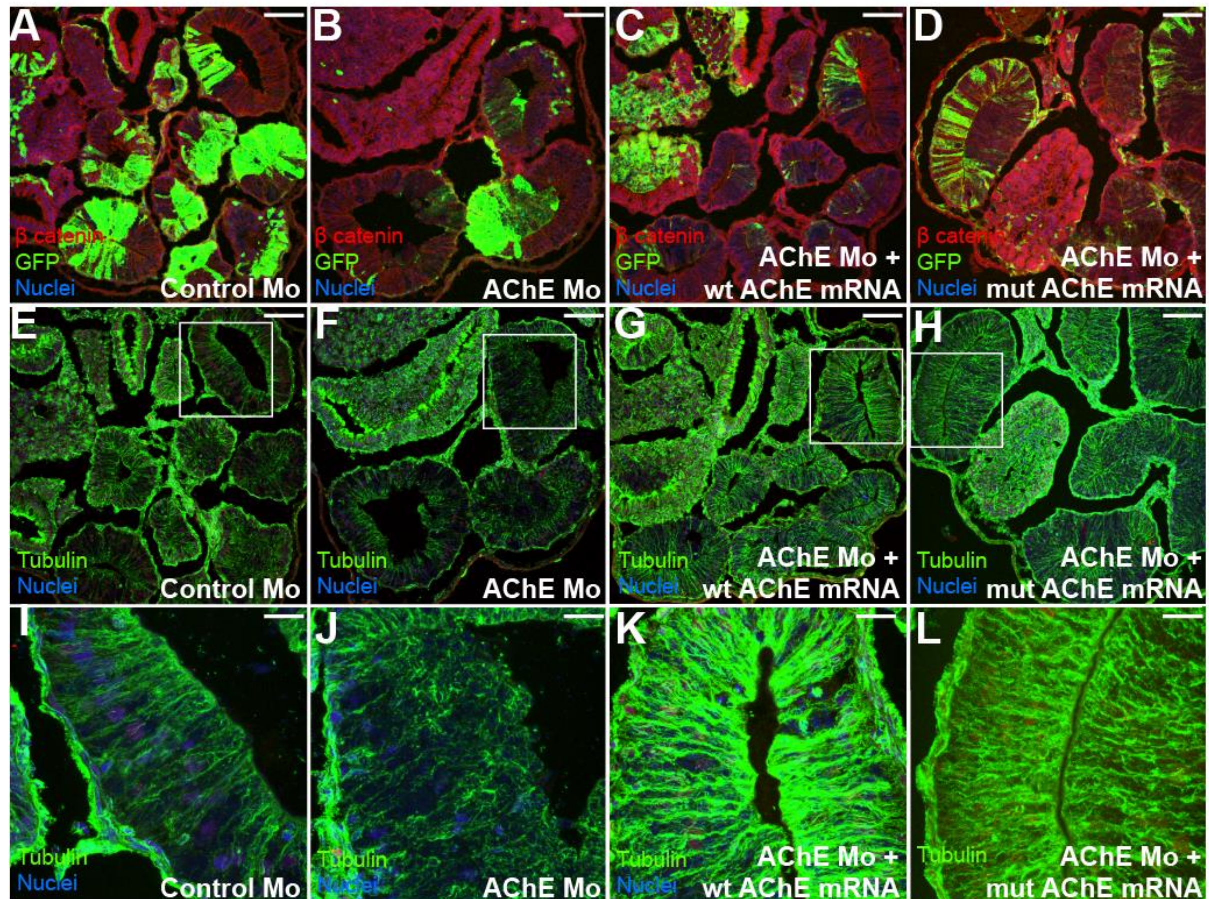

Figure 3.3) AChE is required for microtubule organization.

A-D) Transverse cross-sections through the midguts of NF 46 embryos injected at the 8-cell stage with (A) control morpholino, (B) AChE morpholino, (C) AChE morpholino + wt AChE mRNA, or (D) AChE morpholino + mut AChE mRNA. All morpholinos were co-injected with GFP to identify injected cells (green). β-catenin (red) outlines cell membranes of injected (green) and uninjected intestinal cells. Scale bars = 100 μm.

E-H) Serial sections from the same embryos in A-D immunostained for α-tubulin (green – cell cytoskeleton). Scale bars = 100 μm.

I-L) Higher magnification images of boxed regions in E-H. (I) α-tubulin is normally expressed along the apical-basal axis of columnar epithelial cells, with slight apical enrichment. (J) α-tubulin organization is disrupted when AChE is knocked down in the gut endoderm. Apical-basal organization of α-tubulin is rescued by co-injection of the AChE morpholino with either (K) wt AChE mRNA or (L) mut AChE mRNA. Scale bars = 25 μm.

Figure 3.4) AChE is not required for cell-cell adhesion or for cell substrate adhesion to laminin, but is necessary for cell-substrate adhesion to fibronectin.

A-B) Still images of reaggregation of injected and uninjected endoderm cells from embryos injected with the control or AChE morpholinos at the 8-cell stage. Uninjected and injected (green – GFP positive) cells from both (A) control morpholino and (B) AChE morpholino injected embryos reaggregate 30 minutes after reintroduction of Ca^{2+} ions into the media.

C) Quantification of cell adhesion on fibronectin. Cells from embryos injected with the AChE morpholino are significantly less adherent than control cells on fibronectin. (Bars represent average percentage of adherent cells from 6 independent experiments. Error bars = S.E.M. $p<0.05$, t-test)

D) Quantification of cell adhesion on laminin. There is no difference in cell adhesion between control morpholino and AChE morpholino injected embryos (Bars represent average percentage of adherent cells from 8 independent experiments. Error bars = S.E.M.)

E-F') Transverse cross-sections through and NF 41 embryo midgut immunostained for ECM proteins. (E) Fibronectin (green) is expressed throughout the endoderm. (E') Higher magnification image of boxed region in E, shows an asymmetric distribution of fibronectin, biased toward the basal surface of endoderm cells (arrowsheads). Fibronectin is also expressed at the basement membrane (arrow). (F) Laminin (red) is expressed only at the basement membrane, not throughout the endoderm. (F') Higher magnification image of boxed region in F. Two parallel layers of laminin are expressed at the basement membrane (arrow). (E,F) Scale bar = 100 μm (E',F') Scale bar = 25 μm.

Figure 4

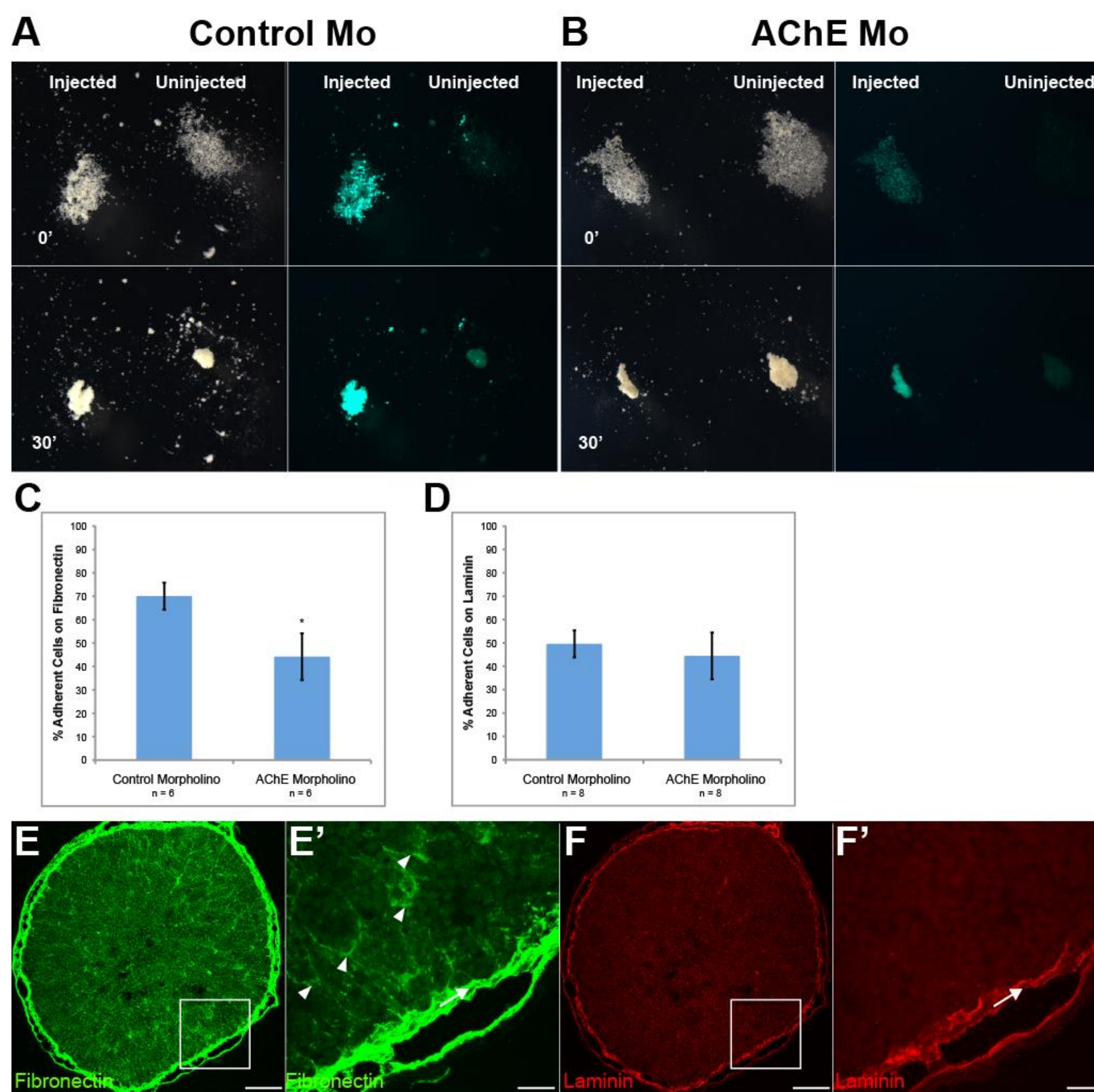

Figure S1

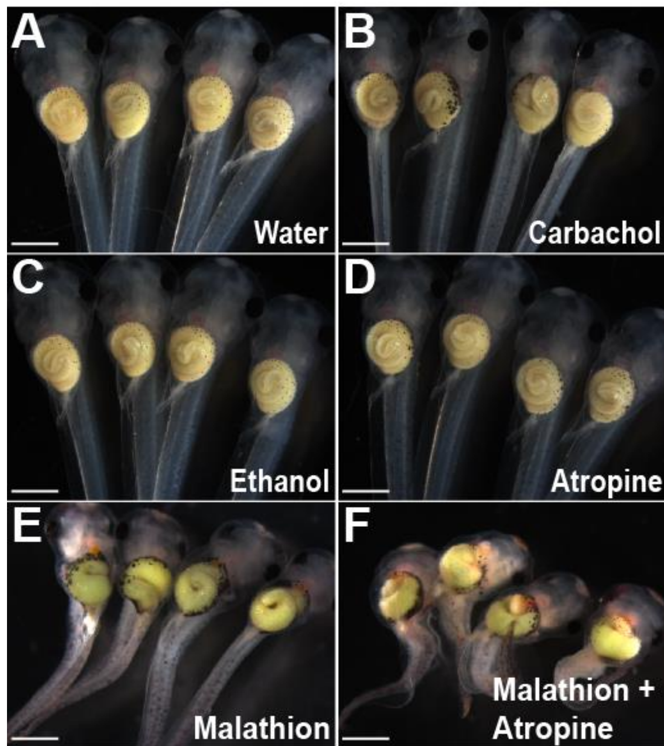

Figure 3.S1) Perturbation of cholinergic signaling does not affect gut morphogenesis. Exposure of *X. laeivs* tadpoles to the cholinergic agonist (B), carbachol, from NF 33-46 does not affect elongation or rotation of the gut relative to (A) solvent controls. Similarly, exposure to the acetylcholine receptor antagonist (D), atropine, has no impact on gut morphogenesis relative to (C) solvent controls. Blocking cholinergic signaling with atropine does not rescue gut elongation in AChE inhibited embryos (E,F). Scale bars = 1000 µm.

Figure 3.S2) AChE chemical inhibitors alter endoderm cell polarity, disrupt microtubule organization, and reduce AChE expression at the apical cell surface. A-I') Transverse cross-sections through midguts of NF 46 tadpoles exposed to DMSO (left column), malation (middle column), or chlorpyrifos-methyl (right column) from NF 33-46. A-C') Exposure to AChE chemical inhibitors (B,B') malathion or (C,C') chlorpyrifos-methyl disrupts aPKC (red) expression at the apical surface of the epithelium compared with normal expression in (A,A') DMSO treated controls. (A-C) Endoderm cells counterstained with integrin (green) to outline cell membranes. Compared with the columnar epithelium in DMSO exposed controls (A), the epithelium appears thicker and less columnar in AChE inhibitor treated embryos (B,C). Scale bar = 100 μm. (A'-C') Higher magnification images of boxed regions in A-C. Arrowheads show normal apical expression in (A') DMSO treated embryos (A'), and patchy aPKC expression in (B',C') chemically inhibited embryos. D-F') AChE inhibition disrupts microtubule arrangement and increases cell death. In (D,D') DMSO treated embryos, α-tubulin (green) is normally arranged along the apical-basal axis of the endoderm cells, with slight apical enrichment. (D) Apoptosis (caspase 3 - red) is low in the gut tube. (E,E') Malathion or (F,F') Chlorpyrifos-methyl exposure disrupts organization of α-tubulin in the endoderm cells. (E,F) Apoptosis (red) is increased in AChE inhibited guts. (D-F) Scale bars = 100 μm. (D'-F') Higher magnification images of the boxed regions in D-F. Scale bars = ???

Figure S2

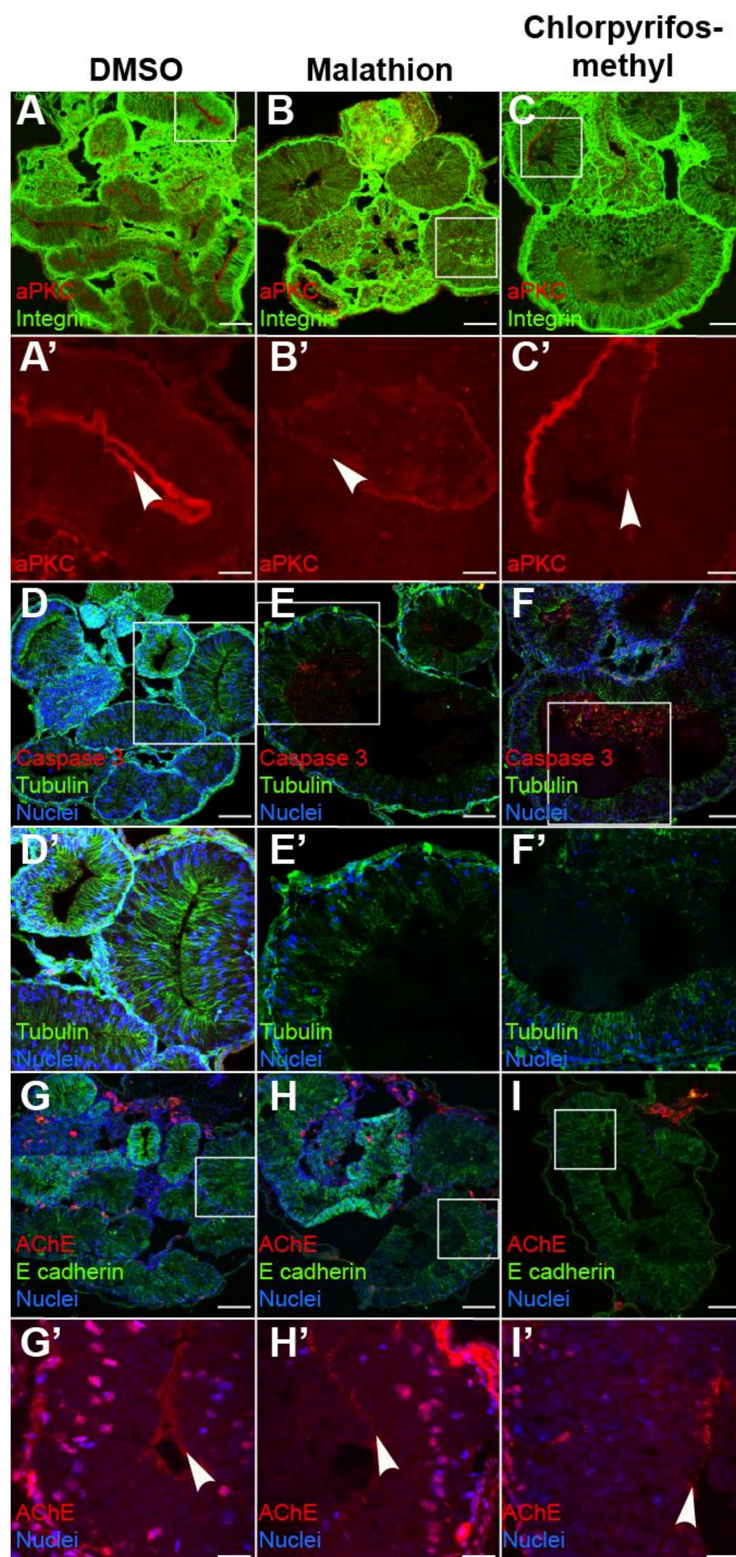

Figure S3

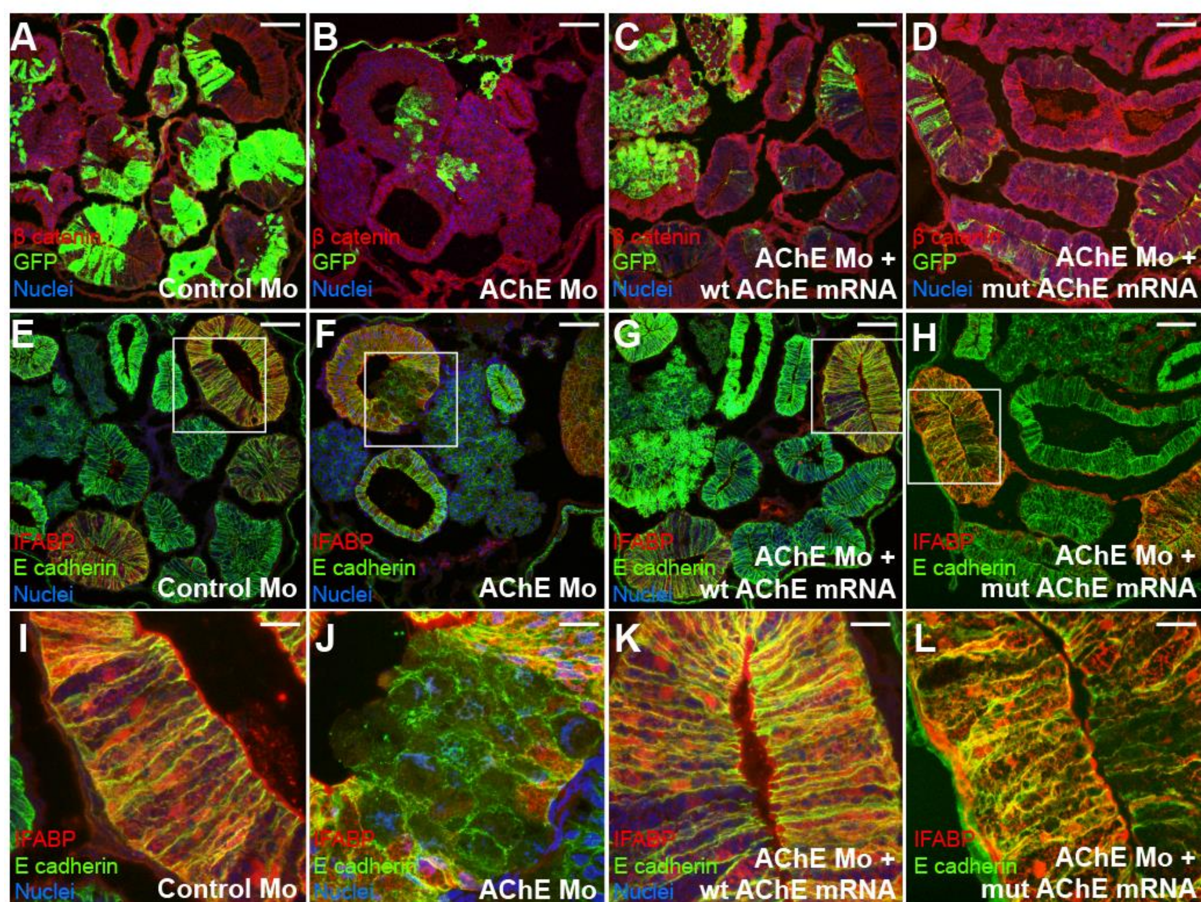

Figure 3.S3) AChE is required for endoderm differentiation.

A-D) Transverse cross-sections through the midguts of NF 46 embryos injected at the 8-cell stage with (A) control morpholino, (B) AChE morpholino, (C) AChE morpholino + wt AChE mRNA, or (D) AChE morpholino + mut AChE mRNA. All morpholinos were co-injected with GFP to identify injected cells (green). β-catenin (red) outlines cell membranes of injected (green) and uninjected intestinal cells. Scale bars = 100 μm.

E-H) Serial sections from the same embryos in A-D immunostained for e-cadherin (green – cell membrane) and intestinal fatty acid binding protein (IFABP)(red – intestinal differentiation). Scale bars = 100 μm.

CHAPTER 4 - DISCUSSION

AChE has a critical enzymatic function in the termination of cholinergic signaling within invertebrate and vertebrate nervous systems. In addition to this esterase activity, AChE has functions in cell-substrate adhesion, cytoskeletal rearrangements, cell survival, and differentiation, *in vitro*. However, observations from AChE knockdowns in vertebrate models have led to speculation that AChE's non-catalytic functions are redundant with other cell-substrate adhesion proteins *in vivo* (Cousin et al., 2005; Johnson et al., 2008). Contrary to this hypothesis, our work presents direct evidence for a non-esterase function of AChE during intestinal morphogenesis in *Xenopus laevis*. Consistent with *in vitro* observations, we provide evidence that AChE is involved in directing cell polarization, cytoskeletal rearrangement, movement, and differentiation of intestinal cells, independently of esterase activity. Additionally, we find that AChE is required for cell adhesion to fibronectin, but not laminin. This suggests a specific interaction between AChE and fibronectin in organogenesis. The importance of cell-substrate interactions in directing differentiation, polarization, cytoskeletal changes, and movement during morphogenesis are well established (Brafman et al., 2013; Davidson et al., 2006; Marsden and DeSimone, 2003; Rozario and DeSimone, 2010; Weber et al., 2012). Thus, we suggest that an AChE – Fibronectin mediated process is essential for altering endoderm cell shape and arrangement during organogenesis

AChE is required for organogenesis

We found that exposure to AChE inhibiting compounds disrupts intestinal elongation and rotation in developing *Xenopus laevis*. We additionally noted spinal defects, edema, and paralysis in these tadpoles. These results are consistent with prior studies in which organophosphate or carbamate exposure resulted in shortened, malformed intestinal tracts, spinal deformities, and edema in frog tadpoles (Bacchetta et al., 2008; Bonfanti et al., 2004; Snawder and Chambers, 1989, 1990). Muscular and notochordal defects have been described in these tadpoles, but the mechanisms underlying intestinal defects have not been pursued. AChE is the primary target of OP and carbamate pesticides and Huperzine A, suggesting a common mechanism of action in teratogenesis of the intestine. Therefore, we knocked down AChE activity/function specifically within the intestine. AChE knockdown resulted in shortened, malrotated intestines similar to those observed with chemical inhibition. These defects were rescued by overexpression of AChE mRNAs, indicating that AChE is required for intestinal morphogenesis and is likely causative of the intestinal defects observed with chemical inhibitors. Organophosphate and carbamate pesticides inhibit AChE esterase activity, increasing the local concentration of ACh. However, esterase inhibition by these compounds also results in conformational changes in AChE's protein structure (Kardos and Sultatos, 2000; Sultatos and Kaushik, 2008) and disrupt functions associated with AChE non-esterase activities *in vitro* (Campanha et al., 2014; Pizzurro et al., 2014; Yang et al., 2008). Significantly, AChE esterase activity is not required for intestinal development to proceed. A mutated AChE, which lacks esterase activity, rescued gut elongation and rotation to the same

degree as wild type AChE. This suggests that AChE has non-esterase functions during morphogenesis of the intestine, which are disrupted by chemical inhibition at the esterase site. This conclusion is also supported by our observation that exposure to the ACh receptor agonist, carbachol, which mimics accumulation of ACh, had no impact on intestinal development.

A non-esterase function is further indicated by the absence of AChE activity in the developing intestine, despite AChE protein expression within this tissue (data not shown). Similarly, AChE activity is very low in fibroblasts, despite expression of the protein (Anderson et al., 2008). AChE has non-esterase roles in polarized cell migration and adhesion in these cells (Anderson et al., 2008). Additional *in vitro* studies report a decrease in AChE esterase activity in cells plated on a laminin substrate (Howard et al., 2005; Johnson and Moore, 2004; Sperling et al., 2012). This may indicate that interactions of AChE's non-esterase peptide sequences with substrate proteins limits access to the active site. Competition between the esterase and adhesive properties is indicated in several cell lines exposed to AChE inhibitors while plated on various substrates (Howard et al., 2005; Johnson and Moore, 2004; Sperling et al., 2012). This might indicate that the esterase and non-esterase functions of AChE are mutually exclusive.

AChE may have a conserved non-esterase function in organogenesis. While there are differences between the cellular movements that drive intestinal and heart development, changes in cell shape, polarization, and adhesiveness are required for morphogenesis of both organs (Rozario and DeSimone, 2010). Indeed many of the signaling pathways that guide

development of these asymmetric, tubular organs are shared (Campione et al., 1999; Onuma et al., 2006). AChE is expressed in heart and intestinal tissue during organogenesis in fish, amphibians, and birds (Bertrand et al., 2001; Downes and Granato, 2004; Drews, 1975). Just as exposure to AChE inhibitors disrupts intestinal development, organophosphate or carbamate pesticide exposure results in size and looping defects in the heart (Pamanji et al., 2015a; Pamanji et al., 2015b; Schock et al., 2012). Pericardial edema was observed in zebrafish AChE mutants, possibly due to underlying tissue defects in the heart (Downes and Granato, 2004). AChE-/- mice do not display gross organ abnormalities at birth, but have multiple abnormalities as they mature (Duysen et al., 2002; Xie et al., 2000). Some of the observed defects can be explained by excessive cholinergic signaling, while others may result from disruption of non-esterase functions (Bytyqi et al., 2004; Duysen et al., 2002). In particular, the mice require high caloric diets to survive, but never grow to the size of their siblings, leading to speculation that there is poor absorption in the digestive tract (Duysen et al., 2002). Based on our current work, AChE may be required for development and/or maintenance of the gastrointestinal tract in mammalian models as well and should be investigated further.

The concentrations of organophosphates used were higher than those found environmentally. However, mixtures of organophosphates and carbamates are frequently detected, which can have an additive effect and alter morphogenesis in native frog species. Interestingly, a second frog species, *L. laevis,* was less sensitive to MTH than *X. laevis*, despite similar levels of endogenous AChE activity and shared homology between the AChE

sequences of the two species. This difference may be due to differences in metabolism or due to inherent differences in the GI tracts of these species. The intestine of the carnivorous *L. laevis*, does not lengthen as extensively as the intestine of the herbivorous *X. laevis*. While all of the intestinal endoderm cells contribute to the mature intestinal epithelium in *X. laevis*, only a subset are involved in *L. laevis* intestinal development, while others undergo apoptosis and shed normally. These differences might make *X. laevis* intestinal morphogenesis more prone to perturbation than *L. laevis*. It would be interesting to determine if the intestines of carnivorous frog species are generally less sensitive to chemical perturbation relative to herbivorous species. Although less sensitive to MTH than *X. laevis*, we did find that higher concentrations of MTH disrupted intestinal elongation in *L. laevis* as well, suggesting that AChE does have a conserved role in amphibian intestinal development.

It is also worth noting that human AChE is far more susceptible to inhibition than *Xenopus* AChE (Shapira et al., 1998*).* Agricultural workers and spouses are often exposed to pesticide mixtures, and cognitive defects have been reported in their children (Arcury et al., 2007; Arcury, 2005; Bouchard et al., 2011; Engel et al., 2007; Huen et al., 2012; Rohlman et al., 2005; Starks et al., 2012; Wilson et al., 2010). Recent epidemiolocial studies also indicate that OP exposure is associated with increased risk for congenital heart defects and may also be association with intestinal stenosis/atresia (Carmichael et al., 2016; Carmichael et al., 2014). With known human exposure to AChE inhibitors and the unknown etiology for many birth defects, both non-esterase and non-esterase functions of AChE should be considered in fetal health and development.

AChE has non-esterase functions required for cell polarization, cytoskeletal reorganization, and movement that drive intestinal morphogenesis

Intestinal endoderm cells give rise to the columnar epithelium, which is the primary absorptive structure of the gastrointestinal tract. These cells occlude the PGT and must undergo changes in shape, polarity, adhesion, and arrangement to produce a mature epithelium and drive elongation of the intestine. In contrast to the single layer of columnar epithelium observed in controls, knockdown of AChE results in rounded endoderm cells that fail to form a single epithelial layer. Additionally, cells lacking AChE have disorganized cytoskeletal structures and fail to polarize. Furthermore, AChE morphant cells do not differentiate, though proliferation and cell death do not appear to be affected. AChE has previously been linked to polarized cell movement, cytoskeletal alterations, and differentiation, but to our knowledge, this is the first time these functions have been confirmed *in vivo*.

Importantly, the defects in endoderm shape, arrangement, polarity, cytoskeletal structure, and differentiation are rescued by co-expression of either wild-type or the enzymatically null mutant AChE. This suggests that AChE influences endoderm cell behaviors independently of its esterase activity *in vivo*. Similar defects in endoderm cell shape, polarity, and cytoskeleton were observed in the intestines of tadpoles exposed to AChE inhibitors, suggesting that inhibition of the active site induces conformational alterations that affect the function of AChE in directing these processes.

Our results are consistent with observations made *in vitro* that suggest a non-esterase function for AChE in a wide variety of cell behaviors. For example, AChE is expressed at the leading edge of fibroblasts, and facilitates cell migration, indicating a role for AChE in polarized cell behavior (Anderson et al., 2008). Antibody sequestration of either AChE or integrin (+ control) disrupted wound healing in this model, whereas excess ACh had no effect on fibroblast movement, suggesting that AChE's role in polarized migration was independent of its esterase activity (Anderson et al., 2008). The defects in endoderm polarity and movement observed with AChE knockdown and rescue with mut AChE, similarly support a non-esterase role for AChE in polarized cell behavior. Cell polarization often involves cytoskeletal rearrangements (Gupton and Gertler, 2010; Weber et al., 2012). For example, neurite outgrowth depends upon polarized cell extensions with extensive cytoskeletal rearrangement. Work in multiple neuronal cell lines suggests that AChE is required for neurite extension (Giordano et al., 2007; Sharma et al., 2001; Sperling et al., 2012; Sternfeld et al., 1998; Yang et al., 2008). AChE expression is also associated with changes in cytoskeletal protein expression and localization in neurons (Chang et al., 2006; Dupree and Bigbee, 1994; Dupree et al., 1995; Keller et al., 2001). Similarly, AChE may be involved in the cytoskeletal changes that drive spicule elongation in sea urchin larvae, as AChE is expressed during morphogenesis of these structures and OP exposure inhibits spicule elongation (Drews, 1975; Ohta et al., 2009). Our data show that AChE is required for organization of the cytoskeleton in intestinal development independently of its esterase

function. The similarities among different cell types and organisms suggest that AChE plays an evolutionarily conserved role in directing cell polarity and cytoskeletal reorganization.

Changes in cell polarity, arrangement, and cytoskeletal structure often accompany differentiation. Our data suggest that AChE has non-esterase functions required for cell differentiation. This is consistent with prior observations associating AChE expression with differentiation in many different tissues and organisms (Drews, 1975; Fossati et al., 2015; Grisaru et al., 1999; Keller et al., 2001). The ability of either wt or catalytically-null mut AChE to rescue endoderm defects in polarity, cytoskeleton, movement and differentiation reinforces the argument that AChE has non-esterase roles in morphogenic processes. Additionally, it validates the observations made *in vitro*, suggesting that the protein functions similarly *in vivo*, and supports an evolutionary function of the protein in influencing multiple cell behaviors.

Our work also provides additional evidence for the importance of endoderm polarity and cytoskeletal dynamics in driving intestinal morphogenesis. Our lab previously reported that perturbation of the Wnt/PCP signaling pathway results in defective endoderm polarity, adhesion, and cytoskeletal structures (Dush and Nascone-Yoder, 2013; Reed et al., 2009). Cells fail to change shape or move between one another, abrogating intestinal elongation and rotation, similar to our observations in the current study (Dush and Nascone-Yoder, 2013; Reed et al., 2009). These cellular defects explain the shortened, malrotated intestines observed at the gross level. It is possible that the Wnt/PCP pathway and AChE directly overlap as organophosphate exposure in either neonatal rats or PC12 cells results in

decreased expression of Wnt pathway gene products (Slotkin et al., 2008). However, it seems more likely that disruption of cell polarity and cytoskeleton dynamics result in similar intestinal defects, through two different proteins that both influence these behaviors. Defects in polarity, adhesion, and/or the cytoskeleton will prevent cellular movements and differentiation, abrogating intestinal development and yielding shortened, malrotated intestines. Importantly, there is a high degree of cross-regulation among the proteins involved in adhesion, polarity, and cytoskeletal arrangement (Davidson et al., 2006; Marsden and DeSimone, 2003; Muhamed et al., 2016). These complex interactions are essential for guiding cell behavior, but can complicate the interpretation of how a single protein acts in morphogenesis. AChE is not a transmembrane protein, but is localized to the extracellular side of cell membranes through interactions with multiple transmembrane proteins depending on the specific isoform of AChE and the tissue it is expressed in. In the developing intestine, AChE is expressed both at the membrane and in the cytoplasm of endoderm cells. Both fractions of AChE may be involved in directing endoderm cell differentiation, as different cell partitions of AChE have previously shown differential responses to cell culture on laminin (membrane portion displays decreased enzymatic activity with no alteration in the activity of soluble AChE) (Sperling et al., 2012). AChE's membrane expression is of particular interest, however, as AChE binds to extracellular matrix proteins and enhances cell-substrate adhesion *in vitro* (Anderson et al., 2008; Inkson et al., 2004; Johnson and Moore, 1999, 2004; Sharma et al., 2001). Significantly, the same peptide sequences involved in cell-substrate adhesion are capable of promoting neuritogenesis to the same degree as full

length AChE (Johnson and Moore, 2004). This indicates that AChE's adhesive motifs are capable of inducing substrate-mediated cytoskeletal rearrangements in differentiating cells. Additionally, the importance of cell-substrate interactions in driving cell polarization and directed protrusion, cytoskeletal rearrangements, differentiation, and movement is well established (Brafman et al., 2013; Davidson et al., 2006; Marsden and DeSimone, 2003; Matsui et al., 2007; Ramos et al., 1996; Trinh and Stainier, 2004; Weber et al., 2012). Thus, AChE's well established adhesive role *in vitro* could explain how this protein influences many cell behaviors both *in vitro* and *in vivo.*

AChE directs endoderm cell changes through interactions with fibronectin

Cell-cell and cell-substrate interactions are essential for morphogenic processes to occur (Davidson et al., 2006; Goto et al., 2005; Marsden and DeSimone, 2001, 2003; Muhamed et al., 2016; Weber et al., 2012). To determine if AChE has a role in cell-cell adhesion, we modified *Xenopus* animal cap assays for intestinal endoderm cells. We found no evidence for a role of AChE in cell-cell adhesion. Neither *in vivo* nor *ex vivo* exposure to AChE inhibitors altered cell reaggregation. Similarly, AChE knockdown had no effect on cell reaggregation, suggesting that AChE is not required for cell-cell adhesion. This is consistent with our IHC data. The adherens junction protein E-cadherin and the associated protein β-catenin, appeared to be expressed at the same levels in control and AChE inhibited/knockdown embryos. This suggests that loss of AChE does not universally disrupt all cell behaviors, and that the observed defects are linked to a specific function of AChE. Similarly, *in vivo* or *ex vivo* exposure of endoderm cells to AChR agonists and antagonists

did not affect cell-cell adhesion. In keratinocytes, antagonism of AChRs reduced cell-cell adhesion, indicating a non-neuronal role for ACh signaling and AChE esterase activity (Nguyen et al., 2003; Nguyen et al., 2000). The absence of any effect in our system further suggests that the non-esterase functions of AChE are of primary importance in the developing intestine.

In order to determine if AChE directs intestinal development through an adhesive mechanism, we isolated gut endoderm cells and plated them on ECM substrates in *ex vivo* assays. Cells from control morpholino injected tadpoles were more adhesive on fibronectin than laminin substrates, which may reflect the differences in expression of these ECM proteins *in vivo.* Morpholino knockdown of AChE did not affect endoderm cell adhesion on laminin, but resulted in a significant decrease in cell adhesion to fibronectin. Preliminary studies also indicated that, similar to laminin, AChE is not required for cell adhesion on collagen. Similarly, MTH exposure did not affect cell-substrate adhesion on laminin or matrigel. This suggests that AChE is required for cell-substrate adhesion on fibronectin, and implies that a specific interaction occurs between AChE and fibronectin during intestinal development. This specific interaction is also supported by the similar distribution of both fibronectin and AChE throughout the endoderm during intestinal cell intercalation, whereas laminin is expressed only at the basement membrane. Such an interaction has previously been suggested in a colon cancer cell line (HT-29). Transfection of these cells with AChE leads to a significant increase in adhesion on fibronectin, but has no effect on adhesion to laminin or collagen (Syed et al., 2008). Additionally, differentiation of human embryonic

stem cells into definitive endoderm (which gives rise to the internal organs, including the intestine) depends upon cell interactions with fibronectin and the closely related protein, vitronectin, but not laminin or other ECM molecules (Brafman et al., 2013). Furthermore, fibronectin knockdown disrupts intestinal development in *Xenopus laevis* (Davidson et al., 2006). Similarly, inhibition of lysyl oxidase disrupts intestinal development (Geach and Dale, 2005). This family of enzymes is involved in the cross-linking of collagen fibrils, but is also required for oxidation of fibronectin (Kraft-Sheleg et al., 2016). This interaction is critical for fibronectin assembly as well as for facilitating myofiber adhesion, stretch, and integrin mediated signaling in developing somites (Kraft-Sheleg et al., 2016). Of relevance, organophosphate exposure is linked with decreased lysyl oxidase activity, providing further evidence for a potential relationship between AChE and ECM in intestinal morphogenesis (Snawder and Chambers, 1993). Additionally, the Wnt/PCP pathway is involved in fibronectin deposition and organization, as well as polarized cell movement in gastrulating *Xenopus* (Goto et al., 2005). Thus, Wnt/PCP and AChE may converge in their interactions with fibronectin, explaining the phenotypic similarities in the gut (Dush and Nascone-Yoder, 2013; Reed et al., 2009). Our results highlight the importance of fibronectin in intestinal development and identify an interaction of AChE with this substrate. Based upon the ability of non-esterase AChE to rescue intestinal phenotypes and the low enzymatic activity detected within the gut, we conclude that AChE has non-esterase functions in cell-substrate adhesion *in vivo.*

Indeed the interactions between cell surface proteins and fibronectin in directing morphogenesis have been well established. For example, integrin interactions with fibronectin, are essential for directing cell shape and movements in *Xenopus laevis* embryos (Davidson et al., 2006; Marsden and DeSimone, 2003). Knocking-down either of these proteins perturbs convergent-extension during gastrulation, with failure of the mesoderm cells to intercalate (Davidson et al., 2006; Marsden and DeSimone, 2003). Furthermore, the normal bi-polar shape of the mesoderm cells is lost with knockdown of either of these proteins, and increased random protrusive activities are observed, indicating alterations of cytoskeletal dynamics (Davidson et al., 2006; Marsden and DeSimone, 2003). Defects in cell shape and movement in fibronectin knockdown cells can be restored when cells are plated on exogenous fibronectin, emphasizing the importance of cell-ECM interactions for development (Davidson et al., 2006). Similarly, fibronectin is involved in epithelial organization, polarization, and midline convergence in the migrating heartfields of zebrafish embryos (Trinh and Stainier, 2004). Mutation of fibronectin results in cardia bifida at a gross level, with decreased expression of the apical markers aPKC and ZO-1 in myocardial precursors, accompanied by altered actin expression and cell shape changes (Trinh and Stainier, 2004). Similarly, a mutation in a sphingosine-1-phosphate receptor, *miles-apart (mil)*, was linked to fibronectin function and myocardial precursor migration (Matsui et al., 2007). These studies highlight the importance of cell-substrate interactions in morphogenesis and reveal that interactions at the cell membrane can influence cell shape, movement, and cytoskeletal structure.

AChE's role in promoting endoderm adhesion on the fibronectin substrate can explain the defects in cell polarity, cytoskeletal structure, and movement observed in the gut endoderm when AChE is inhibited or knocked down. As endoderm knockdown is sufficient to disrupt intestinal elongation without impacting mesodermal structures, it seems likely that AChE acts primarily in the endoderm to promote intestinal elongation and morphogenesis. However, it is interesting that AChE inhibition and knockdown also disrupt mesodermal structures, including laminin. Other studies have established a direct interaction between AChE and laminin *in vitro* (Johnson and Moore, 2004). Although AChE is not essential for intestinal endoderm to adhere to laminin, AChE may play a separate role in the mesoderm where it may contribute to ECM deposition/organization. Similarly, in this study, fibronectin structure appears to be disrupted in the mesoderm of NF 46 embryos in which AChE is knocked down throughout much of the mesoderm. In some cases, we also noted an apparent change in the localization of fibronectin within AChE knockdown endoderm. As opposed to the basal expression of fibronectin in NF 41 endoderm, in AChE knockdown cells we occasionally notice increased fibronectin expression at lateral and apical cell membranes, suggesting the asymmetric distribution is lost. Additional replicates are needed to confirm this altered fibronectin distribution. However, this data indicate that AChE may have a role in organizing the distribution of ECM proteins within the intestine. Such a function could be mediated by direct interaction between AChE and the ECM proteins. *In vitro* work suggests AChE can bind to laminin, collagen, and fibronectin via its PAS (Giordano et al., 2007; Johnson and Moore, 2003, 2004), providing a possible mechanism for AChE in organizing

ECM components. There is some precedent for a function of AChE in ECM deposition within the literature. For instance, diazoxon mediated inhibition of AChE in rat hippocampal neurons results in decreased fibronectin secretion by the cells (Pizzurro et al., 2014). However, this potential function requires additional study.

Models for AChE's non-esterase role in intestinal development

Interactions with the substrate can influence cell behavior through physical changes in force as well as through activation of signal transduction pathways. Therefore, AChE-fibronectin interactions may manifest in several ways. We propose three possible models for how AChE-fibronectin interactions might mediate intestinal development. Additional work needs to be performed to differentiate between these models to understand how AChE guides morphogenesis *in vivo,* though we consider a direct interaction between AChE and fibronectin to be the most likely mechanism in guiding intestinal development. Given AChE's homology with other substrate adhesion proteins and its ability to bind directly to ECM proteins, it seems likely that AChE interacts directly with fibronectin (Giordano et al., 2007; Johnson and Moore, 2003, 2004). This interaction could activate signal transduction through proteins that tether AChE to the membrane or through stabilizing interactions between fibronectin and other transmembrane proteins, such as integrin (Fig. 4.1 A). As already described, cell interactions with the substrate regulate many cell behaviors directly. Additionally, many proteins facilitate fibronectin- cell interactions (e.g. LoxL3, Miles-apart), and AChE may have a similar function in facilitating interactions between fibronectin and integrin, influencing downstream signaling through another cell-membrane protein (Kraft-

Sheleg et al., 2016; Matsui et al., 2007). However, we attempted to rescue AChE knockdown adhesion on fibronectin by activating integrin *ex vivo* with Mn^{2+} ions, with the prediction that if AChE normally facilitates integrin-fibronectin interactions, exogenous integrin activation should overcome defects in cell adhesion. However, Mn^{2+} did not rescue cell adhesion, arguing against an AChE-Fibronectin-Integrin interaction at the cell surface (Fig. 4.2). Alternatively, direct adhesion between AChE and fibronectin may result in differential forces on the cells that trigger changes in cell polarity and cytoskeleton (Fig. 4.1 B). Fibronectin appears to be asymmetrically localized along the A-P axis of NF 41 endoderm cells prior to morphogenic movements. Thus, the interaction between AChE and fibronectin could result in localized differences in force along the membrane. Differential forces have been shown to influence the cytoskeleton and polarity of other motile cells (Houk et al., 2012; Muhamed et al., 2016; Weber et al., 2012). In mesendoderm explants, a pulling force was required to induce polarized cell protrusivity and movement through c-cadherin transmembrane adherens proteins (Weber et al., 2012). This external force was also required for cytoskeletal rearrangements, involved in producing a polarized cell. Additionally, cell interactions with a fibronectin substrate were essential for directing polarized cell movements and cytoskeletal rearrangements, suggesting that tractive forces from the substrate in combination with cell-cell pulling forces are required for morphogenic movements (Weber et al., 2012). Physical forces influence morphogenic processes, and it is possible that AChE's interaction with fibronectin contributes to development through generating such forces (Fig. 4.1 B). A third possibility is that AChE regulates the expression of proteins that interact with fibronectin to

mediate cell signaling, such as integrin (Fig. 4.1 C). AChE is expressed at both the membrane and in the cytoplasm of intestinal endoderm cells. Though AChE is not a transcription factor, it could have cytoplasmic functions involved in regulating gene expression. There is some evidence from colon cancer cells that AChE influences cell-substrate adhesion through regulation of integrin expression, as opposed to a direct interaction between AChE and fibronectin at the cell membrane (Syed et al., 2008). Decreased integrin expression would also result in a decrease in cell adhesion to fibronectin, consistent with our results. We have occasionally noted decreased integrin intensity in endoderm cells lacking AChE, which might support a regulatory function of AChE. However, decreased integrin expression is not observed consistently in endoderm cells lacking AChE, so is unlikely to be the primary target of non-esterase AChE functions *in vivo.* Additional work is needed to reveal how AChE influences cell interactions with fibronectin to direct morphogenesis.

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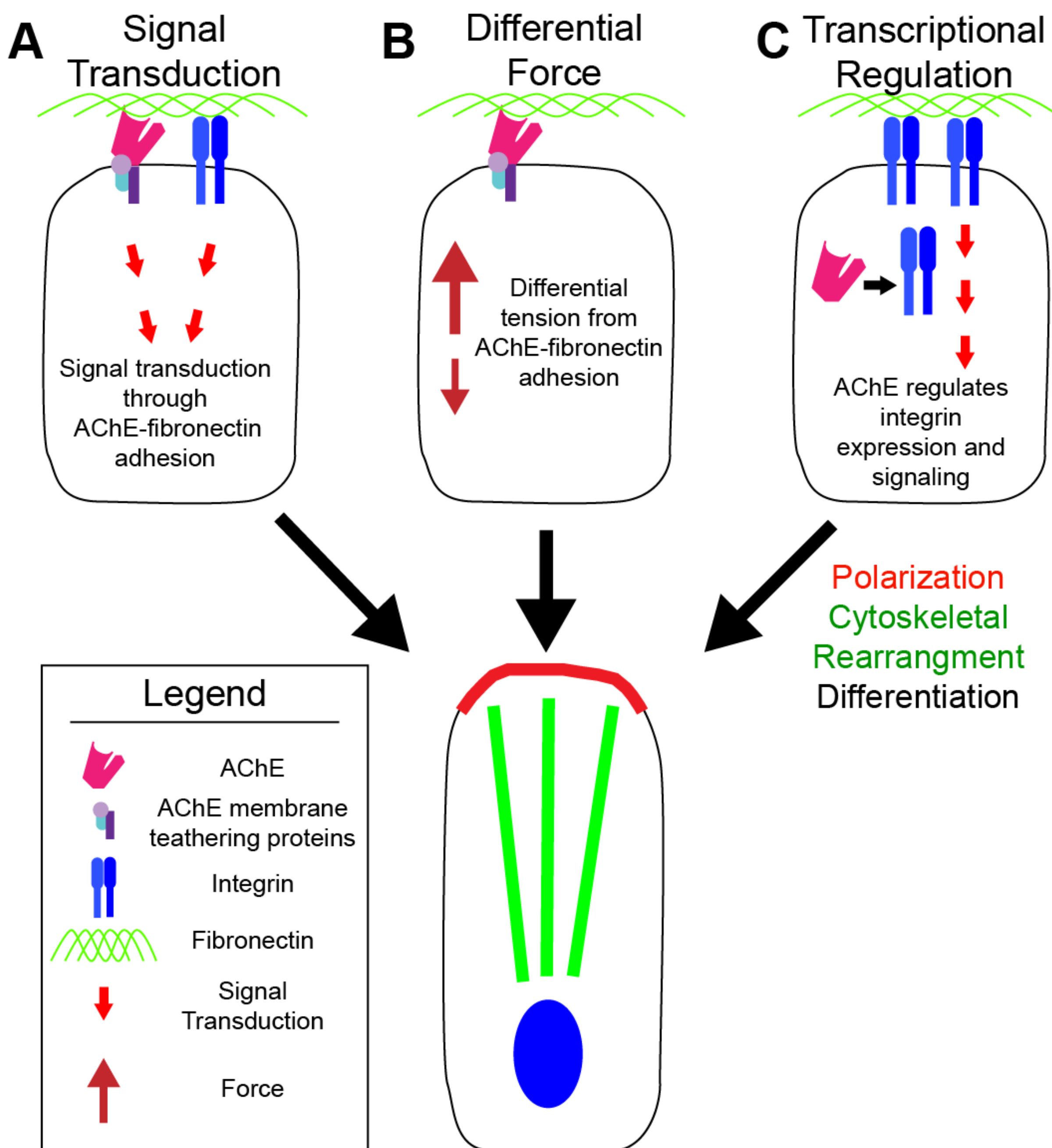
Figure 4.1: AChE mediates endoderm cell adhesion, polarization, and structural changes through fibronectin.

Here we provide three different models for how AChE may mediate these changes. Signal Transduction(A): AChE may bind directly to fibronectin and alter signal transduction through the transmembrane proteins that tether AChE to the cell membrane. Alternatively, AChE adhesion to fibronectin may stabilize interactions between fibronectin and transmembrane proteins, such as integrin, altering signal transduction by facilitating this interaction. Differential Force (B): AChE may bind directly to fibronectin resulting in localized differences in membrane tension. The difference in experienced force on one part of the cell as compared with other regions of the membrane may drive cytoskeletal changes and polarization. Transcriptional regulation (C): AChE may regulate the expression of transmembrane signaling proteins, such as integrin. Upregulation of integrin expression increases cell adhesion to fibronectin and alter signal transduction.


A
Signal Transduction
Signal transduction through AChE-fibronectin adhesion
B
Differential Force
Differential tension from AChE-fibronectin adhesion
C
Transcriptional Regulation
AChE regulates integrin expression and signaling
Polarization
Cytoskeletal Rearrangment
Differentiation
Legend
AChE
AChE membrane teathering proteins
Integrin
Fibronectin
Signal Transduction
Force

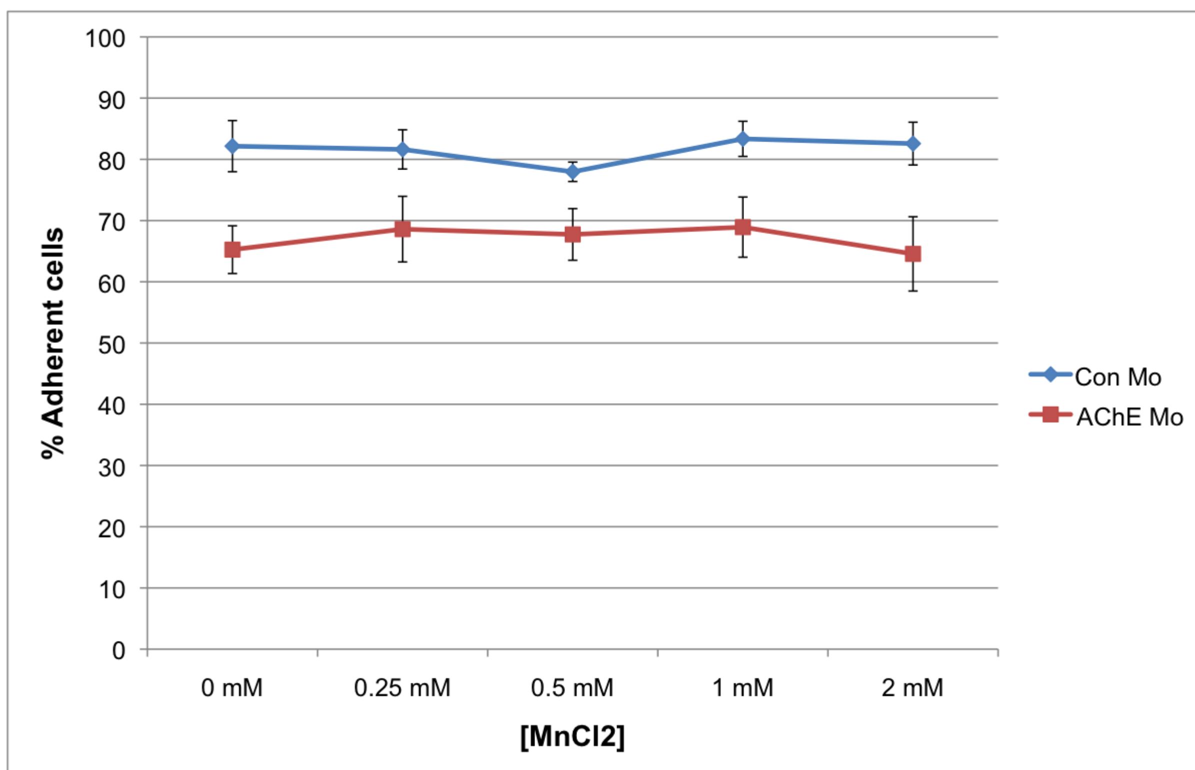


Figure 4.2) AChE does not activate integrin to facilitate integrin-fibronectin interactions and indirectly enhance cell-substrate adhesion.
In order for integrin to interact with fibronectin, it must first be activated by divalent cations. To determine if AChE promotes integrin activation and adhesion to fibronectin, we exposed endoderm cells from control (blue) and AChE knockdown (red) embryos to increasing concentrations of $MnCl_2$, which has previously been used to activate integrin proteins. However, we found no evidence that $MnCl_2$ treatment rescued cell-substrate adhesion on fibronectin, suggesting that AChE does not directly facilitate fibronectin-integrin interactions.

CHAPTER 5 – CONCLUSIONS AND FUTURE DIRECTIONS

AChE is a fascinating protein with both esterase and non-esterase functions. We have shown that AChE is required for intestinal morphogenesis through altering endoderm cell shape, cytoskeletal structure, polarity, and differentiation. These changes are likely mediated through AChE's interaction with the ECM protein, fibronectin, and additional work is necessary to refine our understanding of this interaction. While these non-esterase functions have been observed *in vitro*, ours is the first to provide experimental evidence of non-esterase functions *in vivo*. In humans, AChE expression is associated with several congenital birth defects, and has been implicated in diseases ranging from Alzheimer's and Parkinson's neurodegenerative diseases, to *Myasthenia gravis,* and cancer (Friedrich et al., 1994; Karczmar, 2010; Martucciello et al., 2002; Montenegro et al., 2006; Moore and Johnson, 2005; Slotkin and Seidler, 2011; Small et al., 1996; Syed et al., 2008). AChE inhibiting compounds are widely used as pesticides in industrial and agricultural settings, in warfare, and in the treatment of disease. However, the multiple functions of AChE in development, homeostasis, and disease are still poorly understood. We have shown that AChE inhibitors affect both esterase and non-esterase functions during development. Additionally, we reveal a hitherto unappreciated function for a serine protease in directing cell behaviors during morphogenesis, independently of its esterase activity. We hope that future work will expand our understanding of AChE's non-neuronal roles and protein interactions to influence biological processes *in vivo.*

AChE is an ancient, conserved protein, expressed during the development of non-neural tissues in many organisms. Based on our work, we hypothesize that AChE's non-esterase function in the frog intestine is conserved in other vertebrate species as well. It would be interesting to examine the intestines of AChE mutant zebrafish and mice at the cellular level during organogenesis to determine if AChE is also involved in establishing cell polarity, coordinating microtubule dynamics, and facilitating intestinal elongation in these vertebrate models. This research could improve our understanding of conserved processes underlying organogenesis, potentially shedding light on the etiology of human intestinal defects. AChE is also expressed in the hearts of many vertebrates and OP exposure disrupts heart looping and development in zebrafish and chicks (Pamanji et al., 2015a; Pamanji et al., 2015b; Schock et al., 2012; Wyttenbach and Thompson, 1985), and is associated with congenital heart defects in humans (Carmichael et al., 2014; Romero, 1989; Sherman, 1996). Therefore, we hypothesize that AChE may have a similar role in the morphogenesis of the heart as it does in the intestine. The ECM plays an essential role in the morphogenesis of both organs, and AChE's non-esterase adhesive role may contribute to cellular processes in both tissues. Future studies that investigate AChE's function within the developing heart would also enhance our understanding of AChE's activities *in vivo.* These studies would further contribute to our understanding of evolution in examining both the esterase and non-esterase functions of a conserved protein in multiple tissues and organisms. Investigation into AChE's developmental functions may also identify additional pathways by which OPs and other AChE inhibitors disrupt development. It seems clear that OPs affect both esterase and

non-esterase AChE functions, and their influence on non-esterase functions *in vivo* should be evaluated in addition to their ability to inhibit esterase activity.

Our work also raises the question of how AChE interacts with fibronectin. Biochemical studies indicate direct interaction between AChE and the ECM molecules, laminin and collagen, via protein motifs within and around the PAS (Johnson and Moore, 2003, 2004). Work in a neuronal cell line provides indirect evidence that AChE similarly interacts with fibronectin through the PAS (Giordano et al., 2007). However, this has yet to be directly established and should be determined in a future study. If AChE does not directly bind to fibronectin, then what is the mechanism by which it promotes cell adhesion to this substrate? Additionally, we found evidence that AChE is involved in the distribution and organization of both fibronectin (in endoderm and mesoderm) and laminin (in mesoderm) within the intestine. The mechanism by which AChE contributes to the organization of these proteins remains to be determined, though an adhesive mechanism is a logical hypothesis. Some evidence also suggests that AChE regulates the expression of ECM and cytoskeletal proteins (Giordano et al., 2007; Keller et al., 2001; Pizzurro et al., 2014; Syed et al., 2008), so a regulatory function should also be considered in identifying the mechanism(s) by which AChE mediates ECM and cellular changes. The ECM is a complex environment composed of multiple proteins that may interact with cells and AChE in currently unappreciated ways. Some evidence suggests that the presence or absence of heparan sulfate proteoglycans within the ECM modifies AChE's influences on cellular behavior (Small et al., 1995). Thus, it will

be interesting to consider how ECM dynamics and AChE expression interact to shape cellular phenotypes and processes both *in vitro* and *in vivo.*

An interesting observation from this study, is the endodermal expression pattern of fibronectin. Fibronectin appears to be localized toward the basal cell surface of endoderm cells at NF 41. Fibronectin knockdown has previously been reported to disrupt intestinal organogenesis in *Xenopus,* but its specific function in the intestine has not been elucidated (Marsden and DeSimone, 2001, 2003). The polarized expression of fibronectin is required for the convergent extension of mesendoderm explants (Goto et al., 2005). In some ways, the intestinal endoderm resembles the mesendoderm both in signaling pathways and morphogenic movements. The asymmetric distribution of fibronectin in the gut tube may be a crucial component of the mechanism driving radial intercalation with concurrent narrowing of the intestinal diameter. Future studies should examine how fibronectin becomes asymmetrically organized (by AChE and/or other proteins) and determine the importance of this expression pattern in intestinal morphogenesis. The spatial organization of other ECM components is also likely to contribute to organogenesis. While fibronectin is expressed in both the endoderm and the mesoderm, laminin appears to be expressed only in the mesoderm. A single laminin layer is first detected surrounding the basement membrane of the endoderm. A second, parallel layer rapidly forms during morphogenesis. Laminin is required for intestinal morphognesis, but much remains to be learned about its interactions with cells during this process. We found that endoderm cells are more adhesive to fibronectin than laminin. This might be explained by the difference in the expression of these two ECM

proteins (more endoderm cells have interacted with fibronectin than with laminin *in vivo* when they are plated on the substrates *ex vivo*). In addition to examining cell-substrate adhesion, we also observed cell behavior on these substrates (Appendix 1). Endoderm cells behave differently on these ECM substrates. For example, cells appear rounder and less motile on fibronectin, though they are more adhesive. Cells on laminin are more motile, randomly moving on the substrate. Endoderm cells on laminin also appear protrusive, with both lamellipodia and filipodia-like extensions visualized. These *ex vivo* behaviors need to be better characterized, hopefully with the assistance of semi-automatic image analysis software. The relevance of these *ex vivo* behaviors to *in vivo* processes also needs to be assessed as single cells on a substrate may act differently that multiple cells in a tissue with a complex ECM. Still, the differences observed in our *ex vivo* assays could prove useful for identification of the mechanisms involved in radial intercalation in the intestine, and potentially tissue dynamics in other systems.

Much of our work was performed in the model laboratory frog, *Xenopus laevis*. Its long use as a developmental model and amenability to a wide variety of experimental techniques make it ideal for studying developmental processes. However, there are also advantages to using more than one model organism. Comparison of multiple species can help identify conserved mechanisms of development as well as identify divergence that may reflect different evolutionary pressures to fill a particular niche. For example, the GI systems of the two frogs used in this study are very different, reflecting their different feeding strategies. The long coiled intestine and reduced stomach of *Xenopus* is ideal for processing

the nutrient poor algae tadpoles of this species feed upon. In contrast, *Lepidobatrachus laevis* are carnivorous and have large stomachs with relatively short intestines and minimal coiling. Alteration of signaling pathways have been identified that contribute to the differences between these digestive systems. In this study, we found that AChE inhibition reduces intestinal lengthening in both *X. laevis* and *L. laevis* suggesting that AChE has a conserved role in intestinal elongation in these different amphibian models. Future studies should continue using both models in order to better understand conserved mechanisms of intestinal development, as well as evolutionarily divergent pathways. Using two frogs instead of one is also useful from an ecological perspective. Amphibian populations are declining worldwide. Many factors likely contribute to this reduction, including exposure to pesticides. While FETAX has been developed to assess toxicity, *X. laevis* are often less sensitive to pesticides than other frog species. Thus, the use of multiple frog species is important for evaluating environmental pesticide risk to non-target organisms. Although *L. laevis* appeared less sensitive than *X. laevis* to OPs in this study, they should continue being used for toxicological studies, as their sensitivity to other environmental toxicants is unknown. This data will assist regulatory agencies and policy makers in protecting non-target organisms in the ecosystem.

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APPENDICES

APPENDIX A

Frogs as integrative models for understanding digestive organ development and evolution

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Abstract

The digestive system comprises numerous cells, tissues and organs that are essential for the proper assimilation of nutrients and energy. Many aspects of digestive organ function are highly conserved among vertebrates, yet the final anatomical configuration of the gut varies widely between species, especially those with different diets. Improved understanding of the complex molecular and cellular events that orchestrate digestive organ development is pertinent to many areas of biology and medicine, including the regeneration or replacement of diseased organs, the etiology of digestive organ birth defects, and the evolution of specialized features of digestive anatomy. In this review, we highlight specific examples of how investigations using *Xenopus laevi*s frog embryos have revealed insight into the molecular and cellular dynamics of digestive organ patterning and morphogenesis that would have been difficult to obtain in other animal models. Additionally, we discuss recent studies of gut development in non-model frog species with unique feeding strategies, such as *Lepidobatrachus laevis* and *Eleutherodactylous coqui*, which are beginning to provide glimpses of the evolutionary mechanisms that may generate morphological variation in the digestive tract. The unparalleled experimental versatility of frog embryos make them excellent, integrative models for studying digestive organ development across multiple disciplines.

1. Introduction

The anatomical and physiological complexity of the vertebrate digestive system develops from a simple primitive gut tube (PGT). This PGT undergoes intricate patterning and differentiation events to enable the epithelial lining of the tube to assume the absorptive and secretory functions required of a gastrointestinal (GI) tract, while discrete segments bud off of the original structure to form accessory organs, including the pancreas and liver. Concomitantly, the tube lengthens and rotates, as it transforms from a short, occluded cylinder to a long, hollow conduit arranged in a three dimensional configuration of loops and coils.

Elucidating the mechanisms of digestive organ development has broad implications for many areas of biology and medicine. Some of the most common human birth defects affect the digestive tract, yet the genetic and/or environmental factors that contribute to the etiology of these malformations remain to be discovered. In addition, diseases of the digestive system affect millions worldwide, generating substantial demand for therapeutic interventions; full knowledge of the developmental events that pattern and shape the PGT is likely to be vital for successful regeneration or engineering of human digestive tissues. Finally, although many features of digestive anatomy are highly conserved among vertebrates, the length, compartmentalization and topological orientation of the GI tract can vary tremendously among and between species, especially those with different diets, yet the evolutionary origins of this ecologically-relevant variation are largely unknown.

1.1. The advantages of the frog embryo.

Amphibians have long been used as model organisms for studying embryonic development, and have played instrumental roles in unraveling the intricate events that guide germ layer formation, gastrulation and neurulation (Keller, 2005; Keller et al., 2003; Spemann and Mangold, 2001). Beyond early development, frog embryos also boast several advantages for the study of organ specification and morphogenesis (Blitz et al., 2006). Unlike amniote embryos that are confined to a uterus or shell during development, frog embryos are externally fertilized and can be easily cultured *in vitro*, making them amenable to a wide variety of experimental manipulations. For example, the rate of development of frog embryos can be accelerated or slowed by adjusting temperature, facilitating convenient analyses of any stage of organogenesis (Nieuwkoop, 1994). Moreover, precise fate maps have been generated for the early blastomeres (32-cell stage), allowing loss- and/or gain-of-function (LOF/GOF) reagents and lineage tracers to be targeted to specific organs by standard microinjection technology, enabling gene function to be queried in a tissue-specific manner (Moody, 1987; Moody and Kline, 1990). Furthermore, because frog embryos are relatively large and harbor an innate, intracellular yolk supply, tissue explants can be dissected, recombined and transplanted, or cultured in simple saline, at almost any stage of development, facilitating expedient, inexpensive specification and trans-differentiation studies (Logan and Mohun, 1993; Milet and Monsoro-Burq, 2014; Saint-Jeannet et al., 1994; Sater and Jacobson,

1989, 1990; Smith et al., 1990; Wilson et al., 1989). Finally, the frog embryo's accessibility to chemical agonists/antagonists allows the role of specific signaling pathways to be interrogated during critical windows of organogenesis (i.e., subsequent to earlier developmental events that may also depend on such pathways). In fact, thanks to large clutch sizes, frog embryos provide a powerful platform for high-throughput "chemical genetic" or toxin screening using organ morphology as a phenotypic readout (Dush et al., 2011; Tomlinson et al., 2005; Tomlinson et al., 2009; Wheeler and Brandli, 2009). This experimental amenability makes the frog embryo an ideal model in which to interrogate the mechanisms of organ development.

1.2. More than one frog in the pond.

Amphibian models (mainly urodeles) have been employed in developmental biology research for over a century, but the convenience of *in vitro* fertilization methods made *Xenopus* species the most popular frogs in the laboratory (Callery, 2006). Nonetheless, many non-model frog species are equally amenable to experimentation as *Xenopus*. Comparative "evo-devo" studies utilizing frogs with different reproductive strategies and/or developmental rates (Benitez and Del Pino, 2002) are beginning to provide fascinating insight into the molecular and cellular mechanisms that shape different embryos, while species that fill unique ecological niches or possess intriguing specializations are shedding light on the developmental origins of novel morphologies (Elinson and del Pino, 2012).

In this review, we provide a broad perspective on the ways in which *Xenopus* and emerging frog models have yielded new insight into digestive organ patterning, morphogenesis, and evolution.

2. What can the frog tell us about foregut organ specification?

The developing digestive tract may be divided into foregut (esophagus, stomach, duodenum, liver, pancreas, gall bladder) and midgut/hindgut (intestine) domains. The foregut-derived organs play critical roles in processes such as digestion, glucose homeostasis, and detoxification. Therefore, congenital defects or disease in these organs (e.g., diabetes, pancreatitis, fatty liver disease, biliary atresia, gall stones and gastric/pancreatic cancer) are the cause of substantial morbidity and mortality worldwide (Cano et al., 2007; Desmet, 2005; Hezel et al., 2006; Nair et al., 2007). To ameliorate such afflictions, translational researchers seek to develop regenerative therapies and engineer replacement tissues *in vitro*. Progress in these areas has been profoundly influenced by models of the normal process of foregut organ specification and morphogenesis in the embryo (Wells and Spence, 2014; Zorn and Wells, 2009).

In all vertebrates, the PGT is comprised of an inner endoderm layer, which differentiates into the epithelial lining of the GI tract, surrounded by an outer layer of mesoderm, which will give rise to the visceral muscle and connective tissue. Early in gut development, reciprocal signaling between the endoderm and mesoderm layers gradually distinguishes anterior foregut and posterior hindgut domains (Arterbery

and Bogue, 2014). In addition to digestive tissues, numerous structures with diverse physiological functions are derived from the anterior region of the PGT (including organs of the respiratory and endocrine systems), all of which must undergo morphogenesis in close proximity. The expression of foregut organ-specific genes must therefore be tightly coordinated in time and space to allow individual organs to differentiate appropriately. Moreover, many of the signaling factors involved in foregut organogenesis are re-deployed during different stages of development, and/or evoke contradictory responses depending on their concentration (Zorn and Wells, 2009).

Studying such spatially intricate and seemingly paradoxical signaling dynamics is not trivial in mouse models due to the functional redundancy of the factors involved, and the challenges of achieving tissue-specific or conditional perturbation of gene function to overcome early lethality and pleiotropy. In contrast, the experimental amenability of the *Xenopus* embryo—which enables the pattern, timing and dosage of gene expression to be manipulated in a tissue-specific manner—has provided key insights into the spatiotemporal signaling dynamics that specify region and organ identities in the PGT. We highlight a few salient examples in sections 2.1-2.4 below.

2.1. Complex control of Wnt signaling is required for foregut specification.

Wnt signaling pathways are highly conserved and involved in many fundamental developmental events, including body axis patterning, cell fate specification, cell proliferation, and cell migration. Multiple Wnt ligands stimulate canonical (Wnt/β-catenin) and/or non-canonical (e.g., Wnt/JNK) pathways (Nusse, 2005; van Amerongen and Nusse, 2009). Early in *Xenopus* development, the establishment of the dorso-anterior axis of the embryo is accompanied by high levels of nuclear β-catenin in the anterior endoderm, a readout of canonical Wnt signaling. However, soon afterwards, this same tissue exhibits low levels of nuclear β-catenin, suggesting that Wnt signaling must become restricted from the prospective foregut during gut patterning (McLin et al., 2007). This idea was confirmed by experiments in which the Wnt/β-catenin pathway was ectopically activated in the prospective foregut of the *Xenopus* embryo, resulting in ablation of liver- and pancreas-specific gene expression; in the converse experiment, inhibition of Wnt/β-catenin signaling in the prospective hindgut region expanded liver and pancreas domains at the expense of intestinal tissue (McLin et al., 2007).

The expression of Wnt antagonists in the anterior endoderm also suggests that Wnt signaling is actively suppressed in this region. In *Xenopus,* foregut-specific knockdown of Sfrp5, a secreted antagonist that sequesters Wnt ligands in the extracellular space to prevent their binding to Wnt receptors, reduced liver and

pancreas gene expression (Li et al., 2008). In contrast, ectopic Sfrp5 activity, achieved via targeted injection of synthetic mRNA, expanded the foregut region, inducing massive liver and pancreatic buds(Li et al., 2008). Co-immunoprecipitation assays confirm that Sfrp5 binds and antagonizes Wnt 11 and 5, demonstrating a direct inhibition of the posteriorizing Wnt pathway in the anterior foregut endoderm (Li et al., 2008).

Interestingly, Sfrp molecules have been shown to have biphasic potential, i.e., acting to inhibit Wnt ligands at high concentrations, but improving diffusion and signaling at low concentrations (Mii and Taira, 2009, 2011). The ability to manipulate the effective concentrations of LOF/GOF reagents targeted to specific tissues in *Xenopus* revealed that this biphasic functionality of Sfrp5 is also deployed during foregut specification—high levels of the Wnt inhibitor Sfrp5 decreased levels of the liver-specific transcription factor, *hhex,* but moderate levels increased *hhex* expression (Zhang et al., 2013b). These results suggest that, although Wnt/β-catenin signaling must be suppressed in the prospective foregut region of the PGT, relative to the midgut/hindgut region, low levels of Wnt/β-catenin signaling actually potentiate foregut organ development. Indeed, foregut-targeted knockdown of the Wnt receptor Fzd7 elicits foregut organ hypoplasia (Zhang et al., 2013b). Interestingly, in addition to canonical Wnt/β-catenin signaling, non-canonical Wnt/Jnk mediated cellular morphogenetic pathways were also implicated in this process; Fzd7 knockdown caused the endoderm cells of the developing foregut to be

enlarged and loosely adherent with reduced C-cadherin, β-integrin, cortical β-catenin and F-actin levels and disorganized microtubules (Zhang et al., 2013b).

Importantly, the frog model of Wnt-mediated foregut specification is nicely corroborated by results obtained in mammalian studies. For example, transgenic mice with foregut-specific Wnt overexpression exhibit pancreas agenesis (Heller et al., 2002) and knock out (Matsuyama et al., 2009) or downregulation (Kim et al., 2005) of mouse Sfrps leads to hypoplastic stomach development. Thus, tissue-targeted LOF/GOF assays in frog embryos can provide detailed mechanistic insights into the complex spatiotemporal roles of Wnt signaling in foregut specification (see Figure 1) that are directly relevant to higher vertebrates but would have been difficult to ascertain in such models.

2.2. Concentration and time-dependent FGF signals segregate foregut organs.

Fibroblast Growth Factors (FGFs) are required for multiple developmental processes including mesoderm induction, limb bud development, neural patterning, myogenesis, and organ morphogenesis (Dorey and Amaya, 2010; Pownall and Isaacs, 2010). During gastrulation, FGF signaling specifies posterior fates in the PGT but, shortly thereafter, FGF secreted from the anterior lateral plate and cardiac mesoderm is required to specify anterior foregut organs (pancreas, liver and lung) in the ventral endoderm (Zorn and Wells, 2009). In *in vitro* studies, the induction of different organs by FGF appears to be concentration-dependent, suggesting that

FGF signaling must be tightly regulated during foregut specification. However, it is not known whether the segregation of organ fates *in vivo* is determined by proximity to, or duration of contact with, the neighboring FGF-secreting mesoderm.

The experimental amenability of *Xenopus*, which permits straightforward explant culture of isolated endoderm and mesoderm layers of the PGT (Horb and Slack, 2001), has facilitated a deeper appreciation of the *in vivo* context-dependent regulation of FGF dosage during foregut organ specification. Consistent with higher vertebrates, gut-targeted hyper-activation of FGF signaling in *Xenopus* embryos results in the expansion of liver gene expression and repression of pancreas genes, while inhibition of FGF signaling causes a loss of liver gene expression and expansion of pancreatic markers (Shifley et al., 2012). Mechanistic insight was obtained from *ex vivo* cultures in which removal of the lateral plate mesoderm from explants of ventral endoderm at successive stages of development showed that the pancreas and liver require different time periods of interaction with the neighboring FGF-expressing mesoderm; the liver requires a more prolonged period of incubation to form correctly (Shifley et al., 2012). Chemical inhibitors of both the PI3K and MEK branches of the FGF pathway are capable of eliciting a reduction in liver gene expression, suggesting that both branches are likely required (Shifley et al., 2012). However, in the absence of mesoderm, addition of FGF to cultured foregut endoderm explants was not sufficient to induce liver, suggesting that other signaling pathways must also be involved in ventral foregut organ specification.

This example illustrates how the unique ability to isolate and culture *Xenopus* explants in different tissue-layer and chemical reagent combinations can enable elegant analyses of the concentration and time-dependent nature of conserved growth factor signaling in specifying vertebrate foregut organs (see Figure 1).

2.3. Early retinoic acid signaling is required for dorsal pancreas specification.

The pancreas is formed from three different progenitor populations, one in the dorsal region of the foregut and two (left and right) in the ventral domain. Endocrine cells are initially specified in the dorsal pancreas to make hormones such as Insulin, while the ventral pancreas produces mainly exocrine cells and digestive enzymes. As development proceeds, the dorsal and ventral buds fuse together to form one organ (Blitz et al., 2006; Jarikji et al., 2009; Kelly and Melton, 2000; Pearl et al., 2009), with endocrine and exocrine cells distributed throughout the fused structure. At somite stages, the development of the dorsal (endocrine) pancreas is regulated by signaling molecules secreted from neighboring tissues (e.g., the notochord), including TGF-β and FGF signals, which repress *sonic hedgehog (shh)* expression in the dorsal foregut endoderm, a prerequisite for pancreatic fate (Hebrok, 2003).

The ability to culture early embryonic tissues from *Xenopus* embryos provided some of the first evidence of the key role of another signaling molecule, retinoic acid (RA), in vertebrate pancreas specification. RA is a small, diffusible lipophilic molecule (synthesized from a vitamin A precursor) that acts as a morphogen to exert

concentration-dependent effects on embryonic patterning. RA signals through Retinoic Acid Receptors (RARs), converting them from transcriptional repressors to activators (Niederreither and Dolle, 2008). Utilizing the ability to culture tissue explants from early *Xenopus* embryos, Moriya *et al* showed that treatment of naïve ectoderm tissue from blastulae with a combination of both Activin and RA induces the differentiation of morphological and functional pancreatic tissue (Moriya et al., 2000b). In a related study, slightly later explants from early gastrulae were also induced to form pancreas after exposure to RA (Moriya et al., 2000a). In both cases, the RA-treated explants expressed endocrine hormones, including Insulin, and developed pancreas-like tissue architecture.

The above studies indicate that RA influences pancreas specification very early, during or soon after gastrulation. To determine whether RA signaling is also required at this time for endogenous pancreas specification, *Xenopus* embryos were exposed to RA, chemical RAR inhibitors or injected with mRNA encoding dominant-negative mutant versions of RARs. In all cases, inhibition of RA signaling ablated both exocrine and endocrine gene expression in the dorsal pancreas, while the ventral pancreas was unaffected (Chen et al., 2004; Stafford et al., 2004). This result correlated with ectopic *shh* expression in the dorsal pancreatic endoderm (Chen et al., 2004), suggesting that RA signaling contributes to the exclusion of Shh in the dorsal pancreas field. In the reciprocal experiment, exposing *Xenopus* gastrulae to exogenous RA resulted in enlargement of the pancreas domain. This perturbation

expanded the dorsal endocrine population at the expense of the exocrine population, as indicated by a dose-dependent decrease in exocrine cell markers and an increase in *insulin* expression (Chen et al., 2004).

Consistent with the role of RA in the frog, mice deficient in an enzyme required to synthesize RA (Retinaldehyde dehydrogenase; RALDH) exhibit dorsal pancreas hypoplasia, but retain pancreas markers in the ventral endoderm (Martin et al., 2005; Ostrom et al., 2008). Thus, *Xenopus* explant and pharmacological assays have facilitated clarification of the critical roles of a specific signaling pathway (RA) in pancreas specification and differentiation that are highly relevant to mammalian systems (see Figure 1).

2.4. Genome-wide microarray screens identify new foregut genes.

While sections 2.1-2.3 highlight a few of the key signaling pathways known to be involved in foregut specification and patterning, there is still a paucity of knowledge of all the molecular players and effectors involved in integrating and implementing these signals. Fortunately, microarray analyses of *Xenopus* embryos, explants and developing organs are now being used to identify, on a genome-wide scale, hundreds of new factors likely to play important roles in foregut patterning and organogenesis in all vertebrates. These examples illustrate the power of frog embryos as a platform for unbiased gene discovery.

2.4.1. Microarray analyses of chemically-treated *Xenopus* embryos.

The ability to culture frog embryos in the presence of compounds that modulate specific signaling pathways facilitates straightforward profiling of genes downstream of these pathways. For example, recent microarray profiling of *Xenopus* embryos exposed to an RA inhibitor, identified Ndrg1α as a new RA-responsive factor (Zhang et al., 2013a). Ndrg1α has diverse functions in development and tumorigenesis, but has not previously been associated with digestive organ development (Melotte et al., 2010). Interestingly, Ndrg1α was found to repress the Wnt/β-catenin pathway allowing specification of foregut progenitor cells (Zhang et al., 2013a). This study thereby revealed novel cross-talk between RA and Wnt signaling in foregut development. Given the central role of RA and Wnt signaling in foregut specification, it will be important to ascertain the role of Ndrg1α in the development of the digestive organs of higher vertebrates.

2.4.2. Microarray analyses of *Xenopus* explants.

It is well established that reciprocal signaling between the mesoderm and endoderm layers of the vertebrate PGT is crucial for its regional patterning (Horb and Slack, 2001). To identify new molecules involved in mesoderm-endoderm signaling, Kenny et al cultured isolated endoderm explants versus endoderm/mesoderm explants from the *Xenopus* PGT and conducted microarray analyses to interrogate resultant differences in gene expression (Kenny et al., 2012).

One endoderm gene upregulated in response to mesoderm was the Sfrp-related protein Sizzled (Szl) (Kenny et al., 2012). Szl was found to be required for foregut organ specification downstream of mesodermal BMP signaling, a highly conserved developmental pathway critical for axial patterning and the development of multiple organs (Hogan, 1996) . Like Wnt, BMP also plays dynamic roles in foregut organ specification as BMP signaling initially promotes hindgut development and inhibits foregut fates (Tiso et al., 2002), but is later required to specify foregut lineages (Chung et al., 2008; Wills et al., 2008). Interestingly, Szl maintains BMP signaling by regulating Fibronectin deposition between the endoderm and mesoderm layers of the PGT. In this example, the experimental amenability of *Xenopus* revealed a novel extracellular feedback mechanism that mediates reciprocal Wnt/BMP crosstalk between the endoderm and mesoderm during foregut patterning.

2.4.3. Microarray analyses of *Xenopus* embryonic organs.

It is relatively easy to isolate individual prospective organs within the large and accessible PGT of the frog embryo. This approach has been exploited to discover a trove of new factors and cellular processes involved in the morphogenesis of the pancreas. For example, microarray profiling of *ptf1a*-positive pancreatic endoderm isolated from *Xenopus* embryos revealed putative target genes of this important pancreas-specific transcription factor (Bilogan and Horb, 2012). The genes identified in this analysis contribute to a surprising variety of cellular functions with, as yet, unexplored roles in pancreas morphogenesis, such as

intracellular vesicle docking and fusion, metabolism, cell adhesion, and extracellular matrix stabilization (Bilogan and Horb, 2012). In another study, Jarikji *et al* used microarray technology to identify genes differentially expressed between isolated dorsal and ventral pancreatic buds of the *Xenopus* embryo (Jarikji et al., 2009). This study identified Tetraspanin (Tn4sf3), a transmembrane scaffolding protein that is up-regulated in ventral pancreatic tissue and, intriguingly, required for dorsal and ventral pancreatic fusion (Jarikji et al., 2009).

The above examples suggest that investigation of the molecules and pathways identified by microarray (or RNAseq) analyses in frog embryos could yield profound new insight into the regulatory networks and cellular processes required for the specification and morphogenesis of vertebrate foregut organs. Additional studies are necessary to determine the degree to which the new genes identified in these and other frog studies are conserved in higher vertebrates.

3. What can the frog tell us about intestinal lengthening?

In contrast to the multiple organs specified in the foregut region of the PGT, the posterior (midgut/hindgut) zone is destined to become intestine. This segment of the PGT must undergo dramatic morphogenetic changes, including lumen formation, extensive elongation and counterclockwise rotation while, concomitantly, the visceral mesoderm and epithelial lining of the tract undergo lineage restriction and cellular differentiation. These concurrent events shape and integrate multiple levels of biological organization, from cellular architecture to intricate three-dimensional

anatomy. The mechanisms underlying the complex morphogenesis of the intestine are just beginning to be understood.

Intestinal development is pertinent to a variety of human afflictions. Congenital anomalies of intestinal morphogenesis include narrowing (stenosis) or occlusion (atresia) of the GI tract (Boyden et al., 1967; Carpenter, 1962; Dalla Vecchia et al., 1998; DeLorimier et al., 1969; Seashore et al., 1987), deficits in the normal length of the intestine (congenital short bowel syndrome (Chu et al., 2004; Hasosah et al., 2008; Kern et al., 1990; Palle and Reddy, 2010; Sabharwal et al., 2004; Schalamon et al., 1999; van der Werf et al., 2015)), and intestinal rotation and fixation abnormalities, which occur in as many as 1 in 500 infants (Aslanabadi et al., 2007; Chu et al., 2004; Filston and Kirks, 1981; Ford et al., 1992; Kamal, 2000; Kern et al., 1990; Stewart et al., 1976; Torres and Ziegler, 1993). While malrotation itself is not always symptomatic, it predisposes affected individuals to volvulus, a life-threatening strangulation of the gut tube (Aslanabadi et al., 2007). In addition to birth defects, inflammatory bowel diseases (e.g., Crohn's, ulcerative colitis) are increasingly common chronic conditions in both pediatric and adult populations; progressive complications from these disorders often require surgical resection of the damaged regions of the gut, leading to a shortened GI tract and attendant nutritional issues (Cosnes et al., 2011; O'Sullivan and O'Morain, 2006; Vernier-Massouille et al., 2008). Understanding the events that control normal intestinal development is therefore critical, not only for preventing the causes of common birth

defects, but also for devising strategies to restore normal gut length in congenital or acquired short bowel syndromes.

The concentrically coiled anatomy of the pre-metamorphic *Xenopus* tadpole intestine is relatively simple compared to the visceral anatomy of higher vertebrates, yet it undergoes analogous elongation and rotation events, which occur over the course of only a few days (Chalmers and Slack, 1998; Nieuwkoop, 1994). Because of its internal location, the gut can be challenging to visualize in amniotes, but tadpoles are transparent throughout organogenesis, allowing the cells of the PGT to be labeled and tracked during morphogenesis. Combined with the ability to target LOF/GOF reagents to the gut by microinjection, such studies have led to a deeper understanding of the cellular and molecular events that drive intestine development.

3.1. Endoderm cell rearrangements drive gut lengthening.

Despite early differences in the initial formation of the PGT in amphibian and amniote embryos (Cervantes, 2013; Chalmers and Slack, 2000; Roberts, 2000), there are remarkable similarities in the process of gut elongation. In both frogs and mammals, the early gut tube narrows and elongates coincident with an apparent remodeling of the endoderm (i.e., future epithelial) layer, suggesting that a cell rearrangement process may be involved in vertebrate gut elongation (Matsumoto et al., 2002; Yamada et al., 2010). In the frog, very little cell division is observed during the early stages of gut elongation, supporting the idea that (at least initially), this event is driven almost exclusively by cell rearrangement (Muller et al., 2003; Reed et

al., 2009). In the *Xenopus* embryo, small groups of gut cells can be easily labeled with vital dyes, and their behavior visualized during gut elongation. Such studies revealed that the most central endoderm cells of the PGT radially intercalate during gut morphogenesis (Chalmers and Slack, 2000). As the gut lengthens, the number of endoderm cell layers is reduced from 4-5 cells deep to a single epithelial layer, suggesting that the intercalation of the central cells and concomitant thinning of the epithelium provides the increased surface area necessary to generate length (Chalmers and Slack, 2000; Reed et al., 2009). Moreover, clusters of labeled endoderm cells become aligned in longitudinal tracts along the A-P axis of the lengthening intestine (Chalmers and Slack, 2000; Reed et al., 2009). This indicates that radial intercalation is biased to preferentially occur between A-P neighbors, or is closely coordinated with a convergent extension process, increasing intestinal length, rather than girth (Chalmers and Slack, 2000; Keller et al., 2000; Reed et al., 2009) (Figure 2).

In amphibian embryos, the PGT begins as a solid cylinder full of concentrically stratified endoderm cells, but in amniotes, the endoderm lining of the PGT is already a single-layer, albeit pseudostratified, epithelium(Grosse et al., 2011); thus, multilayer radial intercalation *per se* is not likely to drive gut elongation, but cell rearrangements are likely still involved. It has been hypothesized that the elongation of the mammalian gut tube results from the reorganization of the pseudostratified endoderm layer into a columnar epithelium, but how this results in

anisotropic tissue lengthening is not known (Grosse et al., 2011). Nonetheless, short intestine phenotypes in both frogs and mice are associated with disorganization and stratification of the intestinal epithelium (Cervantes et al., 2009; Grosse et al., 2011; Matsuyama et al., 2009; Yamada et al., 2010), and the genes and pathways found to direct endoderm rearrangement in frogs (by regulating cell shape/polarity/adhesion—discussed in Sections 3.2-3.4 below) also control gut elongation and epithelial morphogenesis in mammalian models. Therefore, despite differences in endoderm tissue architecture in the initial PGT, the processes of gut elongation appear to be conserved at many levels, making the frog embryo a highly relevant model for investigating intestine lengthening.

3.2. Shroom3 mediates endoderm cell shape changes

The PDZ-containing protein Shroom3 is required for directing cell shape changes, including apical constriction and apicobasal cell elongation during neural tube closure (Haigo et al., 2003; Hildebrand and Soriano, 1999; Lee et al., 2007). In *Xenopus*, intestine-targeted microinjection of mRNA encoding a dominant-negative mutant form of Shroom3 (DN-Shroom3) results in severely shortened intestinal tracts, demonstrating a requirement for this protein during gut development (Chung et al., 2010). At the cellular level, DN-Shroom3 expressing cells display reduced apical constriction, or remain rounded and do not intercalate, resulting in a stratified disorganized epithelium; thus, Shroom3 must normally direct cell shape changes in the endoderm that are necessary for both epithelial morphogenesis and gut

elongation (Chung et al., 2010). Interestingly, Pitx transcription factors, which are also required for intestinal elongation (Al Alam et al., 2012; Campione et al., 1999), were found to directly regulate *shroom3* expression, suggesting that Shroom3 directs intestinal morphogenesis downstream of Pitx factors (Chung et al., 2010). These results underscore the relationship between endoderm morphogenesis and gut elongation. Notably, Shroom3 activity has also been correlated with endodermal shape changes and epithelial architecture in the mouse intestine (Grosse et al., 2011; Plageman et al., 2011).

3.3. Non-canonical Wnt/PCP signaling controls intestine lengthening and endoderm rearrangements

In addition to foregut specification, Wnt signaling is also essential for intestinal morphogenesis as knock-out/down of Wnt signaling components results in short, malformed intestinal tracts in all species studied (Cervantes et al., 2009; Dush and Nascone-Yoder, 2013; Matsuyama et al., 2009; Reed et al., 2009; Yamada et al., 2010). However, while canonical Wnt/β-catenin signaling is necessary for cell specification and maintenance of stem cell niches in the intestine, the elongation of the gut tube depends primarily on the non-canonical Planar Cell Polarity (Wnt/PCP) pathway, which involves distinct downstream effectors such as JNK and Rho family GTPases (Chien et al., 2009). Isolating the mechanisms by which Wnt/PCP signaling coordinates cell movements in the gut is non-trivial in murine models due to the redundancy of Wnt signaling components, and the need for stage- and tissue-

specific promoters and/or combinations of mutant alleles (Matsuyama et al., 2009). However, the use of small molecules and gut-specific targeting of LOF reagents in *Xenopus* has contributed a mechanistic understanding of the molecular players and cellular mechanisms involved in gut elongation.

For example, both pharmacological inhibition of RhoA and gut-targeted microinjection of mRNA encoding a dominant negative mutant version of RhoA (DN-RhoA) implicate Rho activity in *Xenopus* gut elongation, as both perturbations abrogate intestinal elongation (Reed et al., 2009). As they prepare to undergo intercalation, endoderm cells normally become polarized and radially oriented, progressing from the most basally located cells toward the center of the PGT (Reed et al., 2009).)However, DN-RhoA-expressing cells remain round in shape, unpolarized and do not intercalate. Rho-inhibited cells also exhibit aberrant Myosin II organization and increased expression of the adherens junction protein, E-cadherin, suggesting that, without Rho activity, endoderm cells are unable to remodel adhesive contacts and, as a result, cannot rearrange or intercalate (Reed et al., 2009). Chemical inhibitors of downstream effectors of Rho kinase (ROCK) and Myosin II phenocopy Rho-deficient intestinal malformations at both the gross and cellular level, suggesting that actomyosin contractility regulated by the Rho/ROCK/Myosin II branch of the Wnt/PCP network is required for the endoderm cell rearrangements that generate gut length (Reed et al., 2009). Interestingly, embryos with less severe gut elongation phenotypes display abnormal intestinal

coiling, revealing a potential mechanistic link between the processes of gut lengthening and rotation.

Non-canonical Wnt/PCP signaling is also mediated by activation of Jun N-terminal kinase (JNK) (Boutros et al., 1998; van Amerongen and Nusse, 2009). Chemical inhibition or gut-targeted knockdown of JNK activity results in shortened intestines, similar to perturbation of Rho/ROCK/Myosin II activity. However, the JNK-deficient phenotype differs at the cellular level, as endoderm cell adhesion is lost in guts lacking JNK activity, in contrast to the increased adhesion observed in ROCK-deficient guts (Dush and Nascone-Yoder, 2013). Abrogation of microtubule polymerization phenocopies loss of JNK activity, and suggests that this arm of the Wnt/PCP signaling cascade mediates cell rearrangement by promoting microtubule polymerization, and maintaining cell adhesion (Dush and Nascone-Yoder, 2013). The amenability of *Xenopus* explants to *ex vivo* cell assays further confirmed that adhesive remodeling is likely to be involved in gut elongation. PGT cells were isolated and dissociated, and their ability to reaggregate was used as an assay for changes in cell adhesion that may be caused by perturbing different branches of the Wnt/PCP cascade. The results confirmed that Rho kinase promotes decreased cell-cell adhesion, while the JNK pathway increases adhesion, suggesting that these two arms of the Wnt/PCP signaling network act in complementary ways to regulate intestinal cell intercalation (Dush and Nascone-Yoder, 2013). Thus, the accessibility

of the frog embryo was instrumental in clarifying Wnt/PCP mediated cellular and molecular dynamics underlying the cell rearrangements that lengthen the gut.

3.4. Hedgehog signals mediate reciprocal mesoderm-endoderm signaling

Hedgehogs (Hhs) are secreted proteins that elicit concentration dependent responses via multi-pass transmembrane receptors (Briscoe and Therond, 2013). Hh signaling plays crucial patterning and morphogenetic roles in ectoderm, mesoderm and endoderm-derived tissues throughout development (Briscoe and Therond, 2013). In mouse models, loss of Hh signaling results in shortened, malrotated gastrointestinal tracts, with disrupted architecture in all three tissue layers (Ramalho-Santos et al., 2000; Sukegawa et al., 2000). *Xenopus* have been instrumental in refining our understanding of how Hh-mediated communication between tissue layers functions in gut development. Hhs are expressed in the endoderm layer of the gut tube but, surprisingly, microinjection of mRNA encoding a constitutively active version of the Smoothened receptor (to induce excessive Hh signaling) does not affect gut development when targeted to the endoderm layer (Zhang et al., 2001). In contrast, ectopic Hh signaling severely disrupts gut elongation and coiling when targeted to the mesoderm layer, indicating that Hh ligands from the endoderm act by binding to receptors in the mesoderm (Zhang et al., 2001). Of significance, this study also showed that Hh-mediated signaling within the mesoderm is essential for the endoderm epithelial rearrangements that elongate

the intestine, revealing insight into the molecular nature of the reciprocal signaling known to be required between the layers of the developing gut (Zhang et al., 2001).

Xenopus experiments also shed light on the role of downstream components of Hh signaling in gut morphogenesis. The transcription factor *foxf1* is upregulated in the gut mesoderm in response to Hh signaling (Madison et al., 2009; Mahlapuu et al., 2001a). However, knockout of *foxf1* is lethal in mice prior to gastrointestinal elongation and looping, precluding use of the murine model for understanding the function of FoxF1 in intestinal development (Mahlapuu et al., 2001b). In contrast, FoxF1 can be directly knocked down in *Xenopus* using targeted microinjection of morpholino oligonucleotides. Morpholino-mediated loss of FoxF1 activity resulted in disruption of mesodermal differentiation and severely reduced the elongation and rotation of the *Xenopus* intestine, confirming that FoxF1 is essential for normal gastrointestinal morphogenesis (Tseng et al., 2004). Subsequent to this work, *foxf2* knockouts and compound *foxf1*$^{+/-}$/*foxf2*$^{+/-}$ mice were generated, which survive to birth (Ormestad et al., 2006). Similar to the observations in *Xenopus,* the intestines of these mice were deformed and showed severe disruption in mesoderm-derived tissues (Ormestad et al., 2006). These examples underscore how the frog embryo can be used to discern the tissue-specific functions of highly conserved genes in directing crucial aspects of gut morphogenesis.

4. Insights from chemical screening.

One of the greatest advantages of externally-fertilized, aquatic embryos, like those of the frog, is that they can be exposed to exogenous chemicals to reveal potential roles for the cellular target of the compound in development. For studies of organ morphogenesis, these are particularly useful reagents because they allow earlier events that might be dependent on the same signaling pathway, to proceed unperturbed. The effects of chemical reagents targeting RA, Wnt, and FGF signaling pathways have been described above, but a few studies using additional small molecules are also worth highlighting (section 4.1). Although the potential mechanisms of action of these compounds in disrupting gut specification or morphogenesis is not yet well understood, these reports nonetheless implicate interesting pathways and processes in digestive organogenesis. Finally, it is important to mention that numerous chemical toxicants, many of which have *unknown* mechanisms of action, also elicit gut phenotypes (section 4.2); these chemicals could provide interesting avenues for future research on both normal and abnormal gut development.

4.1.1 Calcineurin implicates Wnt/Ca^{++} signaling in gut elongation and rotation.

Calcineurin is a calcium/calmodulin-dependent serine/threonine phosphatase which is a component of the non-canonical Wnt/Calcium signaling pathway (Kuhl,

2004). Exposure of *Xenopus* tadpoles to the Calcineurin inhibitors cyclosporine A, FK506, or FK520 for six hour windows beginning at Nieuwkoop and Faber (NF) stage 18, 29/30, 37/38, or 41 resulted in shortened gut tubes often displaying a reversed coiling direction (Yoshida et al., 2004). Given the roles of non-canonical Wnt signaling in gut morphogenesis, it seems possible that Calcineurin regulates endoderm cell properties, such as adhesive or cytoskeletal dynamics, and/or cell polarity. Injection of these inhibitors into dorsal blastomeres at the four cell stage similarly disrupted gut coiling, supporting the idea that Calcineurin plays a specific role in gut development (Yoshida et al., 2004). However, other dorsally derived organs (heart, liver, etc.) were also affected by this injection, and further experimentation is required to determine the importance of Calcineurin in gut morphogenesis *per se*. It will be interesting to determine how Wnt/Calcium signaling is integrated with canonical and/or non-canonical Wnt signaling in this context, using more specific reagents to target Calcineurin activity within the developing intestine.

4.1.2. Lysyl Oxidase and a role for the extracellular matrix in gut elongation.

Lysyl oxidase (Lox) is a copper-dependent enzyme that catalyzes cross-linkage of Collagen and Elastin in the extracellular matrix (ECM). *lox* knockout mice die at birth and have a number of deformities including cleft palate, spinal, cardiovascular and respiratory defects, indicating that Lox is required for normal development in a number of systems (Maki et al., 2002; Maki et al., 2005; Zhang et

al., 2015). In *Xenopus*, exposure to β-aminoproprionitrile (β-APN), a specific inhibitor of the Lox catalytic domain, from NF 6-45 affects many developmental processes, including proper cross-linking of connective tissue fibers in the notochord and somites (Geach and Dale, 2005). Of particular interest, β-APN exposure also results in short, straight gut tubes, suggesting that Lox may also function in gut morphogenesis, but its role in intestinal development has not been specifically investigated. In mice, Lox regulates ECM organization in muscle connective tissue, suggesting that Lox could be required for proper ECM assembly between the mesoderm and endoderm layers of the PGT (Kutchuk et al., 2015). Additional studies that investigate the role of Lox specifically within the gut are necessary to evaluate the potential importance of this protein in intestinal development.

4.1.3. mTOR signaling is implicated in gut elongation

The mTORs are serine/threonine kinases which form complexes with FKBP12 and Raptor (Hara et al., 2002; Kim et al., 2002; Sabers et al., 1995). In *Xenopus*, inhibition of mTOR with rapamycin treatment from NF 2 - 45 results in shorter, fatter gut tubes as compared with controls (Moriyama et al., 2011). Other organs form relatively normally, suggesting that mTORs may be specifically required in gut elongation (Moriyama et al., 2011)(Moriyama et al., 2011)(Moriyama et al., 2011)(Moriyama et al., 2011)(Moriyama et al., 2011)(Moriyama et al., 2011)(Moriyama et al., 2011)(Moriyama et al., 2011)(Moriyama et al., 2011). Consistent with this idea, injection of a dominant-negative Rheb (an upstream positive regulator of mTOR) at the 2 cell stage also decreases intestinal elongation and coiling (Moriyama et al., 2011). As zebrafish exposed to rapamycin also have GI defects,

this pathway may play a conserved role in vertebrate intestinal organogenesis (Makky et al., 2007).

Interestingly, Sirtuin deacetylases (Sirts) are also implicated in regulation of the TOR pathway (Ghosh et al., 2010). Inhibition of Sirt-1 with the specific inhibitor, Ex-527, from the 2 cell stage on also disrupts intestinal elongation and coiling, although Sirt-1 deficient tadpoles exhibit more general defects than those treated with rapamycin, including decreased lengthening along the anterior-posterior body axis, edema, and abnormal eye development (Ohata et al., 2014). As Sirt-1 inactivates p53, and p53 activity is essential for normal embryonic development through regulation of TGF-β signaling (Takebayashi-Suzuki et al., 2003; Wallingford et al., 1997), Sirt-1 could be involved in regulating multiple pathways during gut development. It is important to note that none of these experiments were conducted in a way that specifically evaluates the function(s) of Sirt-1/mTOR in GI morphogenesis, and it is unclear exactly when these reagents may be acting to impact gut development. Thus, additional studies that specifically address the functions of Sirts and TORs in the gut are necessary to determine how this pathway may contribute to intestinal morphogenesis.

4.2 Numerous toxicants perturb gut morphogenesis

The etiology of intestinal malrotation is largely unknown but it is believed to have a multifactorial origin, implicating both genetic and environmental causes. Frog embryos have proven to be excellent models for screening toxicants that impact development, and many studies have implicated exogenous chemicals, including insecticides, nanoparticles and explosives, in digestive tract malformations (Table 1).

Unfortunately, few of these reports attempt to identify the underlying molecular or cellular developmental mechanism(s) disrupted by toxicant exposure. Future research on the mechanism(s) of action of these compounds in *Xenopus* could provide invaluable insight into the pathways required for gut development and potentially reveal environmental factors that contribute to the relatively high incidence of intestinal malrotation and other gut defects in the human population.

Table 1. Anthropogenic toxicants found to disrupt gut development in *Xenopus laevis*.

Chemical Class	Chemicals tested	Uses	References
Azoles	Triadimefon, n-butyl isocaynate, carbendazim	Fungicide	(Lenkowski et al., 2010; Yoon et al., 2008)
Bipyridyliums	Paraquat	Herbicide	(Vismara et al., 2001; Vismara et al., 2000)
Carbamates	Carbaryl	Insecticide	(Bacchetta et al., 2008)
Carboxylic Acids	Valproic acid, pentanoic acid, butyric acid, 2-ethylhexanoic acid	Various: Plasticizer, lubricant	(Dawson, 1994)
Chlorophenoxy Acids	2,4-D	Herbicide	(Lenkowski et al., 2010)
Estrogen	17β - estradiol	Drug	(Sone et al., 2004)
Nanoparticles	CuO, ZnO, polystyrene	Various: semiconductors; drugs, skin care	(Bacchetta et al., 2014; Nations et al., 2011; Tussellino et al., 2015)
Nitroaromatic compounds	TNT, 2ADNT, 4ADNT	Explosive	(Saka, 2004)
Organochlorines	Chlorothalonil, DDT, DDD	Insecticide	(Saka, 2004; Yu et al., 2013)
Organophosphates	Malathion, Malaoxon, Parathion, Paraoxon, Dicrotophos, Monocrotophos, Chlorpyrifos, Diazinon	Insecticide	(Bonfanti et al., 2004; Modra et al., 2011; Snawder and Chambers, 1989, 1990)
Phenols	Bisphenol A, nonylphenol	Various: Plastics, resins, adjuvant	(Sone et al., 2004)
Phosphonoglycines	Glyphosate	Herbicide	(Lenkowski et al., 2010)
Triazines	Atrazine	Herbicide	(Lenkowski and McLaughlin, 2010; Lenkowski et al., 2008; Lenkowski et al., 2010)
Polymer Mixtures	Tire Debris Organic Extract	Tire product	(Mantecca et al., 2007)
Chemical Mixtures	Corexit 9500	Dispersant	(Smith et al., 2012)

5. From Frogs to Humans.

Because the molecules, pathways and processes important for endoderm specification and digestive morphogenesis are conserved across vertebrates, amphibian studies are highly relevant to human gut development. Indeed, frog studies have already informed translational research strategies. For example, knowledge of the hierarchical relationship of factors such as Wnt, Fgf, RA, Bmp, and Hh in *Xenopus* endoderm specification was instrumental in the successful development of protocols to direct human pluripotent stem cells to digestive organ fates and generate digestive "organoids" from human stem cells (Wells and Spence, 2014). Likewise, one of the most important genes in pancreatic development, *pdx1*, was discovered in the *Xenopus model (Wright et al., 1989). Activation of* this pancreatic master regulator is now used in human transdifferentiation protocols to elicit endocrine pancreas fates from extra-pancreatic tissues, such as liver cells, a critical step towards successfully reprogramming adult cells as a cell replacement therapy for diabetes (Horb et al., 2003).

Genes for human congenital GI malformations are just beginning to be identified (Dauve and McLin, 2013). To complement these efforts, frog embryos not only provide excellent models for the discovery of genes critical for normal digestive organ morphogenesis (see section 2.4 above), but facilitate rapid *in vivo* validation of candidate birth defect gene function. For example, the importance of FoxF1 in gut morphogenesis was first demonstrated in *Xenopus*, as discussed above (section

3.4;(Tseng et al., 2004)). Mutations in this gene have recently been detected in human patients with similar malformations, including intestinal malrotation and congenital short bowel (Hilger et al., 2015; Martin and Shaw-Smith, 2010; Stankiewicz et al., 2009). Moreover, trisomy of chromosome 16, which contains the human *foxf1* gene, is also associated with intestinal maladies (Martin and Shaw-Smith, 2010) . Finally, mutations in *zic3*, a transcription factor involved in directing organ laterality in animal models (Kitaguchi et al., 2000), have recently been detected in humans with congenital GI defects (Hilger et al., 2015). In *Xenopus,* overexpression of *zic3*, or injection of a mutant *zic3* mRNA that acts in a dominant-negative manner, disrupt the direction of intestinal looping, providing *in vivo* confirmation of the suspected role of this molecule in organogenesis (Kitaguchi et al., 2000). These examples illustrate the immense potential and relevance of the *Xenopus* model for human biomedical research.

6. What can frogs tell us about digestive organ evolution?

6.1. The diversity of the tadpole gut.

The morphology of the digestive tract determines an organism's ability to assimilate the energy necessary to grow, survive and reproduce--and thus has a profound effect on fitness. For example, the dimensions of the gut tube itself, including its diameter, length and compartmentalization, impact the capacity to digest different food resources. Although there is remarkable disparity between

these parameters in the GI tracts of different vertebrates, the underlying mechanism by which different topologies of the digestive tract evolve is unknown.

Frogs inhabit most of the planet, including every continent except Antarctica. This success is facilitated by a wide array of reproductive strategies which allows them to breed in diverse environments, including terrestrial niches (McDiarmid and Altig, 1999). Because the tadpoles of many terrestrial breeding frogs are derived from eggs laid in environments with limited water and food supplies (e.g., bromeliads, leaves), these species may exhibit unusual larval feeding strategies. For example, in contrast to typical herbivorous tadpoles, terrestrial tadpoles may be carnivorous and feed on unfertilized eggs, invertebrates or even other tadpoles. Alternatively, they may delay or omit the feeding stage entirely by becoming more dependent on maternal yolk stores, as observed in direct-developing species (Altig, 1989). Not surprisingly, these novel feeding (or non-feeding) strategies are complemented by specialized larval gut morphologies. Evo-devo investigations of gut development in two emerging frog models, *Lepidobatrachus laevis* and *Eleutherodactylous coqui*, are providing novel insight into the potential sources of variation that lead to diverse gut morphologies during evolution.

6.2. The evolution of a carnivorous foregut in *Lepidobatrachus.*

The Budgett’s frog, *Lepidobatrachus laevis*, lives in the semi-arid regions of South America (Budgett, 1899). As adults, *Lepidobatrachus* are aggressive, and often cannibalistic, predators, while their tadpole larvae are obligate carnivores that

routinely consume other tadpoles, including siblings (Fabrezi, 2006; Hanken, 1993; Ruibal, 1988). Unlike tadpoles that have a long, un-compartmentalized tract adapted for their nutrient-poor herbivorous diet, *Lepidobatrachus* tadpoles have a large, distendable stomach compartment (Carroll, 1991; Ruibal, 1988). Analysis of foregut morphogenesis in *Lepidobatrachus* has revealed that the development of this unusual anatomy is preceded by a disparity in the proportion of the PGT that is ascribed to foregut versus hindgut, as compared to that observed in *Xenopus* (which is used as a point of comparison to represent the ancestral state (Bloom et al., 2013)). This ultimately results in dramatic differences in stomach morphogenesis and the final anatomical orientation of the gastroduodenal (GD) loop (Figure 3).

To identify potential signaling pathways that may have been involved in the evolution of this novel carnivore morphology, a small molecule screen was conducted in *Xenopus* embryos (Bloom et al., 2013). Compounds targeting known morphogenetic pathways were screened for the ability to transform the more typical herbivore GD loop found in *Xenopus* to resemble that found in the carnivorous *Lepidobatrachus* tadpole. Remarkably, five compounds produced this change, two of which inhibit RA signaling (Bloom et al., 2013). RA plays an early role in dorsal pancreas specification (described in section 2.3 above), but RALDH expression also persists throughout the development of the stomach and duodenum, and perturbations of RA signaling in tailbud stage *Xenopus* embryos implicate RA patterning in GD looping (Lipscomb et al., 2006). Thus decreased RA signaling in

the *Lepidobatrachus* lineage may have led to the unusual carnivore foregut morphology, an idea supported by the formation of a smaller stomach and shallower GD loop (anatomically similar to an ancestral tadpole like *Xenopus)* in *Lepidobatrachus* embryos exposed to exogenous RA (Bloom et al., 2013). This study demonstrated that subtle changes in the levels of a specific foregut signaling factor can lead to anatomical variants that closely mimic extant interspecific variation.

6.3. The evolution of nutritional endoderm in a direct-developing frog.

The epithelial lining of the vertebrate digestive tract arises from the endoderm germ layer of the early embryo. In the frog, the endoderm is derived from the cellularization of the yolky vegetal pole of the egg. In typical tadpoles, all of the endoderm-derived cells rearrange in the gut tube to become the gut epithelium and contribute to intestinal lengthening (see section 3.1 above). In most species, the inherent yolk in the embryonic cells supports development only through the initial tadpole stages, after which the animal needs a functional digestive system to acquire the energy necessary to continue to grow and, ultimately, metamorphose, when the long gut is remodeled into the shorter gut of an adult frog. In contrast, endotrophic (non-feeding, yolk-dependent) species, such as direct developers, often delay or completely skip the formation of a long, coiled gut, since they do not need to

feed (Altig, 1989). Instead, these embryos directly form a short adult-like gut by the time they become a froglet.

The mechanisms by which the processes of yolk utilization and gut development are altered in direct-developing species may provide insight into the origin of novel gut morphologies and feeding strategies during evolution. Indeed, in contrast to species like *Xenopus* that produce feeding tadpoles, in the direct-developing embryo of *Eleutherodactylous coqui*, much of the yolky endoderm does not contribute to the final epithelial lining of the gut tube (Buchholz et al., 2007; Karadge and Elinson, 2013). Instead, it becomes nutritional endoderm (NE) that is utilized solely as a source of energy—the yolk platelets in these cells are metabolized, extruded and, eventually, eliminated as waste from the body. Some of the yolky vegetal cells are specifically set aside for this function during blastula stages, as indicated by the existence of a population of endoderm with reduced signaling/responsiveness in such species (Chatterjee and Elinson, 2014). This alternate fate of the NE is likely related to the modified germ layer patterning often seen in larger eggs (Elinson and Beckham, 2002). The use of endoderm to provide energy to sustain growth, rather than form a longer gut, has interesting implications for the ancient origins of definitive vs. extra-embryonic endoderm, as well as the acellular yolk sac of higher vertebrates (Buchholz et al., 2007).

7. Conclusion/ Future directions.

Amphibian embryos have a rich history as developmental models and have been instrumental in understanding fundamental embryological events, such as gastrulation and neurulation. Here, we argue that the experimental versatility of frog embryos—e.g., the ability to isolate tissue explants, target LOF/GOF reagents, and/or use pharmacological agents to investigate the underlying mechanisms of development—also makes them ideal models in which to examine many facets of digestive organogenesis. Their amenability to these experimental manipulations has enhanced our understanding of the spatiotemporal dynamics of conserved signaling pathways, such as Wnt, FGF, BMP, RA and Hh, in foregut specification and intestinal elongation. In addition, microarray profiling and small molecule/toxicant screening in frog models have revealed novel proteins and pathways likely to play critical roles in normal and abnormal gut morphogenesis. Such information enhances our understanding of gut patterning and morphogenesis in all vertebrates, including humans, making the frog a powerful model for translational embryology.

While many signaling pathways are conserved in gut organogenesis, differences do exist in the size and shapes of digestive organs among species, which is exemplified by the gastrointestinal tracts of tadpoles with different feeding strategies. Studies designed to elucidate differences in gut development in frog species with unique feeding ecologies are beginning to provide intriguing insight into the variety of molecular and cellular mechanisms underlying morphological evolution. Such knowledge has the potential to illuminate specific environmental or

ecological parameters that affect gut development and, therefore, impact survival and fitness. Since frog species are continuing to disappear at an alarming rate, this line of evo-devo research may provide critical information for conservation efforts (Stuart et al., 2004).

Rapid advances in *de novo* transcriptome assembly, proteomics, and genome editing (CRISPR-Cas) continue to make functional genetic studies even more accessible for *Xenopus*, and nearly any frog species (Amin et al., 2014; Blitz et al., 2013; Guo et al., 2014; Rao et al., 2014; Sun et al., 2014; Wuhr et al., 2014). New techniques continue to arise for refining tissue-specific gene manipulation at late stages of organogenesis in the frog, including lipofection (Ohnuma et al., 2002) and electroporation (Chernet and Levin, 2012). Utilizing these cutting-edge technologies to investigate amphibian digestive organ development—integrating aspects of organ specification, morphogenesis, toxicology, and/or evolution—has the potential to advance multiple scientific disciplines.

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Figure 1. Wnt, RA, and FGF pattern the foregut. The primitive gut tube is regionalized along both A-P and D-V axes. During gastrulation, RA (red) is required for dorsal pancreas (DP; purple) specification, likely by inhibiting Shh expression. Slightly later, the foregut is distinguished from the hindgut by a gradient of Wnt signaling (orange). High posterior Wnt specifies the hindgut domain, while low anterior Wnt (yellow; limited by Sfrp5) signals through the Fzd7 receptor to promote foregut fates and initiate cellular morphogenesis. Finally, a gradient of FGF signaling from the neighboring cardiac/lateral plate mesoderm segregates ventral foregut organs; prolonged, higher levels of FGF are needed to specify liver (green) versus ventral pancreas (VP; blue).

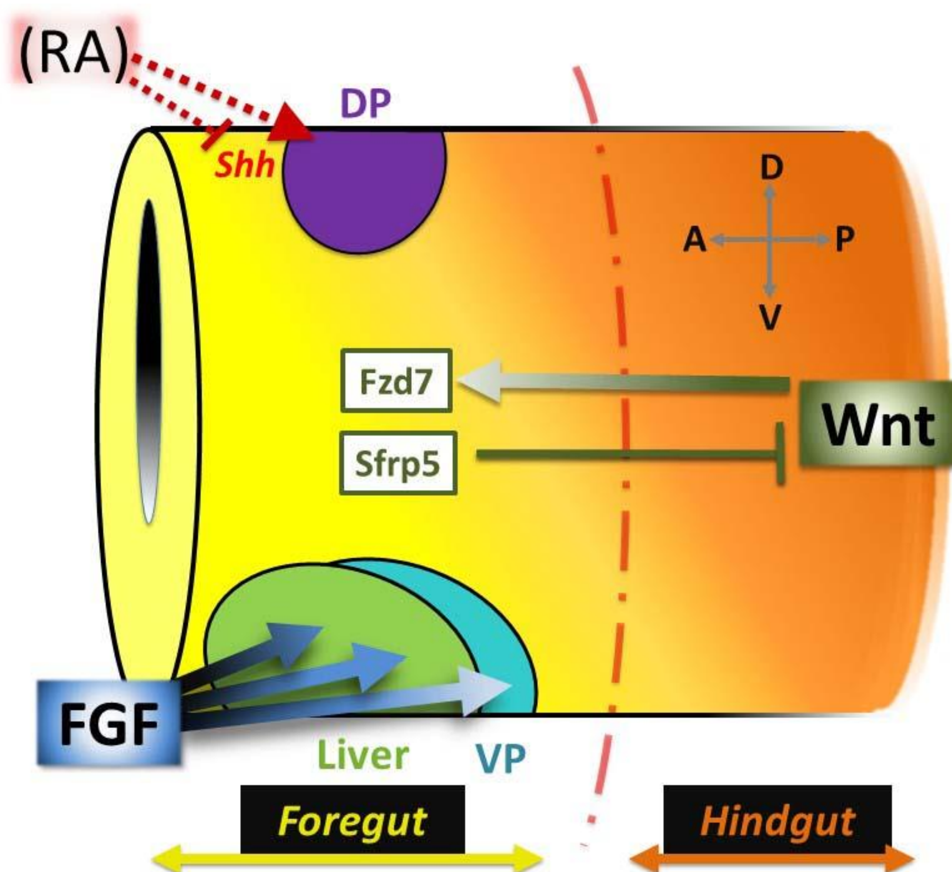

(RA)
DP
Shh
D
A
P
V
Fzd7
Sfrp5
Wnt
FGF
Liver
VP
Foregut
Hindgut

Figure 2. Intestine lengthening involves Hedghog- and Wnt/PCP-mediated endoderm cell polarization, rearrangement and epithelial differentiation. Initially, the endoderm cells of the prospective intestine are rounded, unpolarized and disorganized. Signaling via Hedgehogs (HH; from the endoderm) induces *foxF1* expression in the surrounding mesoderm layer of the gut tube. This facilitates reciprocal signaling from the now differentiating visceral mesoderm, which regulates the rate of epithelial differentiation in the underlying endoderm. Concomitant non-canonical Wnt signals (presumably from the mesoderm) are required for the endoderm cells to become polarized, starting with the outermost (most basal) layers and progressing towards the center of the gut tube. Both actomyosin contractile forces, regulated by ROCK, and microtubule organization, regulated by JNK, are required to dynamically remodel adhesive contacts between the polarized endoderm cells. This enables productive radial intercalation of the most central cells into the outermost layer, resulting in tissue lengthening and the morphogenesis of a single layer of intestinal epithelium.

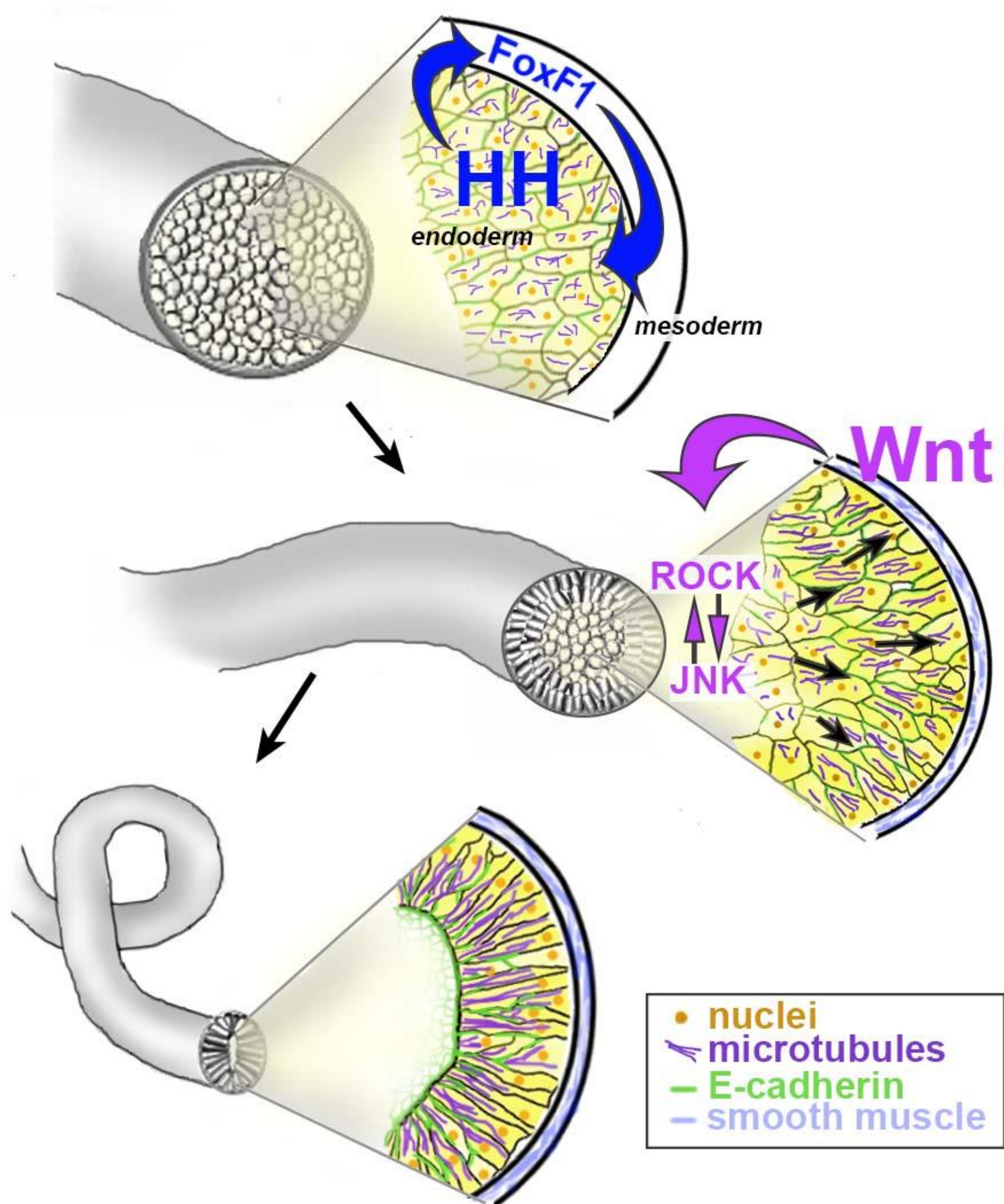

FoxF1
HH
endoderm
mesoderm
Wnt
ROCK
JNK
nuclei
microtubules
E-cadherin
smooth muscle

Figure 3. Altered RA signaling may have led to a novel foregut morphology. A) In the hypothetical ancestral anuran (represented by *Xenopus*), the herbivorous tadpole requires only a rudimentary stomach. The foregut domain of the primitive gut tube is small relative to the hindgut domain, causing the gastroduodenal (GD) loop to form in a relatively anterior position and acquire an acute curvature during later foregut morphogenesis. B) In contrast, in the carnivorous *Lepidobatrachus* tadpole, which requires a capacious stomach, the ratio of foregut to hindgut is greater, and the GD loop forms in a more posterior position. Consequently, the larger carnivore stomach becomes more transversely oriented. This anatomical change may have been dependent on a decrease in RA signaling during foregut development in the *Lepidobatrachus* lineage, since inhibiting RA in *Xenopus* (representative of the ancestral condition) transforms the GD loop to resemble that observed in *Lepidobatrachus*. Conversely, exposing *Lepidobatrachus* embryos to excess RA elicits a more typical foregut configuration.

A *Ancestral anuran*

B *Lepidobatrachus*

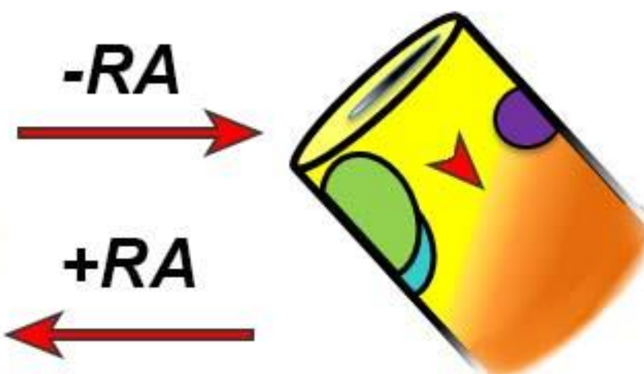
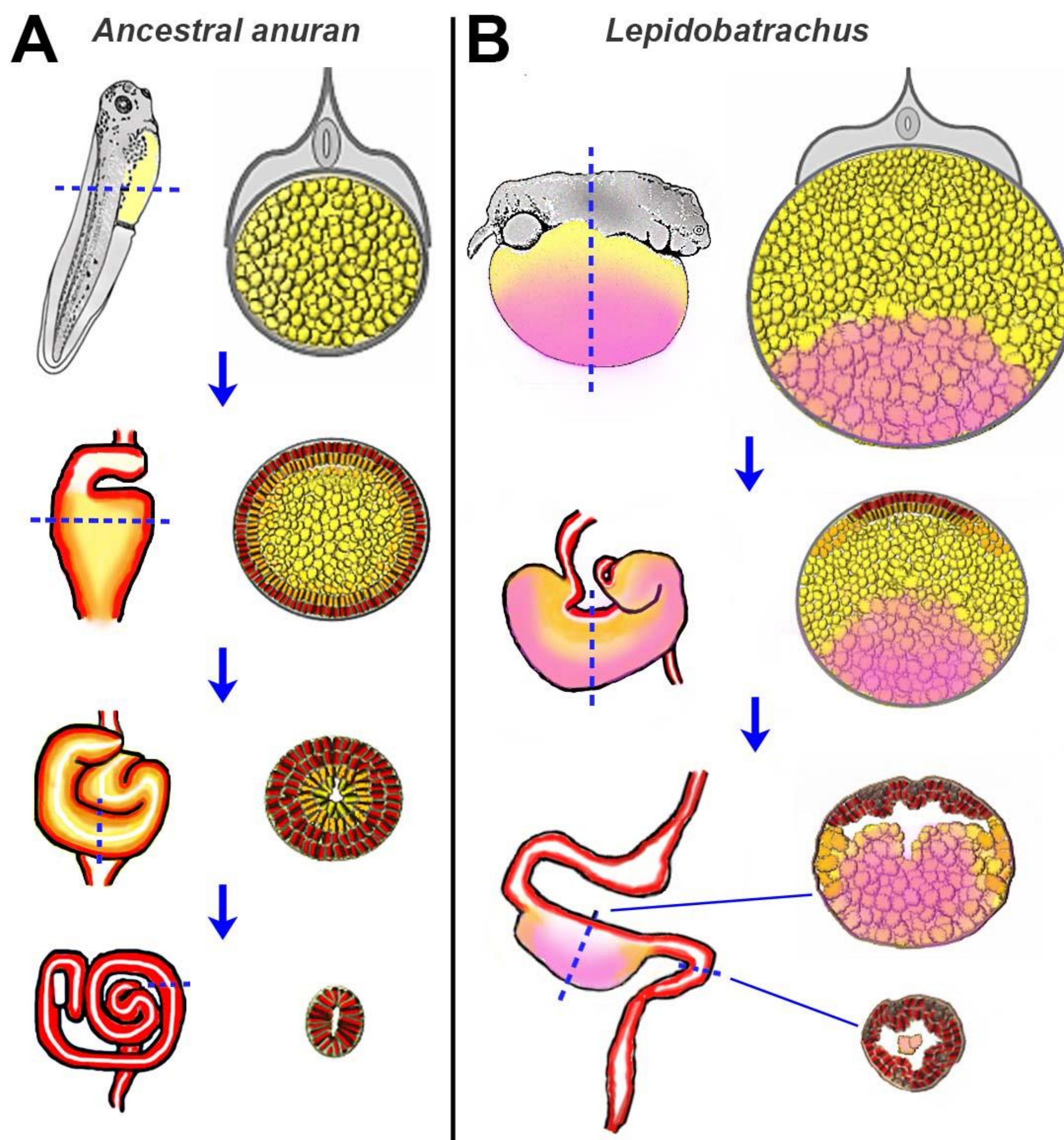


Figure 4. Endoderm morphogenesis in ancestral versus direct-developing frog species. A) In ancestral frogs that produce feeding (exotrophic) tadpoles, all of the yolky vegetal endoderm cells (yellow) in the primitive gut tube (PGT) are used to generate the lining of the tadpole gut. As these cells radially rearrange (see Figure 2) and differentiate into the final digestive epithelium (orange/red), a central lumen is formed and the intestine is lengthened to form a long, coiled tract. The extensive gut is eventually remodeled to a shorter adult tract during metamorphosis (not shown). B) In contrast, in the direct-developing (endotrophic) frog embryo, a subset of the vegetal endoderm cells are fated to become nutritional endoderm (NE; pink), a cell type that does not rearrange nor contribute to the gut epithelium. Instead, these cells are gradually depleted of their yolk, extruded and eliminated as waste. Consequently, the PGT does not generate a long tract, and the developing froglet directly forms a short adult-length intestine.


A
Ancestral anuran
B
Lepidobatrachus