UNIVERSITY OF CALIFORNIA

Santa Barbara

Identifying the Role of T-type Voltage-Gated Ca^{2+} Channels During Chordate Neural Development

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Molecular, Cellular, and Developmental Biology

by

Stephanie M. Khairallah

Committee in charge:

Professor William C. Smith, Committee Chair

Professor Ruth Finkelstein

Professor Stuart Feinstein

Professor Anthony De Tomaso

June 2021

The Dissertation of Stephanie M. Khairallah is approved.

Ruth Finkelstein

Stuart Feinstein

Anthony De Tomaso

William C. Smith, Committee Chair

May 2021

Identifying the Role of T-type Voltage-Gated Ca^{2+} Channels During Chordate Neural Development

Stephanie M. Khairallah

ACKNOWLEDGEMENTS

There are many people I want to thank for my success as a Ph.D. student.

First, I am thankful for my advisor, Dr. William C. Smith. Thank you for being an amazing and patient advisor (even after the number of times I freaked out in your office about this project). Thank you for supporting my crazy experiment ideas and insane literature searches. Additionally, I would like to thank my Ph.D. committee, Dr. Ruth Finkelstein, Dr. Stu Feinstein, and Dr. Tony De Tomaso for their insight, support, and excitement for this project.

Second, I would like to thank my labmates (former and current). You all are an amazing group of people. Erin, thank you so much for your chill and clear guidance. Matt, thank you so much for helping me calm down during my moments of panic and mentorship. Heidi, thanks for always there on FB Messenger when I needed moral support. Lastly, I want to thank Dr. Sarah Abdul-Wajid, Dr. Elijah Spina, and Haley Smith for your experimental insights and collaboration.

Third, I want to thank multiple mentors I have gained during my time as a Ph.D. student. I want to thank Dr. Kathy Foltz, Dr. Lalo Gonzalez, and Dr. Mike Wilton. You all have been instrumental in my development as a future mentor and educator.

Fourth, I thank my health professionals for helping me wrangle my mental health to get me through my Ph.D. program. Thank you to Gigliola Bernardi Sharron, NP, Dr. Bailey, Dr. Zamir, and Dr. Feliciano for listening and helping me through my mental illnesses. I would also like to thank my Ph.D. Qualifying Committee for allowing me to seek professional help when I needed it the most, otherwise I do not know if I would have succeeded in graduate school.

Lastly, I want to thank my family and friends for their unwavering belief in my abilities as a Ph.D. student. Ultimately, I want to thank my father, Michel Sassine Khairallah, for being the biggest advocate for my education. Love you, dad.

VITA OF STEPHANIE M. KHAIRALLAH

Spring 2021

EDUCATION

Bachelor of Science in Cell and Developmental Biology, University of California, Santa Barbara - Spring 2013

Master of Arts in Molecular, Cellular, and Developmental Biology, University of California, Santa Barbara - Spring 2021

Doctor of Philosophy in Molecular, Cellular, and Developmental Biology, University of California, Santa Barbara - Spring 2021

PROFESSIONAL EMPLOYMENT

Cell Biology Laboratory Instructor on Record - Winter 2018 - 2020

Biology Mentoring and Engagement Teaching Assistant - Spring 2021

Developmental Biology Laboratory Teaching Assistant - Winter 2021

Cell Biology Laboratory Teaching Assistant - Winter 2014 - 2017

Introductory Biology Laboratory Teaching Assistant - Fall 2013, Spring 2014, Fall 2015

PUBLICATIONS

Khairallah, Stephanie M., Smith, William C.. Characterization of Cav3.3 during Vertebrate Development. In Preparation.

Smith, Haley M., **Khairallah, Stephanie M.**, Nguyen, Ann H.,Newman-Smith, Erin, Smith, William C. Misregulation of cell adhesion molecules in the Ciona neural tube closure mutant bugeye. In Revision.

Abdul-Wajid, Sarah, Morales-Diaz, Heidi, **Khairallah, Stephanie M.**, and Smith, William C. (2015). T-type Ca^{2+} Channel Regulation of Neural Tube Closure and EphrinA/EPHA Expression. Cell Rep. 13, 829–839.

FIELDS OF STUDY

Developmental Biology and Genetics
Discipline-Based Education Research

Abstract

Identifying the Role of T-type Ca^{2+} channels During Chordate Neural Development

By

Stephanie M. Khairallah

My dissertation focuses on identifying the role of T-type voltage-gated Ca^{2+} channels (VGCC) in neural development. This research uses invertebrate and vertebrate models - specifically, the ascidian *Ciona* and the frog *Xenopus*, to span chordate development. Our lab previously identified a *Ciona savignyi* (*C. savignyi*) mutant, *bugeye,* that phenocopies exencephaly in humans by displaying an open brain. In *C. savignyi bugeye*, we see a dramatic decrease of *Cav3* expression using qPCR. The *Cav3* gene encodes for the single T-type VGCC in the *Ciona* genome. T-type VGCCs are part of a larger family of VGCCs, and are distinguished as being "low-voltage" because they are activated by small depolarizations in membrane potential to increase Ca^{2+} permeability. T-type VGCCs had not been implicated in embryonic development prior to these findings. Moreover, our lab found that EphrinA-d, a cell repulsion protein, was overexpressed in

bugeye embryos, and thus was likely regulated by Ca^{2+}. We found that overexpression of EphrinA-d phenocopied the open brain phenotype in wild-type embryos.

More recently the Smith lab discovered a new exencephaly mutant in a related species, *Ciona robusta* (*C. robusta)*. To identify the causative gene, I performed linkage analysis and a complementation test which pointed toward *Cav3*, the causative gene in *C. savignyi bugeye*. RT-PCR analysis of *Cav3* indicated no change in expression levels compared to wild-type siblings. However, sequence analysis of the mutant *C. savignyi Cav3* gene revealed multiple amino acid changes, many in areas of functional importance. The Smith lab followed up on these observations and performed RNAseq on both *C. savignyi bugeye* and *C. robusta bugeye embryos t*o identify novel genes involved in neural tube closure.

I extended research on Cav3 by examining a vertebrate model, the African clawed frog *Xenopus laevis*, which provided more tools to study morphogenesis. In *Xenopus*, I found that morpholino oligonucleotide knockdown of Cav3.3 caused a wide range of developmental defects, including delays in gastrulation and neurulation, craniofacial defects, a dorsal flexure phenotype, and an apparent mis-migration of melanocytes. To identify a cellular pathway, I investigated Calpain2, a Ca^{2+} -dependent

protease involved in the Wnt/Ca^{2+} pathway during gastrulation and neurulation. Knockdown of Calpain2 shows similar phenotypes as knockdown of Cav3.3, and I observed abolishment of tagged Calpain2L fluorescence, mislocalization of Calpain2L in nearby cells, and increased surface area for cells affected by the knockdown. Furthermore, we found loss of apical actin localization along the hinge points of the folding neural tube. This apical constriction is essential for normal neural tube closure. Our results indicate that Cav3.3 is necessary for neural induction and/or differentiation and mechanical aspects of gastrulation and neurulation. Future research will aim to identify the mechanisms through which Cav3.3 affects these events.

TABLE OF CONTENTS

I: Introduction

My dissertation work focuses on the role of Ca^{2+} in chordate development - specifically the role played by a particular Ca^{2+} channel, a T-type voltage-gated Ca^{2+} channel (abbr. VGCC, also known as Cav3). In this introduction, I will first review the particular developmental stages that will be examined: gastrulation and neurulation, and also the various roles played by the Wnt pathways during these developmental events, because my findings appear to link T-type VGCCs and the Wnt pathways. I will then provide an overview of the VGCCs before explaining Ca^{2+}'s known roles in the regulation of neural development. Finally, I will present my hypothesis for the role of T-type VGCCs in chordate gastrulation and neurulation.

Neural Induction and Differentiation in the Gastrula and Neurula Chordate Embryo

Neural Induction

Neural induction is the process by which the neural plate is specified from ectoderm on the dorsal side of the embryo. In *Xenopus*, neural induction starts in the late blastula embryo and proceeds through gastrulation (Stern, 2005). Gastrulation is a morphogenetic process involving extensive cell migration during which the embryo forms the

primary germ layers (endoderm, mesoderm, ectoderm), and establishes the anterior-posterior axis of the embryo (Solnica-Krezel and Sepich, 2012; Wallingford and Harland, 2001). Neural-inducing bone morphogenetic protein (BMP)-antagonists, such as Noggin, are secreted from group of cells located at the blastopore lip known as the organizer (called the Spemann Organizer in amphibians and Hensen's node in birds and mammals) (Stern, 2005). BMP is inhibited along the dorsal ectoderm, inducing neural fate and the formation of the neural plate (Brafman and Willert, 2017; Stern, 2005). The Wnt/β-catenin pathway is also thought to be involved with the induction of the neural plate, potentially inhibiting BMP, although there is no consensus on its role, as explained later in this chapter (Brafman and Willert, 2017).

Neural Differentiation

Neural induction is followed by neural differentiation, which I will define as the regionalization of the developing central nervous system (CNS) (*e.g.*, differentiation between the anterior and posterior neural plate and, later, the forebrain, midbrain, etc.), and the specification of distinct cell types within the neural tube. In *Xenopus*, the posterior and anterior regions of the neural plate differentiate at different times, with the posterior neural plate, containing the future hindbrain and spinal cord,

being specified at the start of gastrulation, while the anterior neural plate, which contains the future forebrain and midbrain, remains proliferative and undifferentiated until neural tube closure (NTC) is completed (Andreazzoli et al., 2003; Papalopulu and Kintner, 1996). Neural differentiation continues within the neural tube, which is thought to be driven by gradients of morphogens, with opposing signals emanating from the neural floor plate, neural roof plate, notochord, and lateral mesoderm (Brafman and Willert, 2017; Sagner and Briscoe, 2019).

Morphogenesis of the chordate embryo: gastrulation and neurulation

Tissue Movement

The morphogenetic events of gastrulation and neurulation are highly regulated processes with multiple molecular mechanisms at play (Kühl et al., 2001; Nikolopoulou et al., 2017; Ossipova et al., 2015b; Rolo et al., 2018; Skoglund et al., 2008; Solnica-Krezel and Sepich, 2012; Wallingford et al., 2001; Wallingford and Harland, 2001; Zanardelli et al., 2013). In *Xenopus*, gastrulation is first evident with the formation of apically-constricted cells, known as bottle cells, at the forming blastopore (Keller et al., 2003; Solnica-Krezel and Sepich, 2012). This signals the start of the

involution of the mesoderm along the blastocoel wall to where it comes to underlie the developing neuroectoderm. During gastrulation, convergent extension, by which mesoderm and posterior neuroectoderm cells intercalate medially, creates the force extending the anterior-posterior axis of the embryo (Wallingford and Harland, 2001) (Fig. 1). Both Wnt signaling and Ca^{2+} are essential during these critical steps in morphogenesis, as discussed in later sections.

After gastrulation is complete, the embryo enters neurulation and undergoes NTC. NTC involves a tightly choreographed series of

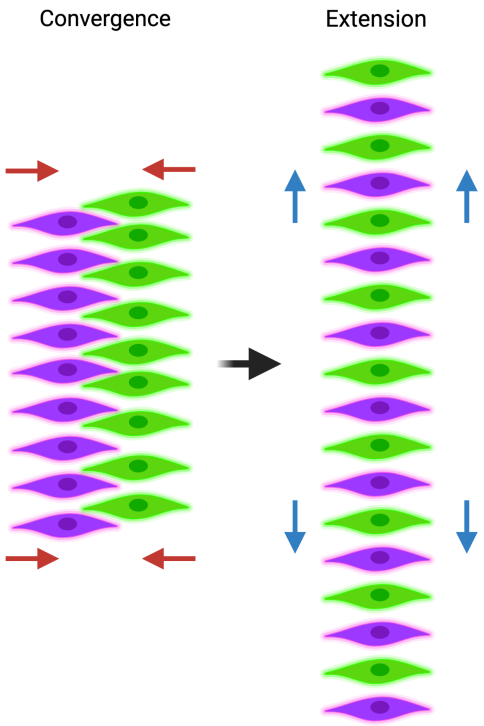


Fig. 1. Convergent Extension. Cells, typically mediolaterally, intercalate (converge) and lengthen (extend) the tissue, typically anterior-posteriorly. Created with BioRender.com

constriction, expansion, bending, adhesion, and repulsion that converts the flat neural plate to the closed neural tube. Although NTC varies between

chordate species (*e.g.*, mammals, amphibians, ascidians), there are features of NTC morphogenesis that are universal (Nikolopoulou et al., 2017). During NTC, the ensuing convergence, extension and collective bending of the neural plate brings the lateral edges together to fuse at the midline (Fig.2) (Nikolopoulou et al., 2017). Once NTC is completed, the embryo has internalized the entirety of its developing central nervous system.

Neural Fold Fusion

The mechanics of neural fold fusion, in which the edges of the neural folds adhere and fuse at the midline while separating from the lateral ectoderm, is still poorly understood (Fig. 2). It has been observed that in all vertebrate models studied, cellular protrusions extend out between the neural folds to facilitate fusion (Nikolopoulou et al., 2017). In mouse spinal NTC, Rac1, a Rho GTPase, appears to facilitate proper protrusion formation. However, these protrusions may be only necessary from the non-neural ectoderm, as ablation of Rac1 from the non-neural ectoderm causes NTC defects but not if removed from the neural ectoderm (Nikolopoulou et al., 2017). Other non-neural ectoderm genes, Grainyhead-like (Grhl) 2 and 3 regulate adhesion genes and loss of either or both causes neural tube defects (NTD) in mice models (Ray and Niswander, 2012).

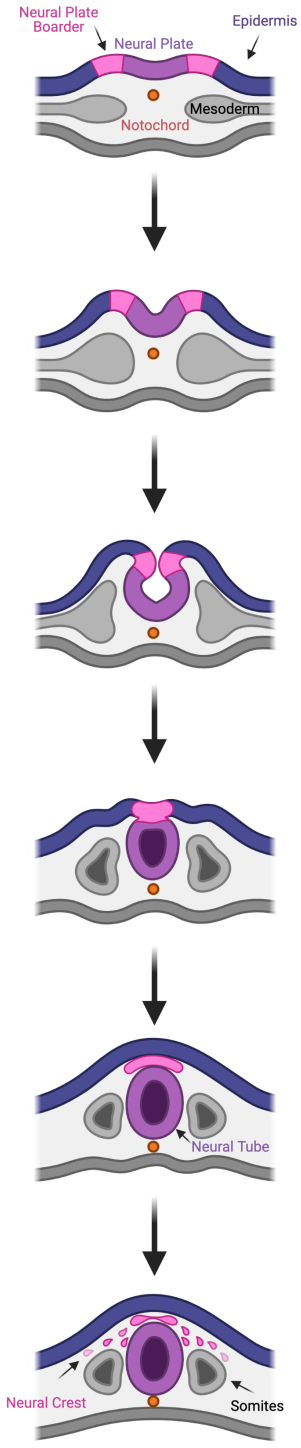


Fig. 2. Neural Tube Closure. Neural tube closure begins with the bending of the neural plate (purple) and subsequent elevation and bending of the neural folds. The folds meet along the dorsal midline and fuse. Created with BioRender.com

Our lab has shown that in the *C. savignyi* exencephaly mutant, *bugeye*, proper differentiation and neural tube closure occurs; however, fusion appears to fail as the anterior neural fold reopens and the brain is exposed (Abdul-Wajid et al., 2015). We found that adhesion/repulsion molecule EphrinA-d is upregulated in *bugeye* mutants, and its experimental overexpression in wild-type embryos mimics the *bugeye* phenotype; thus, we hypothesize that EphrinA-d expression is down-regulated by Ca^{2+}-induced signaling

in wild-type embryos at the completion of NTC (Abdul-Wajid et al., 2015). Ephrin and Ephrin receptors (Ephs) are involved in both repulsion and adhesion for proper cell migration, fusion, and axon guidance with downstream targets such as Rho GTPases involved in actin regulation (Cramer and Miko, 2016). Ephrins have been implicated in mouse neural fold fusion (Abdul-Aziz et al., 2009). The vast number of Ephrins and Ephs combinations available during development, as well as their overlapping expression, make them quite difficult to study due to potential redundancy, as well their pleiotropic effects (Fagotto et al., 2014).

The Wnt Pathways (Wnt/β-catenin, Wnt/PCP/and Wnt/Ca^{2+}) in Gastrulation and Neurulation

The Wnt/β-catenin Pathway Is Essential for Neural Induction and Differentiation

Neural Induction

The Wnt pathway regulates a variety of different cellular responses, depending on which Wnt ligands, receptors, and downstream targets are activated. The standard three Wnt pathways (Wnt/β-catenin, Wnt/Planar Cell Polarity (PCP), and Wnt/Ca^{2+}) perform multiple functions during development, including neural induction and convergent extension

(Brafman and Willert, 2017; Miller and McCrea, 2010). The Wnt/β-catenin pathway, which regulates transcription of target genes, is implicated in both neural induction and differentiation. With respect to induction, there are conflicting data on whether the Wnt/β-catenin pathway activates or inhibits neural induction. For example, in *Xenopus*, the Wnt/β-catenin pathway has been shown to inhibit BMP, thus allowing neural inducing factors, such as FGF, to activate neural induction; moreover, the Wnt/β-catenin pathway has been shown to promote expression of BMP antagonists (Brafman and Willert, 2017). Conversely, there have been other studies in *Xenopus* and chick that indicate the opposite: that Wnt signaling inhibits neural induction, thus promoting epidermal fate (Brafman and Willert, 2017). These interactions appear to be species and timing dependent (Brafman and Willert, 2017; Stern, 2005). Again, while the precise roles of Wnt/β-catenin pathway are still being investigated, it is clear this pathway plays an important role in neural development.

Neural Differentiation

During neural differentiation, the Wnt/β-catenin pathway is activated and deactivated spatially within the developing CNS to specify fates of the developing neural plate and tube (Brafman and Willert, 2017; Mulligan and Cheyette, 2012). For example, multiple experiments performed to identify the role of the pathway revealed that a Wnt/β-catenin gradient is required

for proper posterior neural differentiation but needs to be inhibited anteriorly for the anterior neural plate to form properly (Mulligan and Cheyette, 2012). Later in development, the Wnt/β-catenin pathway is also involved in neural tube regionalization, such as in the midbrain-hindbrain boundary (Brafman and Willert, 2017). Ultimately, the Wnt/β-catenin pathway controls multiple regions of the developing neural tube.

Wnt Pathways and Tissue Morphogenesis

During gastrulation, the mesoderm and overlying posterior neuroectoderm undergo convergent extension, elongating the axis of the embryo through Wnt pathway regulation (Kühl et al., 2001; Roszko et al., 2009; Wallingford and Harland, 2001). The Wnt/Ca^{2+} pathway is thought to inhibit the Wnt/β-catenin pathway during gastrulation for proper mesoderm convergent extension (Kühl et al., 2001). Classically, the Wnt/Ca^{2+} regulates downstream cellular behaviors (e.g. adhesion and convergent extension) through activation of secondary messengers such as Ca^{2+}/Calmodulin-dependant Protein Kinase II (CamKII) (Kühl et al., 2000; Zanardelli et al., 2013). The Wnt/PCP pathway, which regulates cell polarity and migration, is also required for proper convergent extension of the posterior neural ectoderm and mesoderm during gastrulation; it regulates proper apical constriction and mediolateral cell intercalation

(Darken et al., 2002; Miller and McCrea, 2010; Ossipova et al., 2015b; Wallingford and Harland, 2001).

The Wnt/Ca^{2+} and Wnt/PCP pathways are also required for proper convergent extension of the neural plate during neurulation (Wallingford and Harland, 2002; Zanardelli et al., 2013). Knockdown of Dishevelled, a Wnt pathway protein, resulted in wider neural plates than normal, and their inability to fuse (Wallingford and Harland, 2002). The Wnt/Ca^{2+} pathway and Dishevelled are required for proper Calpain2 - a Ca^{2+}-dependent protease - localization, which is required for proper convergent extension of the neural plate (Zanardelli et al., 2013). Similarly, actomyosin dynamics are extensively involved in both gastrula and neurula stage convergent extension, as well as in bending of the neural plate. While the neural plate thins and lengthens, Myosin-IIB drives midline intercalation through actin cytoskeleton reorganization and bending of the neural plate through apical actin constriction (Rolo et al., 2009). Apical constriction occurs when the apical surface of the cell constricts, causing the cell to take on a wedge-like shape (Wallingford John B., 2005). Xenopus studies support actomyosin dynamics as the driver of apical constriction during gastrulation and neurulation, although other modes have been proposed (Kinoshita et al., 2008; Ossipova et al., 2015a; Rolo et al., 2009; Wallingford John B., 2005). Furthermore, the Wnt/PCP and actomyosin pathways are

intertwined as Myosin-IIB and RhoA (an upstream activator of Myosin-IIB) are both activated downstream of the Wnt/PCP pathway (Kinoshita et al., 2008; Ossipova et al., 2015b). These movements ultimately lead to formation of the neural tube.

Ca^{2+}

In this section, I will first describe VGCCs before discussing Ca^{2+}'s role in neural development.

Voltage-Gated Ca^{2+} Channels

How can a single ion (Ca^{2+}) be involved in so many different processes during development? Researchers suspect that Ca^{2+} is able to perform multiple roles during development due to the ability of the cell to spatially localize Ca^{2+} based on Ca^{2+} channel distribution (Toth et al., 2016). Cytoplasmic Ca^{2+} buffers (Ca^{2+} binding proteins that localize Ca^{2+} and regulate Ca^{2+} signaling) and Ca^{2+} pumps help to compartmentalize Ca^{2+} into "Ca^{2+} microdomains" (Schwaller, 2010; Toth et al., 2016). VGCCs can be found by these microdomains. For example, L-Type VGCC derived Ca^{2+} microdomains are found within neurons (Parekh, 2008).

My research focuses on T-type VGCC's role in regulating intracellular Ca^{2+} during development. VGCCs fall into three subfamilies, Cav1, 2 and 3 (Catterall, 2000). Any one species will have numerous paralogs encoding VGCCs of each type (e.g., Cav1.1, Cav1.2, etc.). Cav1 encodes L-type VGCCs and L denotes "long-lasting", due to their large conductance (Dolphin, 2018). They also need greater depolarization to be activated in comparison to T-type VGCCs, and are found in both neuronal and muscle cells (Catterall, 2000). Cav2 encodes various neuron exclusive VGCCs (N-type, Q-type, P-type, and R-type) (Dolphin, 2018). T-type VGCCs, encoded by Cav3 genes, are "low voltage activated" and labeled T-type to their transient opening and closing (not to be confused with a Ca^{2+} transient, which is an umbrella term). They require lower depolarization events and are quicker to close than other voltage-gated Ca^{2+} channels. They are found in multiple tissue types such as muscle and neurons, and are involved in cardiac pacemaker activity (Catterall, 2000; Sousa et al., 2015).

All VCGGs have a similar structure: four repeated 6-transmembrane subunits with S5 and S6 forming the pore of the channel with a membrane-associated loop between them (Catterall, 2000; Zhang et al., 2013). T-type VGCCs are tightly regulated by inhibitors and enhancers, such as Calmodulin and PKA, to control the amount of Ca^{2+} within the cell (Lee et

al., 2017; Zhang et al., 2013). Proper regulation of VGCCs allows for fine-tuning Ca^{2+} entry into the cell (Chemin et al., 2017; Hering et al., 2018). Interestingly, the Wnt/Ca^{2+} and Wnt/PCP pathways contain many T-type VGCCs modulators and downstream targets, such as ROCK and CamKII (CamKII can both enhance and be activated by T-type VGCCs) (Asmara et al., 2017; Iftinca et al., 2007; Park et al., 2006; Sheldahl et al., 2003; Zhang et al., 2013). Furthermore, the Wnt/Ca^{2+} pathway, through Wnt5a, the same Wnt that is upstream of Calpain2, has been found to interact with VGCCs to regulate neuronal excitability and trafficking (Christodoulou and Skourides, 2015; McQuate et al., 2017; Zanardelli et al., 2013).

Ca^{2+}-dependent signaling pathways in chordate gastrulation and neurulation

Ca^{2+}'s role during neural induction and differentiation

Like Wnt, Ca^{2+} is also thought to play a key role in neural induction and increased Ca^{2+} concentration alone is able to induce neural fate (Leclerc et al., 2012). Ca^{2+} transients are observed throughout the anterior dorsal ectoderm prior to neural induction (Leclerc et al., 2000). Many studies have indicated that L-type VGCCs are involved in neural induction through the use of drug inhibition. Drug inhibition of L-type VGCCs from blastula through early neural stages led to loss of Ca^{2+} transients during

gastrulation and defects in the anterior nervous system (Leclerc et al., 2000). Furthermore, authors found that L-type VGCCs regulate the expression of early neural induction genes, *Zic3* and *geminin* (Leclerc et al., 2000). Moreover, the FGF pathway is thought to regulate neural induction through L-type VGCCs (Lee et al., 2009). In *Xenopus* animal caps, Noggin, which is expressed downstream of the Wnt/β-catenin pathway, has been shown to activate L-type VGCCs, leading to the expression of transcription factor *c-fos* and its implication during neural development (Ding et al., 2017; Leclerc et al., 1999). Ca^{2+} is also believed to play a role in neural differentiation. For example, Ca^{2+} transients are seen in the midbrain-hindbrain region in zebrafish, similar to those we observed in Ciona embryos (Abdul-Wajid et al., 2015; Créton et al., 1998; Webb and Miller, 2003). Through drug studies, T-type VGCCs have been implicated for proper gene expression during spinal neuron differentiation (Webb and Miller, 2003). This previous work stresses the importance and indispensability of Ca^{2+} during neural development.

As discussed above, L-type VGCC inhibitors have been found to inhibit neural induction; this does not negate a role for T-type VGCCs, potentially as triggers opening of L-type Ca^{2+} channels, as seen in pacemaker cells (Su, 2014). T-type VGCCs activate early during small depolarizations of the cell, bringing in Ca^{2+} to increase depolarization and

leading to the activation of L-type VGCCs (Su, 2014). The modulation of T-type VGCCs can determine the cells' sensitivity. Furthermore, some of these studies have used nifedipine, a L-type VGCC inhibitor, that also appears to target T-type Ca^{2+} channels, complicating analysis of the results (Arnoult et al., 1998; Leclerc et al., 2003; Lee et al., 2009; Shcheglovitov et al., 2005; Suzuki et al., 2017). Thus, I hypothesize that T-type VGCCs functions as a Ca^{2+} tuning mechanism, allowing for the minute control and regulation of various sensitive processes during neural development.

Ca^{2+}'s role during morphogenesis

Xenopus and zebrafish gastrulation movements are thought to require Ca^{2+}, as Ca^{2+} waves correlate to convergent extension in both models, although Ca^{2+} appears to operate independently of the non-canonical Wnt pathways (Markova and Lenne, 2012; Tada and Concha, 2001; Wallingford et al., 2001). However, as discussed in the *Wnt Pathways and Tissue Morphogenesis* section, the Wnt/PCP and Wnt/Ca^{2+} pathways have been shown to be required for proper convergent extension during gastrulation and authors speculate these Ca^{2+} waves are involved in a parallel pathway to control convergent extension (Wallingford et al., 2001; Wallingford and Harland, 2001; Zanardelli et al., 2013).

Ca^{2+} transients are seen as both waves and cell autonomous Ca^{2+} transients across the neural plate during neural tube closure and have been shown to control apical actin constriction (Suzuki et al., 2017). Suzuki et al. 2017 inhibited VGCCs with nifedipine and saw that cell autonomous Ca^{2+} transients were inhibited. However, the authors admitted that although cell autonomous Ca^{2+} transients in the neural plate were inhibited, they couldn't definitively say whether VGCCs were involved in multicellular wave transients, and that VGCCs "*preferentially"* regulate cell autonomous Ca^{2+} transients (Suzuki et al., 2017). It is important to note that the authors of this study never specified which subgroup of VGCCs were being inhibited by nifedipine, although it is implied that they are L-type. However, in this study, nifedipine was used at a high enough concentration to also target T-type VGCCs (Shcheglovitov et al., 2005; Suzuki et al., 2017). Furthermore, morpholino knockdown of Cav3.2 in *Xenopus*, one of the three T-type VGCCs in vertebrates, was shown to inhibit Ca^{2+} transients, which appear to be Ca^{2+} waves, in the developing neural plate (Abdul-Wajid et al., 2015). In a related study, Christodoulou et al. 2015 found that Calpain2 is a regulator of the actin-driven apical constriction induced by cell autonomous Ca^{2+} transients in the neural plate (Christodoulou and Skourides, 2015). Distinguishing which VGCCs are performing what functions during development must be resolved within the field.

Specific Goals

My aims were to identify the underlying role of Cav3 in *Ciona* and Cav3.3 in *Xenopus laevis* during neural development. In *Ciona,* our lab has previously identified a *Ciona savignyi* mutant, *bugeye*, in which Cav3, a T-type VGCC, has drastically decreased expression due to an 81 basepair insertion mutation in its cis-regulatory region (Abdul-Wajid et al., 2014). The loss of Cav3 leads to failure of the neural tube to remain closed and the brain develops outside of the head of the embryo, phenocopying a human developmental defect, exencephaly. It was found that loss of Cav3 causes an increase of EphrinA-d, a repulsion protein, and that overexpression of EphrinA-d is sufficient to cause the open brain phenotype in wild-type embryos (Abdul-Wajid et al., 2015). Furthermore, in *Ciona robusta*, a sister species to *Ciona savignyi*, we found a new exencephaly mutant with tight linkage to Cav3. Unlike Cav3 in *Ciona savignyi bugeye*, we found that *Cav3* was not misexpressed in the *Ciona robusta* mutant; however, there were multiple amino acid changes found in known functional regions of the channel and we suspect that these cause Cav3 to be functionally null. This work has gone on to a larger RNAseq study to identify other genes, such as *Flrt*, that are misexpressed due to loss of Cav3 during neural development (manuscript in review).

In *Xenopus*, Cav3.3 has never been detected via *in situ* hybridization or implicated for function during early neural development, potentially due to its extremely low level of expression (Lewis et al., 2009; Lory et al., 2006; Zhang et al., 2013). As discussed previously, Ca^{2+} is a powerful signaling messenger, with the ability to modulate gene expression, contraction, and other cellular processes (Bootman, 2012). Cav3.3 may play a part in multiple pathways necessary for proper neural initiation, differentiation, and morphogenetic movements, such as convergent extension, during gastrulation and neural tube closure. As such, we see, similar to what is seen in *Ciona bugeye*, that Cav3.3 regulates neural tube closure. However, we also have evidence that Cav3s play a larger role in vertebrates during gastrulation. In both stages of development, we find that Cav3.3 may be involved in neural fate determination and morphogenesis.

II. Ciona savignyi bugeye EphrinA-d Overexpression and Ciona robusta Cav3 mutant characterization

A. Introduction

During neurulation the elongation, folding, and fusion of the neural plate forms the neural tube. Successful completion of this central aspect of chordate morphogenesis involves a multitude of coordinated processes, such as the control and regulation of the actomyosin and PCP pathways during folding and convergent extension (Nikolopoulou et al., 2017). Disruption of any of these processes can lead to failure of neural tube closure (NTC) (Nikolopoulou et al., 2017; Wallingford et al., 2013). While a majority of neural tube closure defects in humans are due to folate deficiency and avoided with folate supplementation during pregnancy, approximately 30% are potentially due to genetic mutations (Wallingford et al., 2013).

Ciona, an invertebrate chordate, has a relatively simple nervous system with about 177 neurons (Ryan et al., 2016). As in vertebrates, the *Ciona* neural plate undergoes differentiation, folding, and fusion of the neural tube. *Ciona's* neural plate undergoes neurulation, first zippering up the spinal cord, but then switching to global purse-string closure in the brain (Hashimoto et al., 2015). Additionally, *Ciona* is a hermaphrodite and

particularly amenable to forward genetic screens of mutants, revealing recessive mutant alleles through self-fertilization and subsequent observation of offspring (Veeman et al., 2011). Tunicates diverged before the whole genome duplication events which characterize the vertebrate lineage, so while a typical vertebrate may have three *Cav3* paralogs (*3.1*, *3.2*, and *3.3*), *Ciona* has a single *Cav3* gene. Consequently, a mutation or knockout of the *Ciona* Cav3 is not likely to be rescued by redundant functions of the other paralogs, as in vertebrates, simplifying analysis of gene pathways. Our lab discovered a *Ciona savignyi (C. savignyi)* excencephaly mutant using this method and named the mutant *bugeye* (Abdul-Wajid et al., 2014). The mutation was mapped to the promoter region of a T-type Ca^{2+} Channel (T-type VGCC), Cav3, which caused a significant decrease in *Cav3* expression during development.

CRISPR knockdown of *Cav3* also phenocopied *bugeye* in wild-type (WT) embryos, indicating directly that the loss of *Cav3* expression during neurulation leads to anterior NTC failure. *Cav3 in situ* analysis showed low expression along the neural tube and strong staining in the midbrain-hindbrain region, identified due to expression overlapping with that of engrailed. Morpholino oligonucleotide (MO) knockdown of Cav3.2 caused NTC delay, unfused neural folds beneath the epidermis which caused

malformed, and reduced Ca^{2+} transients along the neural plate during neural tube closure (Abdul-Wajid et al., 2015).

In *bugeye*, the folds of the neural plate appear to converge medially and come together properly, but fail to fuse along the anterior dorsal midline (Abdul-Wajid et al., 2015). It was found that neither neural differentiation, based upon proper opsin and arrestin staining, nor the actin cytoskeleton, based upon phalloidin staining, were disrupted in the brain. However, EphrinA-d, a repulsion/adhesion protein, was found to have higher levels of transcript in *bugeye* embryos via qRT-PCR. Overexpression of *EphrinA-d* in WT embryos caused an open brain phenotype, similar to the *bugeye* mutant. Moreover, expression of a dominant negative form of Eph3, an Ephrin receptor, rescued the open brain phenotype in *bugeye* embryos. It was also found that *EphA2* was overexpressed in Cav3.2 knockdown embryos, implicating the cause of the unfused neural tube beneath the closed epidermis. Overall, we showed that Cav3 is necessary for proper neural tube closure, and that its misexpression resulted in overexpression of EphrinA-d.

In this chapter, I describe my work on EphrinA-d and a newly discovered *Ciona robusta (C. robusta)* recessive mutant with an open brain phenotype similar to that of the *bugeye* mutant in *C. savignyi.* This mutant

was found during a forward genetic screen of wild caught *Ciona* and selfing them to find pre-existing mutations. We suspected *Cav3* was the mutated gene due to the similarity to the *C. savignyi bugeye* phenotype. Linkage analysis, complementation failure between *C. savignyi bugeye* and *C. robusta* mutants, and multiple mutations found throughout *Cav3* in the *C. robusta* mutant lead us to believe that the mutation causes a nonfunctional Cav3 to be expressed. This mutant was subsequently used in RNAseq studies in the lab (manuscript in revision).

B. Materials and Methods

EphrinA-d overexpression

EphrinA-d plasmid construction and electroporation methods were described previously (Abdul-Wajid et al., 2015). Wild-type embryos were electroporated at the 1-cell stage with either 50 µg of ETR>EphrinA-d and 15 µg of ETR>H2B:GFP, or 65 ug of ETR>H2B:GFP as a control.

Cross-species complementation test

C. robusta bugeye eggs were mixed with either *C. robusta bugeye* sperm or *C. savignyi bugeye* sperm. Embryos were first dechorionated (Veeman et al., 2011; Zeller, 2018) to allow for cross-species fertilization and then analyzed for the *bugeye* phenotype at late tailbud stage. The presence of embryos with the *bugeye* phenotype at the expected frequency for a recessive mutation (25%) would indicate that the two mutations are in the same complementation group.

Linkage analysis

Two heterozygous *C. robusta* adults were crossed and the resulting progeny were analyzed post-hatching. The progeny were then sorted based on the presence of the *bugeye* phenotype. Progeny with closed (normal) neural tube could be either wt/wt or *bugeye*/wt, while the open brain progeny are *bugeye/bugeye*. Genomic DNA was collected from separate pools of 20 closed and open neural tube larvae, as previously described

(Veeman et al., 2011). Primers targeting flanking exons of intron 16 were used to amplify the region for SNP detection (primers listed in Table 1). Amplicons were sent for sequencing to UC Berkeley DNA Sequencing Facility.

Cav3 Expression in C. robusta bugeye

For RT-PCR analysis of Cav3 expression, *C. robusta bugeye* closed and open brain larvae were selected as described in the previous section. *C. robusta bugeye* larval RNA was extracted and cDNA generated for RT-PCR analysis of *Cav3* expression, as previously described (Abdul-Wajid et al., 2015). *RPS27A* was used as a loading control. Primers are listed in Table 1.

cDNA Sequencing

For cDNA analysis, *C. robusta bugeye* larval RNA was extracted from progeny exhibiting the open brain phenotype and cDNA generated, as previously described (Abdul-Wajid et al., 2015). cDNA cloning primers are listed in Table 1. For cDNA sequencing, *Cav3* cDNA amplicons were TA-cloned into pCR8 vector using pCR8/GW/TOPO TA Cloning Kit (Invitrogen). *Cav3* cDNA was sequenced using M13 forward primer, T7 forward primer, or internal primers listed in Table 1. Sequencing was performed by UC Berkeley DNA Sequencing Facility. *C. robusta Cav3* WT transmembrane

structure was manually plotted and visualized by Protter (http://wlab.ethz.ch/protter/) (Omasits et al., 2014).

C. Results

EphrinA-d overexpression causes open brain phenotype in wild-type embryos

Based on qRT-PCR data, *EphrinA-d* was overexpressed in *C. savignyi bugeye (bug)* embryos (Abdul-Wajid et al., 2015). Considering EphrinA-d is a repulsion protein and the neural folds come together along the dorsal midline before reopening in *C. savignyi bug*, EphrinA-d is an attractive candidate for Cav3 downstream regulation of neural tube closure. To identify if EphrinA-d overexpression in wild-type (WT) embryos could cause an open brain (OB) phenotype alone, WT embryos were electroporated at the 1-cell stage with an ETR promoter driving an *EphrinA-d* cDNA construct. Compared to controls electroporated with ETR>H2B:GFP, we saw a significant increase in embryos with the OB phenotype (15.2% versus 4.8% in controls, Fisher exact test p=0.0006) (refer to Figure 6D in Abdul-Wajid et. al 2015). As loss of Cav3 leads to overexpression of EphrinA-d in mutant embryos, and forced Ephrin overexpression in WT embryos is sufficient to cause the OB phenotype, we concluded that Cav3 is upstream of *EphrinA-d* expression.

A Ciona robusta Cav3 mutant

During a screen for spontaneous developmental mutants in *Ciona*

robusta (C. robusta), we identified a mutant line exhibiting a recessive open brain (OB) phenotype (provisionally named *C. robusta ob*), similar to *C. savignyi bug* (Abdul-Wajid et al., 2015). We performed a complementation test between *C. savignyi bug* and *C. robusta ob*, using a method described previously for *Ciona* pigmentation mutants (Salas et al., 2018). To characterize this mutant, we crossed heterozygotic *C. robusta ob* eggs with heterozygotic *C. savignyi bug* sperm. As controls, we crossed *C. robusta ob* eggs and sperm as well as *C. robusta* WT eggs and WT sperm. For crosses using gametes from the *ob* and *bug* adults, the OB phenotype was observed at the approximate percentages expected for non-complementing recessive mutations [31% for *C. robusta ob* x *C. savignyi bug* (n = 55), and 26% for *C. robusta ob* x C. robusta *ob* (n =53)], while the occurrence of OB was very low in the negative controls (Fig. 1A). Representative images of the OB phenotypes from both crosses are shown in Fig. 1B. To further investigate *C. robusta ob*, we tested for linkage of the OB phenotype to the *Cav3* locus using intronic allelic-specific single nucleotide polymorphisms (SNPs), as described previously (Veeman et al., 2011). Two *C. robusta* adults heterozygous for the OB phenotype were crossed, and genomic DNA was collected from pools of 20 OB larvae and 20 closed brain (CB) larvae. PCR was used to amplify the 16th intron of *Cav3* from the two pools and sequenced. Fig. 1C shows a segment of the intron sequence containing a SNP observed in the CB pool, which consists

of both homozygous WT as well as heterozygous progeny (arrow). However, in the genomic DNA pool from the OB progeny only one allele is present (arrow), consistent with this allele of *Cav3* being linked to the causative mutation. Taken together, the similarity of phenotype to *C. savignyi bug*, and the complementation and linkage results, lead us to conclude that the *C. robusta ob* is in the same gene as *C. savignyi bug*, and will be called from here on *C. robusta bugeye* (bug).

In *C. savignyi bug*, *Cav3* has an 81 bp insertion in the cis-regulatory region that was hypothesized to result in the observed reduced expression of *Cav3* (Abdul-Wajid et al., 2014). By contrast, we did not observe a decrease in *Cav3* mRNA levels in *C. robusta bug* embryos by RT-PCR (Fig.1D). We sequenced *Cav3* cDNA from *C. robusta bug* and found six amino acid changes throughout the sequence in comparison to the published sequence (XP_018667819.1) (Fig. 1E). To characterize these amino acid substitutions, the predicted amino acid sequence for WT Cav3 was mapped to the known transmembrane topology of *Homo sapiens* Cav3.2 (Fig. 1E). We found multiple amino acid substitutions throughout *C. robusta bug* Cav3. Notably, we found multiple amino acid changes in the I-II loop and C-terminus, both regions known for having important Cav3 functional domains. Due to the number of mutations in known functional

regions of Cav3, we suspect that they likely lead to improper function of Cav3.

D. Discussion

Our lab has previously shown T-type Ca^{2+} voltage-gated channel (T-type VGCC) involvement during neural tube closure (NTC) in the basal chordate, *Ciona savignyi (C. savignyi)* (Abdul-Wajid et al., 2015). In Abdul-Wajid et al. 2015, we showed that loss of *Cav3* expression causes overexpression of *EphrinA-d,* a repulsion protein. Ephrins are known to be involved during vertebrate NTC and are used during development for proper tissue organization (Abdul-Aziz et al., 2009; Fagotto et al., 2014; Holmberg et al., 2000). Here, we extend the importance of Ephrins in chordate neurulation by showing that overexpression of EphrinA-d in wild-type (WT) *Ciona* embryos causes the open-brain phenotype, indicating a role for Cav3 through regulation of *EphrinA-d* expression. Ephrins interact with opposing EphA receptors and signal for cellular repulsion based upon their interactions. In *C. savignyi bugeye* (*bug*) embryos, we suspected that overexpression of EphrinA-d causes improper separation of adjoining neural folds and leads to exencephaly. The finding that overexpression of EphrinA-d in WT embryos results in a similar phenotype, supports the involvement of EphrinA during neural tube closure and indicates Cav3's role in the regulation gene expression.

A newly discovered exencephaly mutant in *Ciona robusta* was found to exhibit the same phenotype as *C. savignyi bug*. We suspected Cav3

involvement after complementation failed when we crossed *C. savignyi bug* sperm and *C. robusta bug eggs*. *Ciona* is highly polymorphic, with an intronic single nucleotide polymorphism (SNP) found every 200 base pairs on average (Veeman et al., 2011). Linkage between phenotype and SNP occurrence can be used as a tool to help identify the genetic locus responsible for mutant phenotype, although it does not confirm the gene directly, but identifies the region in which the mutation is localized. Based upon the intronic SNP in exon 16, *Cav3* had clear linkage to the *bug* open brain phenotype. However, unlike *C. savignyi bugeye* which shows lower expression of *Cav3* transcription than in *C. savignyi* wild-type (WT) embryos, *Cav3* is expressed at similar levels to WT siblings. This indicates that the mutation may not cause a decrease in mRNA expression but potentially disrupts or alters Cav3 protein function. Accordingly, cDNA analysis of *C. robusta bug* indicates multiple amino acid changes within Cav3. Notably, a majority of the amino acid changes were found in the I-II loop, which is important for proper trafficking and channel gating (Vitko et al., 2007; Zhang et al., 2013). There is also a non-conserved amino acid substitution (T-I) in the C-terminal tail, a region which is important for Cav3 regulation and interactions with other proteins for downstream events (Anderson et al., 2010; Asmara et al., 2017; Jurkovicova-Tarabova et al., 2019; Lee et al., 2017; Smith et al., 2017; Weiss et al., 2012).

Since we have previously shown that loss of Cav3 function leads to EphrinA-d overexpression, we assumed multiple genes were affected downstream of Cav3. We suspect Cav3 may signal the completion of neural tube closure. *C. savignyi bug* and *C. robusta bug* mutants were collected alongside WT siblings and RNAseq analysis was performed to determine differential gene expression between the two samples. From the RNAseq analysis we found multiple differentially expressed (DE) genes, both over- and under-expressed in the *bug* mutants. Several of these have been characterized by both gain and loss of function analysis. We have also found that a number of these DE genes are expressed during neural tube closure, supporting their involvement in neurulation. For example, *Flrt*, a cell adhesion protein, was found to be overexpressed in *bug* embryos. Forced overexpression of *Flrt* in WT embryos causes the open brain phenotype, similar to that observed in *bug* mutants. Furthermore, *in situ* hybridization analysis of *Flrt* identifies its expression in the neural plate during neurulation (manuscript in preparation). This work will go on further to find more novel genes involved during neural tube closure and to characterize their function in development. Furthermore, future experiments could investigate the potential importance of the amino acid substitutions we see in the *C. robusta bug* by a directed mutation approach.

E. Figures and Figure Legends

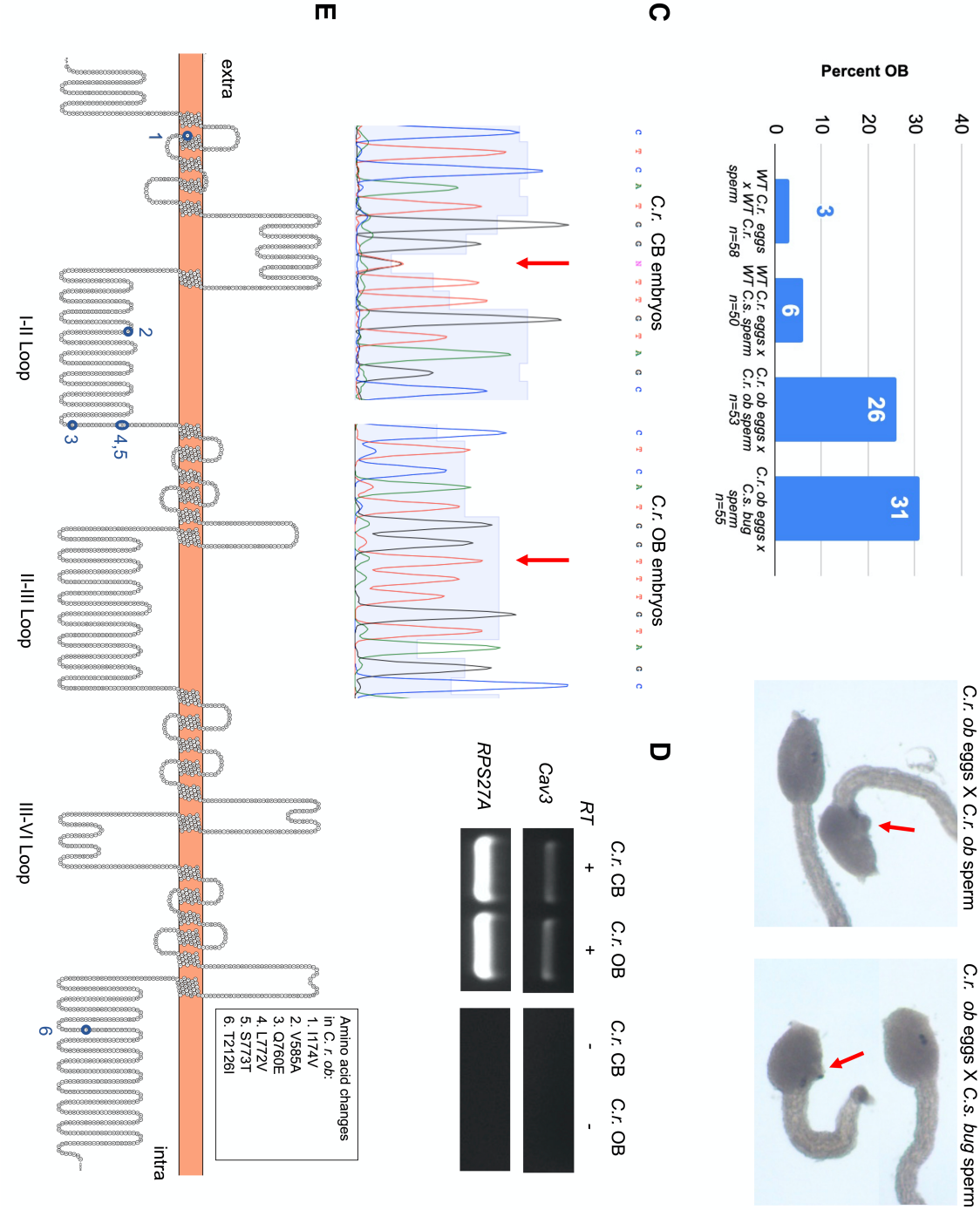


Figure 1. *Ciona robusta* open brain mutant (ob). **A.** Complementation test between *C. robusta* ob and *C. savignyi bug* mutants. Open brain (OB) phenotypes were observed from crosses of *C. robusta ob* eggs to C. robusta ob sperm, and from crosses of *C. robusta ob* eggs to *C. savignyi bug* sperm at frequencies of 26% and 31%, respectively. **B.** Representative examples of OB (red arrow) and closed brain (CB) phenotypes. **C.** Linkage analysis of *C. robusta ob* to the *Cav3* locus using progeny of crossed *C. robusta ob* heterozygous adults. Red arrows indicate a single nucleotide polymorphism that is present in CB larvae, but that resolves to a single nucleotide in OB siblings. **D.** Expression of *Cav3* by RT-PCR in CB and OB *C. robusta* ob larvae. **E.** Structure prediction of C. robusta Cav3. Amino acid changes found in the ob allele are indicated in the sequence. Multiple amino acid substitutions were observed in the I-II loop. Structure manually plotted and image generated using Protter (Omasits et al., 2014).

Table 1: *Ciona robusta* primers

NAME	SEQUENCE	GENE MODEL ID
Linkage analysis primers		
Cav3 F	TTGTCCAAAATCGTGGCATA	KH.C6.65
Cav3 R	TAAAAGTCGCCACGTTGTCC	
Cav3 cDNA primers		
Section 1F	GCGAACCCATATCCTTTTCA	
Section 1R	TGCAATTACATCTGCTTTTGTTT	
Section 2F	CAGAACAAGAAATTGAAATCAAAA	
Section 2R	CTCACGAATTGTGTCCTTCA	
Section 3F	CCGGTCACTGCTGGAAAC	
Section 3R	GCTAACCAAATGATAGGCCGTAA	
Internal Sequencing primer F1	AGCGACTCAGTTCTCAGAAACA	
Internal Sequencing primer F2	ATACACTAAGTATGGGCATTGAACA	
Internal Sequencing primer F3	TACAATGTTTTCGACGCACTT	
Internal Sequencing primer R1	CTCGTTTGATCTCGACTGATTC	
RT-PCR primers		
Cav3 RT	AGGACAAGGTGCAACAAACTG	KH.C6.65
	TGTGTGCCTGCTGCTATTTC	
RPS27A RT	CACCCTTGAGGTTGAAGCAT	KH.C10.239
	GGTTCTCAGGTTTGAACACG	

III. Cav3.3 is required for proper vertebrate neural development

A. Introduction

As was discussed in the Chapter I: Introduction, the role of Ca^{2+} during neurulation is vast as this single ion acts in a multitude of different processes, potentially through the use of Ca^{2+} microdomains (Parekh, 2008; Toth et al., 2016). In the *Ciona bugeye* mutants and Cav3.2 morpholino oligonucleotide (MO)-knockdown embryos, it appears that Cav3 is necessary for proper gene expression in neural tube fusion (Abdul-Wajid et al., 2015). However, I have found that Cav3.2 plays a larger role during neural tube closure (NTC) apart from neural fold fusion during vertebrate development. Furthermore, I investigated another T-type voltage-gated Ca^{2+} channel (VGCC), Cav3.3, during neural development and found a multitude of developmental defects as discussed below. First, there are some specifics to the *Xenopus* project I will go over more in depth in this introduction.

Xenopus laevis is allotetraploid

To address the two knockdown genes I primarily focus on in my *Xenopus* project, *Cav3.3L* and *Cav3.3S*, I need to explain the allotetraploidy of *Xenopus laevis* (Session et al., 2016). It was produced from the combination of two diploid *Xenopus* species. *Xenopus laevis*

contains two subgenomes (L and S for long and short) with more than 56% of all genes retained in both genomes. L and S chromosomes are homoeologues, orthologous chromosomes that were retained during a polyploidization event. Genes in homoeologues can be called either homoeologous genes or alloalleles. *Cav3.3L* and *Cav3.3S* are alloalleles. Interestingly, the S subgenome has lost significantly more genes (31.5%) than the L subgenome (8.3%). Furthermore, some of these genes have lost expression even when present within the genome. Thousands of genes that were expressed were found to have either different expression levels or different spatiotemporal patterns compared to their partner alloallele. Typically, L alloalleles were found to be higher expressed than S alloalleles. My gene of interest, *Cav3.3*, is found on both subgenomes, and I have investigated the role of each during neural development (Karimi et al., 2018).

A little more info on neural developmental pathways

As a refresher, there are multiple pathways that are required for proper neural induction, differentiation, convergent extension, and apical actin constriction. The Wnt/β-catenin pathway plays a central role in proper neural induction through downstream secreted factors like Noggin, and also plays a role in neural differentiation of the neural plate and neural tube

(Brafman and Willert, 2017; Ding et al., 2017; Mulligan and Cheyette, 2012). The Wnt/Ca^{2+} and Wnt/PCP pathways are involved in proper convergent extension during gastrulation and NTC; the Wnt/PCP pathway is also implicated for proper apical constriction during gastrulation and neurulation (Kinoshita et al., 2008; Kühl et al., 2001; Ossipova et al., 2015a; Roszko et al., 2009; Wallingford and Harland, 2002; Zanardelli et al., 2013).

Diving a little more into the mechanics behind neural development, there appears to be tight control of morphogenetic processes. First, Wnt/Ca^{2+} inhibits Wnt/β-catenin at multiple points in its pathway to regulate gene expression for proper convergent extension during gastrulation (Kühl et al., 2001). In mice, apical actin constriction is dispensable more posteriorly, along the spinal cord (Rolo et al., 2009). However, unrestricted actin accumulation causes defects along the entire length of the neural tube and requires the RhoA/Rock pathway for proper actin turnover (Escuin et al., 2015). This is thought to occur because with too little actin, the floppy neural folds can still come together but with too much actin accumulation, the folds are too stiff to come together and fuse (Rolo et al., 2018). In *Xenopus*, it was shown that RhoA accumulates along the apical surface of the neural plate downstream of Wnt/PCP activation and inhibition of the Wnt/PCP pathway causes decrease of RhoA along the

apical surface and increased surface area of the neural plate cells (Kinoshita et al., 2008). It was speculated that this was due to the loss of apical actomyosin constriction. The proper balance of neural tube closure processes, including convergent extension, appears to be a common theme for Wnt pathway components. For example, inhibition and overactivation of Calpain2 causes inhibition of convergent extension (Zanardelli et al., 2013).

Neural Crest Cells

There is another player involved with proper neural development, the neural crest. Neural crest cells are derived from the neural plate border, which is situated between the neural and non-neural ectoderm (Klymkowsky et al., 2010). In *Xenopus,* they begin to migrate out of the neural plate border toward their target areas late in NTC through mid-tailbud (Barriga and Theveneau, 2020; Sadaghiani and Thiébaud, 1987; Theveneau and Mayor, 2012). Neural crest cells go on to differentiate into various tissue types such as peripheral neurons, cartilage and bone for facial structures, pigment cells like melanocytes, and, in *Xenopus*, fin development (Milet and Monsoro-Burq, 2012; Szabó and Mayor, 2018; Tucker and Slack, 2004). Loss of neural crest cells can lead to craniofacial defects, the primary cause of infant mortality (Trainor, 2010).

My Findings

With the above information in mind, this leads into the story of Cav3.3's role during neural development and potential pathways that it regulates through Ca^{2+}. Our lab has identified multiple functions of Cav3.3 during gastrulation and neurulation in *Xenopus laevis*. We have found that knockdown of Cav3.3 causes delays in gastrulation and NTC, misregulation of actomyosin related proteins, craniofacial defects, a dorsal flexure phenotype, and abnormal fin and melanocyte development. Our data shows that Cav3.3, as a source of Ca^{2+} influx, is able to regulate multiple processes during development. Furthermore, we have found that the previously reported Cav3.2 MO-knockdown embryos display an unreported phenotype, the dorsal flexure phenotype.

B: Materials and Methods

Animals

Xenopus laevis females were induced for ovulation using human chorionic gonadotropin (Sigma-Aldrich or MERCK) injected subcutaneously as described (Sive et al., 2000). Eggs and sperm were collected for *in vitro* fertilization, also as described (Sive et al., 2000). Embryos were staged according to Neiuwkoop and Faber (Nieuwkoop and Faber, 1994).

Morpholino oligonucleotide (MO) microinjections

Xenopus embryos were injected at the 1-, 4-, or 8-cell stage, depending on experimental requirements. Targeted injections were performed to minimize the effects of Cav3.3 knockdown in other tissues. MO (GeneTools) were injected from 6.25-80ng depending on experimental requirements. Sequences for *Xenopus laevis* Cav3.3L and Cav3.3S were obtained from Xenbase genome version 9.1 (http://www.xenbase.org/, RRID:SCR_003280). Cav3.2 MO 1 and MO 2 sequences were used previously (Abdul-Wajid et al., 2015). Cav3.3 morpholinos were designed by Gene Tools (gene-tools.com). Two types of controls were used, a Gene Tools standard control MO (Gene Tools, CTL-MO), as well as injections with experimental MOs into non-target blastomeres on the ventral side of the animal pole. Sequences for all MOs are listed in Table 1. Embryos were

injected with either fluorescein tagged MOs or tracer dyes to ensure proper blastomeres were injected (Fast Green (Sigma) 1.25-2.5 ng total, Texas Red Dextran 10,000 MW (Molecular Probes) 5ng total, Dextran Alexa Fluor 488 10,000 MW (Invitrogen) 10-20ngtotal, Dextran Alexa Fluor 568 10,000 MW (Invitrogen) 1-2ng total). Embryo development was recorded by time lapse microscopy from early neurula (~stage 12.5) to tailbud stages. The number of embryos with a delay in neural tube closure was counted when a majority of control embryos neural folds were joined along the midline. Dorsal flexure phenotype was determined by comparing embryos to the angle of a slightly curled control. Embryos were either fixed in 4% PFA in 1X MEM salts or lysed in Trizole (Invitrogen) for mRNA extraction for expression analysis. Knockdown of the MO targets was confirmed by RT-PCR, and the primers sequences are listed in Table 1. P values for all statistical tests (Fisher exact test and Wilcoxon rank-sum tests) were determined with one-tailed hypotheses.

Plasmid construction and mRNA synthesis

Calpain2L cDNA was generated from pCMV-SPORT6 *Xenopus laevis* Calpain2.2L (Dhermacon, CloneId: 3399721) without the stop codon. The amplicon was cloned into pDONR-211, and recombined into plasmid pSPE3-Rfa-Venus (Roure et al., 2007). pSPE3-Calpain2L-Venus was linearized

with S*fiI* and purified with QIAquick PCR Purification kit (Qiagen). T3 mMessage mMachine (Invitrogen) was used to *in vitro* synthesize Calpn2L-Venus mRNA, which was then resuspended in molecular-grade water for injections.

Immunohistochemistry

For Calpain2L studies, embryos received two injections. First, the embryos were injected at the 1-cell stage with 1 ng Calpn2L-Venus mRNA; second, at the 8-cell stage they were injected in 1 dorsal blastomere with 10ng of Cav3.3S MO. Embryos were grown until mid-neurula (stage 14/15) and were bisected with a blade, preserving the neural plate region. Bisected embryos were fixed in 3.7% PFA solution in 1/3X Modified Frog Ringer's solution (MR). *Xenopus* whole-mount immunostaining was performed as described previously, using 1:500 Mouse anti-GFP and 1:500 Alexa Fluor 488 anti-mouse (Invitrogen) before final washes and clearing (Lee et al., 2008). For all fluorescent microscopy experiments, embryos were imaged on an inverted Olympus Fluoview 1000 Spectral Confocal microscope and analyzed with Imaris and ImageJ. Images are Z-stack projections.

To visualize actin localization, embryos were injected at the 8-cell stage in a single dorsal blastomere with 10ng of Cav3.3L. At mid-neural

(stage 16/17) embryos were cut transversely with a scalpel, fixed in 3.7% PFA solution in 1/3X MR and stained with 5U/mL Rhodamine Phalloidin (Invitrogen) overnight at 4^{O}C before final washes and clearing. Embryos were dehydrated in an isopropyl alcohol series before clearing in Murray's clear solution (2 parts benzyl benzoate: 1 part benzyl alcohol).

Drug treatments

ML218 (Sigma-Aldrich) was reconstituted in DMSO and stored at 4^{O}C. Equivalent amount of DMSO was used for control studies. Drugs were diluted into working concentrations (300-350 μM) in 1/3X MR and added to embryos at late gastrula/early neurula stages before taking a timelapse of development through neurulation. The number of embryos with a delay in neural tube closure was counted when a majority of control embryos neural folds were joined along the midline.

C. Results

Knockdown of Cav3.2 causes dorsal flexure phenotype in Xenopus embryos

Previously, our lab described a Cav3.2 morpholino oligonucleotide (MO) knockdown which caused a delay in neural tube closure (NTC), loss of fusion of the neural plate despite proper fusion of the overlying ectoderm, and results in malformed heads (Abdul-Wajid et al., 2015). Our lab also showed that EphA, a receptor for EphrinA repulsion/adhesion proteins, is overexpressed in Cav3.2 knockdown embryos. I have expanded on these findings as well as recapitulating that Cav3.2 MO-injected embryos have a delay in NTC (93.3%), compared to 7.1% in the control embryos (Fig. 1A,C). Most of my work will be on a related T-type Ca^{2+} voltage-gated channel (T-type VGCC, Cav3.3; see below), but I also wanted to take a closer look at the Cav3.2 knockdown phenotype. The phenotype appears to be more extensive than originally thought, as Cav3.2 MO-injected embryos displayed a dorsal flexure phenotype, a phenotype that has not been reported previously from Cav3.2 knockdown embryos (Fig. 1B). Dorsal flexure of *Xenopus* embryos occurs when the overlying dorsal tissue fails to properly undergo convergent extension while the underlying ventral tissue extends normally, causing the rostral region of the embryo to curl backward (Rolo et al., 2009; Wallingford and Harland, 2001). Since Cav3.2 causes a delay in NTC, it was not surprising to find

that Cav3.2 has additional roles outside of neural fold fusion. This dorsal flexure phenotype is similar to that observed from knockdown of Myosin-IIB and Calpain2 (Rolo et al., 2009; Zanardelli et al., 2013), and indicates that Cav3.2 is necessary for proper convergent extension of the neural plate.

Cav3.3 knockdown causes disruption to vertebrate gastrulation

Convergent extension during gastrulation requires the PCP pathway and actomyosin dynamics for proper movement (Skoglund et al., 2008; Wallingford et al., 2000). Additionally, it was found that a knockdown of Calpain2 caused delays during gastrulation, potentially due to loss of convergent extension (Zanardelli et al., 2013). To examine the effect of loss of Cav3.3 on gastrulation, I injected 1-cell embryos with control MO, Cav3.3S MO, Cav3.3L MO, or both MOs in combination. All Cav3.3 MOs caused significant delays in gastrulation, varying from moderate to severe compared to control MO-injected embryos (Fig. 2). Furthermore, in embryos injected at 4/8-cell stage with a combination of the two MOs (Cav3.3S+L) targeted to the neural precursors (i.e., away from the blastopore), the defect was catastrophic, with the cavity of the embryo exposed down the midline (Fig. 3H,I). My findings show that Cav3.3 is an important component for proper gastrulation, potentially due to loss of

convergent extension of the posterior neuroectoderm and/or mesoderm through the Wnt/PCP, Wnt/Ca^{2+}, or actomyosin pathway (Habas et al., 2001; Skoglund et al., 2008; Zanardelli et al., 2013).

Cav3.3 knockdown causes disruption to vertebrate neural development

Previous studies have supported a role for T-type VGCCs in vertebrate neural development. Ca^{2+} transients that have been observed both during gastrulation and in the developing neural plate, most likely through L-type VGCCs, and which are thought to be necessary for proper neural induction, differentiation, and neural plate apical constriction (Leclerc et al., 2006; Suzuki et al., 2017; Toth et al., 2016; Webb and Miller, 2003). Calpain2, a Ca^{2+} -dependent protease, was shown to be necessary for proper NTC, acting downstream of the Wnt/Ca^{2+} pathway and potentially interacting with the Wnt/PCP pathway for proper convergent extension (Zanardelli et al., 2013). Calpain2 has also been shown to stabilize actin-driven apical constriction induced by cell-autonomous Ca^{2+} transients within the neural plate (the same transients that are dependent on nifedipine sensitive VGCCs) (Christodoulou and Skourides, 2015; Zanardelli et al., 2013). I injected embryos with Cav3.3S or L MOs at the 4/8-cell stage, targeting the dorsal animal cells (i.e. the

neural plate precursors), as above. Using time-lapse microscopy, I observed a delay of neural fold formation during NTC (Fig. 3A,D,E,G).

A second Cav3.3L MO (Cav3.3L MO 2, which targets a different site on the transcript) also showed a delay in NTC when injected into 1-cell embryos (Fig. 4E,F). It should be noted that Ca3.3L MO 2 may also hit Cav3.3S as the 23 of the 25 oligonucleotides match to Cav3.3S' sequence. RT-PCR confirmed knockdown of *Cav3.3* transcripts by Cav3.3S MO, Cav3.3L MO and Cav3.3L MO 2 (Fig. 4B-D). Furthermore, we used RT-PCR to detect *Cav3.3L* expression in unmanipulated embryos during the stages of interest (*i.e.*, during NTC) from late blastula-mid-tailbud (stage 9-stage 26) (Fig. 4A). Cav3.3S is also expressed at these times, as indicated by RNAseq data (Fortriede et al., 2020; Session et al., 2016). Cav3.3 is expressed at a very low level and I attempted to detect *Cav3.3* using in *situ hybridization* probes that bind to both alloalleles. Unfortunately, due to *Cav3.3*'s low expression levels, I was unable to detect it by *in situ* hybridization during NTC and tailbud stages.

Furthermore, in mid-tailbud stage embryos, I observed a dorsal flexure phenotype similar to that seen with Myosin-IIB knockdown embryos (Rolo et al., 2009) (Fig. 3B,D,F,G). In an additional experiment, 1 dorsal cell at 4-cell stage was injected with Cav3.3L MO knockdown and these

embryos displayed a curve toward the side of injection. This phenotype indicates that the embryo was unable to undergo proper convergent extension of the neural tube during neurulation. Furthermore, I observed craniofacial defects including abnormally small heads and malformed eyes (Fig. 3B,D,F,G). This may indicate defects in neural induction or differentiation and/or misregulation of neural crest cells.

To ensure this delay in NTC was not due to the gastrulation delay, as we still saw a slight delay in gastrulation in targeted MO-injections, I treated late gastrula/early neurula embryos (stage 12-13 embryos) with the pharmacological agent ML218, a T-type VGCC inhibitor. I still observed a delay in NTC (Fig. 3J,K). These results indicate that the delay during gastrulation and NTC may be separate processes, and that Cav3.3 is important for both gastrulation and neurulation.

To further support the hypothesis that Cav3.3 is involved in neural crest formation and migration, Cav3.3S MO knockdown shows a distinctive spikey dorsal fin phenotype (hereafter called ridgeback), suggesting neural crest involvement, as neural crest cells are known to migrate into the dorsal fin during development (green bracket in Fig. 3F) (Tucker and Slack, 2004). However, the ridgeback phenotype appears before known neural crest migration into the dorsal fin around mid-tailbud (~stage 25/26 vs late

tailbud, stage 31), which may indicate that neural crest cells migrate earlier than previously appreciated (Tucker and Slack, 2004). The dorsal ectoderm is also induced to develop as dorsal fin by neural crest, starting around late neurula, but the fin does not form until late tailbud stages (Tucker and Slack, 2004).

Conversely, the ridgeback phenotype was not nearly as prominent in the Cav3.3L MO-injected embryos (Fig. 3B,D). This may be due to differing roles of the S and L alleles of *Cav3.3*, although it could also reflect differences in the efficacy of the MOs. However, I believe that the first possibility is more likely, and that Cav3.3L and Cav3.3S may be playing separate roles during development, individually playing roles to different extents depending on the process. That ridgeback phenotype may result from misregulation of neural crest cell migration, further supported by mislocalization and decrease of melanocytes, a neural crest derivative. This was around the base of the head and along the body of Cav3.3S MO knockdown embryos at late tailbud stage (stage 35/36) (Fig. 3L). In summary, it appears that Cav3.3 is necessary for both neural induction, differentiation, neural plate convergent extension and folding, and neural crest migration and differentiation.

Cav3.3 knockdown causes disruption to Apical Actin and Calpain2 localization

Cav3.3 knockdown phenocopies loss-of-function phenotypes of a number of gene products known to be important for NTC, such as Myosin-IIB and Calpain2 (Rolo et al., 2009; Zanardelli et al., 2013). Calpain2 is a Ca^{2+}-dependent protease, knockdown of which has been shown to cause gastrulation and neurulation defects similar to Cav3.3. To study the molecular effect that knockdown of Cav3.3 causes during NTC, I first injected 1-cell stage embryos with mRNA encoding Venus tagged Calpain2L, then our targeted Cav3.3S MO injection into a single blastomere at the 8-cell stage. I observed that with knockdown of Cav3.3S there was a threefold effect on Calpain2L and MO-targeted cells. First, I observed that MO-targeted cells (indicated with a co-injected tracer dye) resulted in no Calpain2L fluorescence within the same cells (Fig.5A). Second, cells adjacent to the MO-targeted cells expressed Calpain2L at a lower level and appear to have lost Calpain2L membrane localization; and third, MO-targeted cells had a larger surface area than those seen with Calpain2L expression on the control side (Fig. 5A). The larger surface area is indicative of loss of apical actin constriction of the plasma membrane, a similar phenotype seen in knockdown of genes required for proper apical constriction (Christodoulou and Skourides, 2015; Kinoshita et al., 2008).

Furthermore, since Cav3.3 causes a similar dorsal flexure phenotype to that of Myosin-IIB knockdown embryos, I injected one blastomere at the 8-cell stage with Cav3.3 MO and stained for actin with rhodamine phalloidin. A previous study has shown that knockdown of Myosin-IIB leads to loss of apical actin accumulation in the neural plate (Rolo et al., 2009). I observed that knockdown of Cav3.3 also results in a loss of apical actin formation in the neural fold (Fig. 5B, arrow). This result indicates that Cav3.3 may play a role with either the expression, localization, or activation of proteins necessary for proper NTC, as Cav3.3 is very unlikely to directly interact with the actin localization machinery. In summary, I suspect that Cav3.3 plays a regulatory role that finely tunes multiple pathways required for proper neural development (Fig. 6).

D. Discussion

To the best of my knowledge, the role of Cav3.3 has not been studied in the context of early vertebrate development. I have discovered a range of developmental defects that arise following the knockdown of Cav3.3 with MOs. Moreover, the phenotypes uncovered resemble those resulting from disruption of well-characterized developmental pathways for neural induction and differentiation, actomyosin dynamics, convergent extension, and neural crest development, indicating widespread importance of T-type voltage-gated Ca^{2+} channels (T-type VGCCs) signaling in early development. Specifically, I have found that Cav3.3 is necessary for proper neural development during vertebrate gastrulation and neurulation.

Cav3.3S and L indicates differences expression and function of alleles within the allotetraploid model, Xenopus

As presented in the Introduction, *Xenopus laevis* is allotetraploid, and thus has two alloalleles of *Cav3.3*: *Cav3.3L* and *Cav3.3S*. It has been reported that that L subgenome genes typically were expressed higher than S subgenome alloalleles (Session et al., 2016). This contrasts with RNAseq data which shows *Cav3.3S* is expressed at lower levels than *Cav3.3L* prior to neurulation, but higher during neurula stages and beyond (Fortriede et al., 2020; Session et al., 2016). Furthermore, *Cav3.3S* has higher

expression in the adult brain and eye than *Cav3.3L*. Very few studies have attempted to tease apart the roles of alloalleles, as I have done here. My findings indicate that the two *Cav3.3* alloalleles have different, but overlapping, roles in development. For example, knockdown of Cav3.3S causes a ridgeback phenotype that is not seen significantly in Cav3.3L knockdown embryos - despite the fact that I find that the Cav3.3L MO gives very strong phenotypes in other contexts, indicating the the difference in producing the ridgeback phenotypes is not simply quantitative. Consistent with this, *Cav3.3L* and *Cav3.3S* are differentially expressed during development (Fortriede et al., 2020; Session et al., 2016), suggesting that Cav3.3S may be playing a larger role in fin development than Cav3.3L.

My findings on the differences between the L and S versions of *Cav3.3* should be of interest for all *Xenopus laevis* researchers, who typically have studied only one of the two alleles, or have unknowingly studied both using methods targeting both allolalles. To my knowledge, I am one of the first to study the alloalleles separately. It will be interesting to see if other alloalleles differ functionally, as appears to be occurring with *Cav3.3L* and *Cav3.3S*.

Cav3.2 displays another phenotype not previously reported

Cav3.3L and *Cav3.3S* are highly expressed during gastrulation stages, and decrease during neural tube closure (NTC) (Fig. 4A) (Fortriede et al., 2020; Karimi et al., 2018; Session et al., 2016). Furthermore, the related T-type VGCC *Cav3.2* has a different time course of expression through development than *Cav3.3*, as it is expressed at low levels prior to NTC, and then increases drastically, starting around late gastrula (stage 12) (Karimi et al., 2018; Session et al., 2016). This suggests that Cav3.3 primarily functions during neural induction, actomyosin dynamics, and convergent extension during gastrulation, while Cav3.2 activity is more prominent during NTC for convergent extension, neural fold formation, and neural fold fusion.

However, *Cav3.3* is still expressed during neurulation. Cav3.2 knockdown appears to decrease waves of Ca^{2+} along the anterior neural plate (Abdul-Wajid et al., 2015). Thus Cav3.2 may be playing a larger, more expansive role during NTC, while Cav3.3 fine-tunes the response in apical constriction at the single cell level (Abdul-Wajid et al., 2015). For example, cell autonomous Ca^{2+} transients are thought to be involved with regulating proper spatiotemporal apical constriction along the neural plate during NTC (Christodoulou and Skourides, 2015). Through the use of

nifedipine, VGCCs have been implicated to be the source of calcium for these cell autonomous transients (Christodoulou and Skourides, 2015; Suzuki et al., 2017). Cav3.3 is a great candidate for fine-tuning pathways within the cell at its low expression levels and ability to bring in quick bursts of Ca^{2+} within the cell.

My observations suggest that Cav3.3 plays a role in both gastrulation and neurulation. Cav3.3 knockdown delay in NTC appears independent of the observed gastrulation defect because ML218, a T-type VGCC inhibitor, causes delay of NTC when added to late-gastula/early neurula embryos (stage 12/13). However, unfortunately, I can not parse out the difference between the effect of the drug on Cav3.2 and Cav3.3 as this drug inhibits both channels (Xiang et al., 2011).

Cav3.3 may regulate multiple processes during neural development

The first defects detectable with Cav3.3 MO knockdown is the delay in gastrulation and NTC. Disruptions in convergent extension can lead to delay in either gastrulation and neurulation, as seen with disruption of the Wnt/PCP, Wnt/Ca^{2+}, and actomyosin pathways in *Xenopus* embryos (Habas et al., 2001; Rolo et al., 2009; Skoglund et al., 2008; Wallingford and Harland, 2002; Zanardelli et al., 2013). Another piece of evidence that

Cav3.3 is involved in convergent extension is the dorsal flexure phenotype. This defect can occur with loss of both neural ectoderm and mesoderm convergent extension. However, based on literature, the neural ectoderm must be affected to produce the dorsal flexure phenotype (Wallingford and Harland, 2001). Both Cav3.3S and L MO-knockdowns significantly displayed this phenotype (see figure 3B and F). However, it is possible that the neural ectoderm (and potentially mesoderm) did not differentiate properly, causing the delays and dorsal flexure phenotype seen in Cav3.3 knockdown embryos.

To further support Cav3.3's role during convergent extension, I wanted to observe if Cav3.3 MO knockdown would cause loss of proper localization of Calpain2L to the plasma membrane. Oddly, I observed that MO-targeted cells (indicated with co-injected tracer dye) had no Calpain2L fluorescence. It is possible that knockdown of Cav3.3 perturbs a pathway that leads to the instability of Calpain2L mRNA or protein. Unsure of what has occurred, there is still plenty to infer from this experiment. The cells surrounded by MO-targeted cells appear to have lost Calpain2L plasma membrane localization, indicating that Calpain2L localization requires crosstalk between cells. A small area of the neural plate appears to have both MO and Calpain2L (white outline, Fig. 5A), and Calpain2L appears to have lost proper membrane localization within this area. Additionally, cells

on the MO-injected side appeared to have a larger surface area than the control side, indicating loss of apical actin constriction that is typically seen all along the neural plate (Balashova et al., 2017; Christodoulou and Skourides, 2015; Kinoshita et al., 2008). Further supporting Cav3.3's involvement in apical actin constriction, Cav3.3L MO knockdown causes loss of apical actin along the neural plate, likely the underlying cause of the delayed NTC, much like that seen in knockdown of Myosin-IIB in *Xenopus* embryos (Rolo et al., 2009). Inhibition of convergent extension can also cause this delay, as seen with knockdown of Calpain2 (as well as Dishevelled) in *Xenopus* embryos (Wallingford and Harland, 2002; Zanardelli et al., 2013). As before, loss of proper neural induction and/or differentiation could also be the reason apical actin constriction is lost along the neural plate.

With knockdown of both Cav3.3S and L, I also observed smaller heads, eyes and other craniofacial defects relative to control embryos, indicating potential loss of proper neural induction and/or differentiation (Fig. 3B,F,L). Disruptions to both of these events caused anterior developmental defects. In Spemann organizer transplant experiments, knockdown of Chrodin caused loss of central nervous systems and smaller eyes (Oelgeschläger et al., 2003). Knockdown of Sox19b during zebrafish neural differentiation can cause smaller heads (Li et al., 2019, p. 19).

Inhibition of L-type VGCCs during neural induction also leads to deformed heads and reduced or loss of eye formation (Leclerc et al., 2000).

I wanted to see the effect of targeted double knockdown on *Xenopus* embryos to identify if we would see complete NTC failure, considering the two alloalleles may be redundant. Unfortunately, this caused failure of gastrulation along the animal dorsal side of the embryo, leaving the cavity of the embryo open. This result potentially indicates that Cav3.3 is required for proper cell survival and cell viability, and the embryo was not able to compensate for the loss of both alloalleles. T-type VGCCs have been implicated in cell viability as researchers found that inhibition of Cav3.1 induced apoptosis in rat neural progenitor cells obtained from the cerebral cortex of rat embryos (embryonic day 14, NTC occurs embryonic day 11) (Kim et al., 2015; Smith and Wood, 1989).

My observations indicate that Cav3.3 may also be involved in neural crest differentiation and migration. As neural crest forms cranial cartilage and eye structures, the observed craniofacial defects could be in part due to loss of proper neural crest differentiation or migration. Knockdown of Msx1 and Pax3, which are required for proper neural crest differentiation, caused craniofacial defects (Monsoro-Burq et al., 2005). Knockdown of neural crest gene *kdm3a*, a lysine demethylase, led to defects in neural

crest migration and subsequently smaller heads and eyes in *Xenopus* (Lee et al., 2019). Furthermore, Calpain2 also displayed smaller eyes and loss of head structures (Zanardelli et al., 2013). Authors speculated it could be due to impaired neural crest cell migration but did not investigate the phenotypes further.

Although highly speculative, as we did not stain for neural crest cells within the fin, further support of Cav3.3's involvement with neural crest development is the ridgeback phenotype. I have never seen this phenotype described in the literature. The ridges appear before proposed fin development, indicating that the neural crest migration is disrupted (Tucker and Slack, 2004). Furthermore, to support Cav3.3's role during neural cell differentiation and migration, L-type VGCC's have been implicated in neural crest cell differentiation and migration (Moran, 1991). Similar to inhibition of L-type VGCCs in neural crest development, I observed loss of proper melanocyte formation and migration in Cav3.3S knockdown embryos. Knockdown of Msx1 also caused decreased pigmentation with loss of proper neural crest differentiation (Monsoro-Burq et al., 2005). My results indicate that either neural crest cells were unable to differentiate and/or unable to properly migrate to the cranium and flanks of the developing embryo (Fig. 3L)

Proposed Cav3.3 Pathway

With pleiotropic effects of Cav3.3 S and L knockdown on neural development, I propose the following pathway, which is based on known mechanisms of gastrulation and neurulation, as well as speculation based on literature in other systems.

The best known role for T-type VGCCs, due to their low voltage sensitivity, are to modulate the responsiveness of cells to external signals. In other words, they can trigger cellular responses, but are not the downstream effectuators. Cav3.3 is situated to play a similar role in neural development, by fine tuning multiple processes, and may either be acting alone or upstream of L-type VGCCs - as seen in pacemaker cells and developing neurons in culture (Lory et al., 2006; Su, 2014). There is the "cell-help-hypothesis" which states that spikes of Ca^{2+} transients could be used to help fine tune individual cell behavior during massive tissue reorganization (Markova and Lenne, 2012). Nifedipine-sensitive cell autonomous Ca^{2+} transients are thought to be involved with regulating proper spatiotemporal apical constriction and the extent at which cells constrict along the neural plate (Christodoulou and Skourides, 2015; Markova and Lenne, 2012; Suzuki et al., 2017). It is speculated that having

the ability to quickly establish and stop Ca^{2+} influx could help control local cytoskeletal dynamics (Markova and Lenne, 2012).

While my MO experiments show that Cav3.3 is necessary, it is expressed at very low levels. I speculate that low expression of *Cav3.3*, with Ca^{2+} microdomains, allows Cav3.3 to fine-tune specific cellular processes during neural development. As discussed prior, many pathways during neural development require tight control for proper morphogenesis (Escuin et al., 2015; Kühl et al., 2001; Zanardelli et al., 2013). T-type VGCC can be tightly regulated for proper calcium influx into the cell (Cazade et al., 2017; Chemin et al., 2017). T-type VGCC's have already been implicated in proper neural differentiation of *Xenopus* spinal neurons where VGCC drug-targeted inhibition of T-type VGCC's caused loss of Ca^{2+} spikes (Gu et al., 1994). Thus it is reasonable to hypothesize that Cav3.3 modulates Ca^{2+} transients, either in cell-autonomous transients or multicellular waves, ensuring proper cellular responses such as cellular constriction and gene expression.

Downstream of Wnt/β-catenin pathway activity, the neural inducers, FGF and Noggin have been shown to be upstream of L-type VGCC activation for downstream neural gene expression (Brafman and Willert, 2017; Leclerc et al., 1999; Lee et al., 2009). The activation of the L-type VGCCs

is essential for normal neural development (Leclerc et al., 2000). The activation of T-type VGCCs was not addressed in this study, but the two channel types can act in concert, with T-type VGCCs controlling the depolarization events that led to L-types VGCCs activation (Su, 2014). As in pacemaker cells, I speculate that Cav3.3 could be activated first to trigger a depolarization event to activate L-type VGCCs, which in turn lead to cellular responses, including gene expression (Leclerc et al., 1999; Lee et al., 2009; Su, 2014). Additionally, it is possible that Cav3.3 and the Wnt/β-catenin pathway negatively regulate each other during neural induction for proper neural gene expression. As mentioned prior, the Wnt/β-catenin pathway and the Wnt/Ca^{2+} pathway work antagonistically to regulate proper convergent extension movements during gastrulation through regulation of β-catenin-induced gene expression (Kühl et al., 2001). Furthermore, the Wnt/β-catenin pathway downregulates *Cav3.1* expression in cardiomyocytes and decreases Cav3.1 current (Florczak, 2021). This indicates that Cav3.3 could be regulating neural induction, potentially inhibiting the Wnt/β-catenin for proper levels of proneural gene expression. In neural differentiation, T-type VGCC's currents have been recorded along the floorplate of the neural tube (Lory et al., 2006). Furthermore, they have been implicated in spinal neuron differentiation in culture and, in general, Ca^{2+} transients have been implicated in proper differentiation and electrical maturation of developing neurons (Gu et al.,

1994; Lory et al., 2006; Rosenberg and Spitzer, 2011). Both my own observations, and known roles of VGCCs during neural development, indicate that Cav3.3 is essential for the proper neural induction and differentiation.

Cav3.3 may play a role within the Wnt/Ca^{2+} pathway, parallel to Wnt/PCP pathway (which will be later discussed). The Wnt5A/Ca^{2+} pathway, acting through Calpain2 - a Ca^{2+} dependent protease- regulates convergent extension (Zanardelli et al., 2013). In addition, Calpain2 was found to stabilize actin-driven apical constriction induced by nifedipine-sensitive cell autonomous Ca^{2+} transients (Christodoulou and Skourides, 2015; Suzuki et al., 2017). Furthermore, the Wnt5A/Ca^{2+} pathway requires L-type VGCCs for increased trafficking of N-methyl-D-aspartate receptors to the plasma membrane in hippocampal neurons, indicating the use of VGCCs for localization of players in the Wnt signaling pathways (McQuate et al., 2017). I hypothesize that Wnt activation leads to Cav3.3 influx of Ca^{2+}, which then binds to Calpain2 for proper localization to the plasma membrane, allowing Calpain2 to properly regulate convergent extension and apical actin stabilization within the neural plate.

One of the primary publications in this area, Suzuki et al. 2017, speculated "...it is also possible that non-muscle myosin II is activated by

Ca^{2+} transients through known pathways such as calmodulin-dependent kinase (CaMKII)-mediated RhoA and myosin light chain kinase (MLCK) activation". As stated above, Cav3.3 could be a source of Ca^{2+} that activates another Ca^{2+}-dependent protein, Calmodulin. Ca^{2+}-Calmodulin activates both CamKII and MLCK for downstream actomyosin activation (Haws et al., 2016). In chick, Calmodulin was thought to be involved in apical constriction and neural fold formation (Webb and Miller, 2003). It has been shown that Calmodulin binds to the C-terminal tail of Cav3.3, and, when Ca^{2+} influx occurs, Ca^{2+}-Calmodulin changes the conformation of Cav3.3 to regulate the amount of Ca^{2+} influx (Lee et al., 2017). Furthermore, direct binding of T-type VGCCs and Calmodulin has been shown to activate CamKII; also, CamKII been shown to increase T-type VGCC currents (Asmara et al., 2017; Zhang et al., 2013). Building further upon my hypothesis, CamKII is also a component of the Wnt/Ca^{2+} pathway (Kühl et al., 2000).

I hypothesize that Calmodulin works in parallel with the Wnt/PCP pathway to control actomyosin activation, and that regulation of Cav3.3 Ca^{2+} influx from components of both pathways leads to proper downstream processes. As mentioned above, in mice, it has been shown that RhoA/Rock controls proper actin turnover during spinal NTC, and loss of this regulation causes increased actin accumulation (Rolo et al., 2018). This occurs

because, even with loss of ROCK-mediated MLC activation, MLCK can still phosphorylate MLC and increase apical actomyosin (Escuin et al., 2015; Rolo et al., 2018). In *Xenopus*, inhibition of the Wnt/PCP pathway led to loss of RhoA accumulation along the apical surface of the neural plate and an increase in cell surface area, potentially due to loss of actomyosin dynamics from decreased RhoA activation (Kinoshita et al., 2008). In *Xenopus*, Myosin-IIB is required for actomyosin dynamics during both gastrulation and neurulation (Rolo et al., 2009; Skoglund et al., 2008). PCP protein Vangl2 requires activated Myosin-II for proper localization and also leads to downstream activation of Myosin-II through ROCK during NTC (Ossipova et al., 2015b). Vangl2 is speculated to be required for proper apical constriction during neural tube closure and it also functions during apical constriction of bottle cells during gastrulation (Ossipova et al., 2015b, 2015a). Rock and other Wnt pathway components have been shown to inhibit Cav3.3 in other systems (Iftinca et al., 2007; Zhang et al., 2013).

With all of the above literature as support, my proposed Cav3.3 pathway, which I hypothesize is downstream of the Wnt/Ca^{2+} pathway, regulates both gene expression in neural induction and differentiation, and convergent extension and apical constriction (Fig 6). It is possible that Cav3.3 is working through a parallel pathway (denoted by ? in Fig. 6) as,

for instance, Ca^{2+} waves have been shown to be independent of non-canonical Wnt signaling (Wallingford et al., 2001). It is absolutely fascinating that a single, low expression Ca^{2+} channel has the potential to control so many pathways during neural development. From my data and proposed pathway, there are many research avenues to take this project further.

Future Directions

There are so many future directions to study the role of Cav3.3 in neural development. Since Cav3.3 brings Ca^{2+} within the cell, and Ca^{2+} plays a large part in multiple processes within the cell, it is not surprising to find that Cav3.3 acts in multiple pathways. There are multiple experiments that could be performed to extend my observations. First, further support Cav3.3's involvement during neural induction and differentiation, *in situ* hybridization against genes involved with neural induction and differentiation should be examined in Cav3.3 MO-knockdown embryos, conditionally for neural differentiation. Repeating the *Xenopus* spinal neuron experiments with direct knockdown of Cav3.3 would also reveal if Cav3.3 is the responsible VGCC (Gu et al., 1994). Second, conditional knockdown of Cav3.3 during NTC would also further support my findings that Cav3.3 is involved in convergent extension and apical

constriction during NTC. Keller sandwiches, isolated dorsal tissue that contains the neuroectoderm and mesoderm, can be performed to identify if Cav3.3 directly affects convergent extension of the neuroectoderm and/or mesoderm. Third, the involvement of neural crest cells within the fin is highly speculative and its role in craniofacial and melanocyte development can be directly visualized by staining against neural crest markers in Cav3.3 MO knockdown embryos. Lastly, this work supports others who have shown that the two alloalleles in *Xenopus laevis* may be functionally different and previously studied single alloalleles, or even those studied unknowingly in combination should be revisited (Murato et al., 2007; Session et al., 2016).

Another area of Cav3.3 neural development research to explore is distinguishing whether or not T-type VGCCs and L-type VGCCs are working in unison, or separately, during various stages of development. Older studies claiming L-type VGCC's involvement during early development primarily used nifedipine at high concentrations, which also targets T-type VGCCs. However, some of these studies did use R(+)Bay K 8644, a L-Type VGCC inhibitor, and S(−)Bay K 8644, a L-type VGCC agonist which have not been shown to inhibit or activate T-type VGCCs (Leclerc et al., 2000, 1999). It would be interesting to see if L-type VGCCs and T-type VGCCs were working together, such that T-type VGCCs fine tune Ca^{2+} influx and

potentially activate L-type channels for larger bursts of Ca^{2+} if necessary.

Ultimately, the role of Cav3.3 appears to be vast and there are multiple avenues where to take this research. Cav3.3 was quite difficult to study but it is fascinating to see a gene expressed at such a low level can cause such large defects when knocked down. As technologies for low expression genes improve, it will be interesting to see what other genes with low expression are required for major developmental processes.

E. Figures and Figure Legends

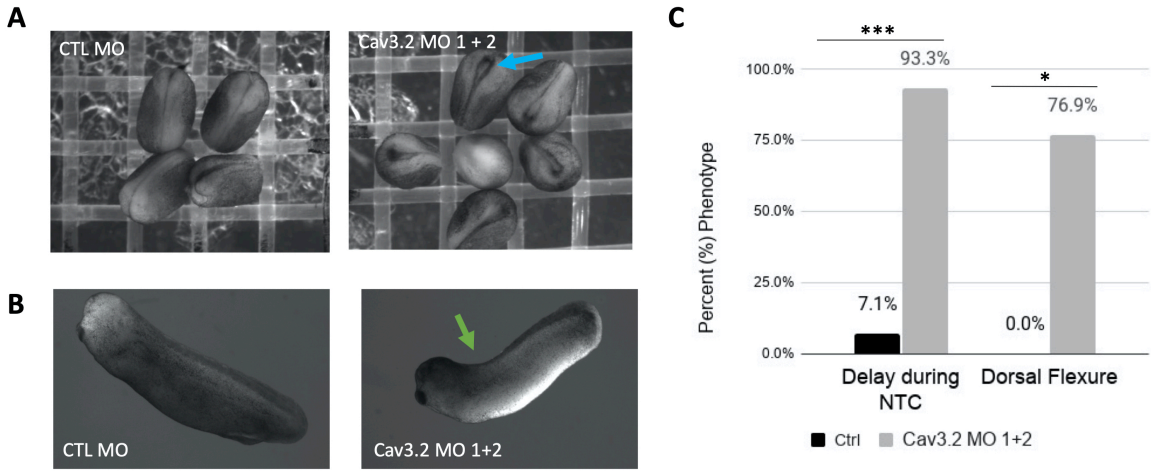


Figure 1. Cav3.2 morpholino oligonucleotide (MO) knockdown causes delay in neural tube closure (NTC) (blue arrow) and dorsal flexure phenotype (green arrow). Embryos were injected with 20-40ng of each Cav3.2 MO 1 and Cav3.2 MO 2 at the 1 cell stage, or an equivalent amount of control MO (40-80ng). A) Representative images of embryos during NTC with either control (CTL) or Cav3.2 MO 1+2 injected at the 1-cell stage. **B)** Representative embryos of Cav3.2 MO-injected tailbuds exhibiting dorsal flexure phenotype when compared to CTL. **C)** Graphical representation of Cav3.2 MO phenotypes compared to CTL. 93.3% (28/30) of Cav3.2 MO knockdown embryos compared to 7.1% (1/14) of CTL embryos display a delay in NTC (Fisher exact test, $P < 0.0001$). 76.9% (10/13) of Cav3.2 MO knockdown embryos display dorsal flexure phenotype compared to 0% (0/3) of the CTL embryos (Fisher exact test, $P = 0.0357$). (* = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$)

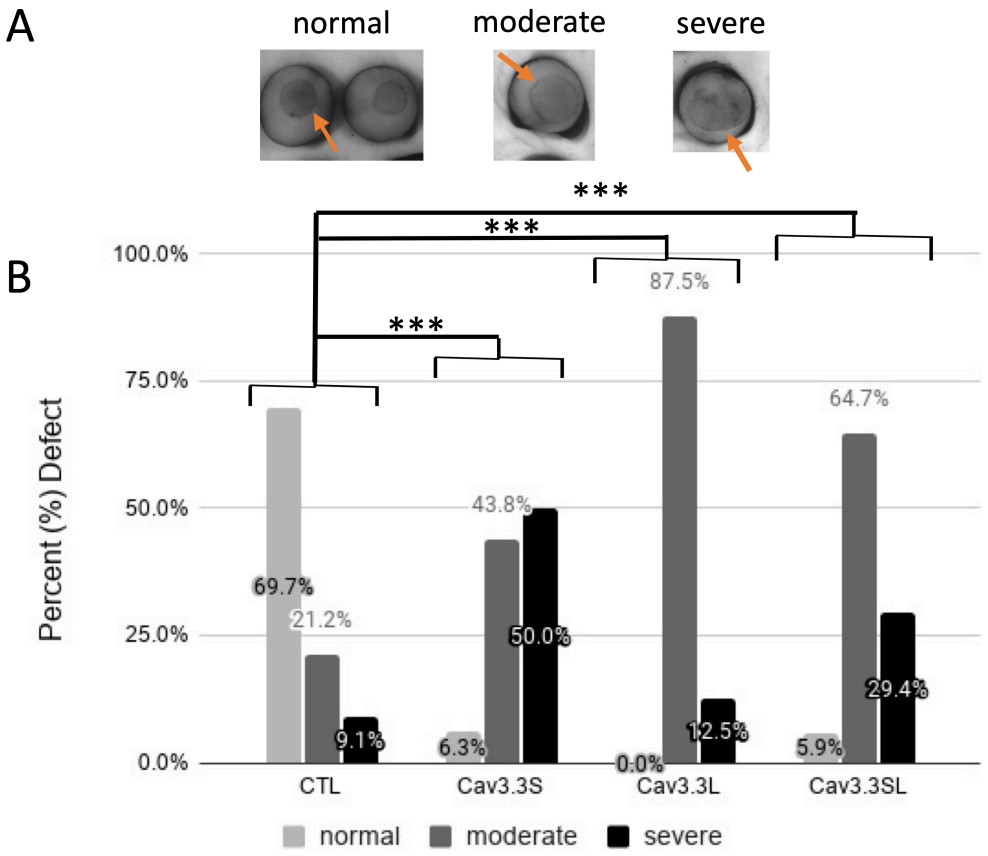


Figure 2. Cav3.3 Morpholino (MO) knockdown causes gastrulation delay. Embryos were injected with 40 ng of Control, Cav3.3S, and Cav3.3L MO at 1-cell stage. For Cav3.3SL MO knockdown, embryos were injected with 20 ng of each Cav3.3S MO and Cav3.3L MO at 1-cell stage. **A)** Representative images of normal embryos compared to moderate, and severely delayed embryos. Blastopore indicated with orange arrow. **B)** Graphical representation of CTL MO-injected embryos displaying 69.7% (23/33), 21.2% (7/33), and 9.1% (3/33) for normal, moderate, and severe embryos, respectively. Cav3.3S MO-injected embryos displaying 6.3% (2/32), 43.8% (14/32), and 50% (16/32) for normal, moderate, and

severe embryos, respectively. Cav3.3L MO-injected embryos displaying 0% (0/32), 87.5% (28/32), and 12.5% (4/32) for normal, moderate, and severe embryos, respectively. Cav3.3SL MO-injected embryos displaying 5.9% (2/34), 64.7% (22/34), and 29.4% (10/34) for normal, moderate, and severe embryos, respectively. All Cav3.3 MO-injected embryos had significant delays in gastrulation compared to CTL (Wilcoxon rank-sum test, $P < 0.00001$ for all Cav3.3 experiments compared to CTL). (* = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$)

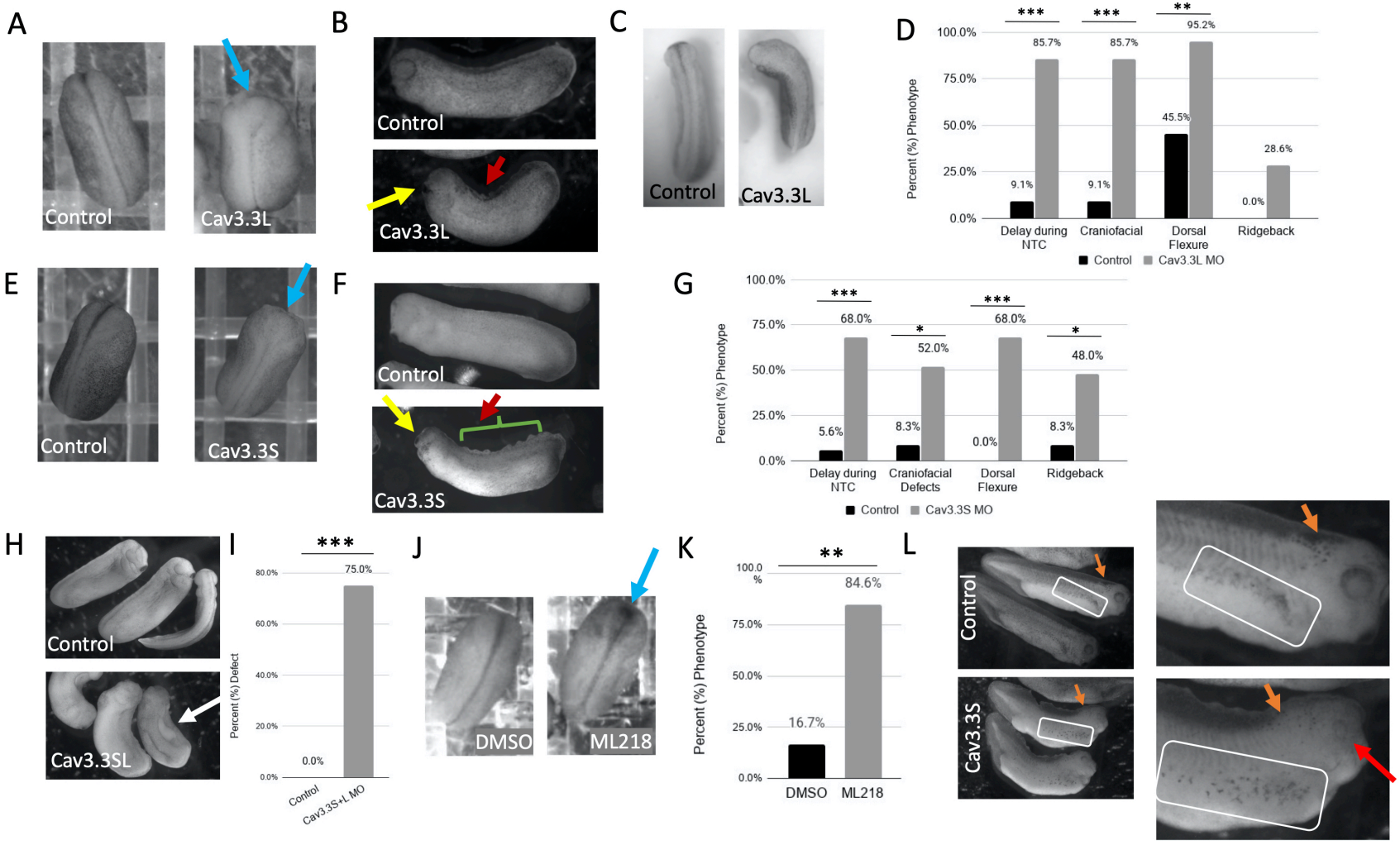

A
Control
Cav3.3L
B
Control
Cav3.3L
C
Control
Cav3.3L
D
***
***
**
100.0%
75.0%
50.0%
25.0%
0.0%
Percent (%) Phenotype
85.7%
85.7%
95.2%
45.5%
9.1%
9.1%
28.6%
0.0%
Delay during NTC
Craniofacial
Dorsal Flexure
Ridgeback
Control
Cav3.3L MO
E
Control
Cav3.3S
F
Control
Cav3.3S
G
***
*
***
*
100.0%
75.0%
50.0%
25.0%
0.0%
Percent (%) Phenotype
68.0%
52.0%
68.0%
48.0%
5.6%
8.3%
0.0%
8.3%
Delay during NTC
Craniofacial Defects
Dorsal Flexure
Ridgeback
Control
Cav3.3S MO
H
Control
Cav3.3SL
I
***
80.0%
60.0%
40.0%
20.0%
0.0%
Percent (%) Defect
75.0%
0.0%
Control
Cav3.3S+L MO
J
DMSO
ML218
K
**
100.0 %
75.0%
50.0%
25.0%
0.0%
Percent (%) Phenotype
84.6%
16.7%
DMSO
ML218
L
Control
Cav3.3S

Figure 3. Knockdown of Cav3.3 causes neural tube and neural crest defects. **A-G)** Embryos injected with 10ng of MO at the 4/8-cell stage. **A)** Representative image of Cav3.3L MO-injected embryo compared to control embryo during neural tube closure (NTC). Notice that the anterior neural plate is wider in the Cav3.3L MO-injected embryos (blue arrows) **B)** Representative image of Cav3.3L MO-injected embryo compared to control embryos at mid-tailbud (stage 26), showing craniofacial defects (yellow arrow) and dorsal flexure phenotype (red arrow). **C)** Embryos injected in a single 4-cell dorsal cell with Cav3.3L MO curve toward the side of injection, indicating bilaterally-asymmetric elongation. **D)** Graphical representation of Cav3.3L MO-injected embryos compared to control embryos. 85.7% (18/21) of Cav3.3L MO-Injected embryos displayed a delay in NTC compared to 9.1% (1/11) of control embryos (Fisher's exact test, $P < 0.0001$). 85.7% (18/21) of Cav3.3L MO-Injected embryos displayed craniofacial defects compared to 9.1% (1/11) of control embryos (Fisher's exact test, $P < 0.0001$). 95.2% (20/21) of Cav3.3L MO-injected embryos displayed the dorsal flexure phenotype (red arrow) compared to 45.5% (5/11) of control embryos (Fisher's exact test, $P = 0.003$). 28.6% (6/21) of Cav3.3L MO-Injected embryos displayed the ridgeback phenotype compared to 0% (0/11) control embryos (Fisher's exact test, $P = 0.0599$). **E)** Representative image of Cav3.3S MO-injected embryo compared to control embryo during neural tube closure (NTC), showing a delay in NTC.

F) Representative image of Cav3.3S MO-injected embryo compared to control embryos at mid-tailbud (stage 26), showing craniofacial defects (yellow arrow), dorsal flexure phenotype (red arrow), and ridgeback phenotype (green bracket). **G)** Graphical representation of Cav3.3S MO-injected embryos compared to control embryos. 68.0% (17/25) of Cav3.3S MO-Injected embryos displayed a delay in NTC compared to 5.6% (1/18) of control embryos (Fisher's exact test, $P < 0.0001$). 52.0% (13/25) of Cav3.3S MO-Injected embryos displayed craniofacial defects compared to 8.3% (1/12) of control embryos (Fisher's exact test, $P = 0.0109$). 68.0% (17/25) of Cav3.3S MO-Injected embryos displayed the dorsal flexure phenotype compared to 0% (0/12) of control embryos (Fisher's exact test, $P < 0.0001$). 48.0% (12/25) of Cav3.3S MO-injected embryos displayed the ridgeback phenotype compared to 8.3% (1/12) control embryos (Fisher's exact test, $P = 0.019$). **H)** Embryos were injected with 6.25-10ng of each Cav3.3S and Cav3.3L MO at the 4/8 cell stage, or an equivalent amount of control MO (12.5-20ng). Representative images of Cav3.3SL MO-injected embryos and control embryos, showing open dorsal tissue into the body cavity. **I)** 75.0% (9/12) of Cav3.3SL embryos display complete gastrulation failure (white arrow) compared to 0.0% (0/15) of control embryos (Fisher's exact test, $P < 0.0001$). **J)** Representative images of embryos treated with 300-350 μM ML218 or vehicle control (DMSO). **K)** 84.6% (11/13) of ML218 embryos displayed delayed NTC compared to

16.7% (2/12) of DMSO treated embryos (Fisher's exact test, P=0.001). **L)** Cav3.3S MO-injected display decreased and mislocalized head and flank melanocytes (white box and orange arrow), compared to control embryos (stage 35/36). Cav3.3S knockdown embryos also have smaller heads, eyes, and loss of pigment in the eye (red arrow). Fin development is also disrupted and jagged, displaying ridgeback phenotype (green brackets). (* = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$)

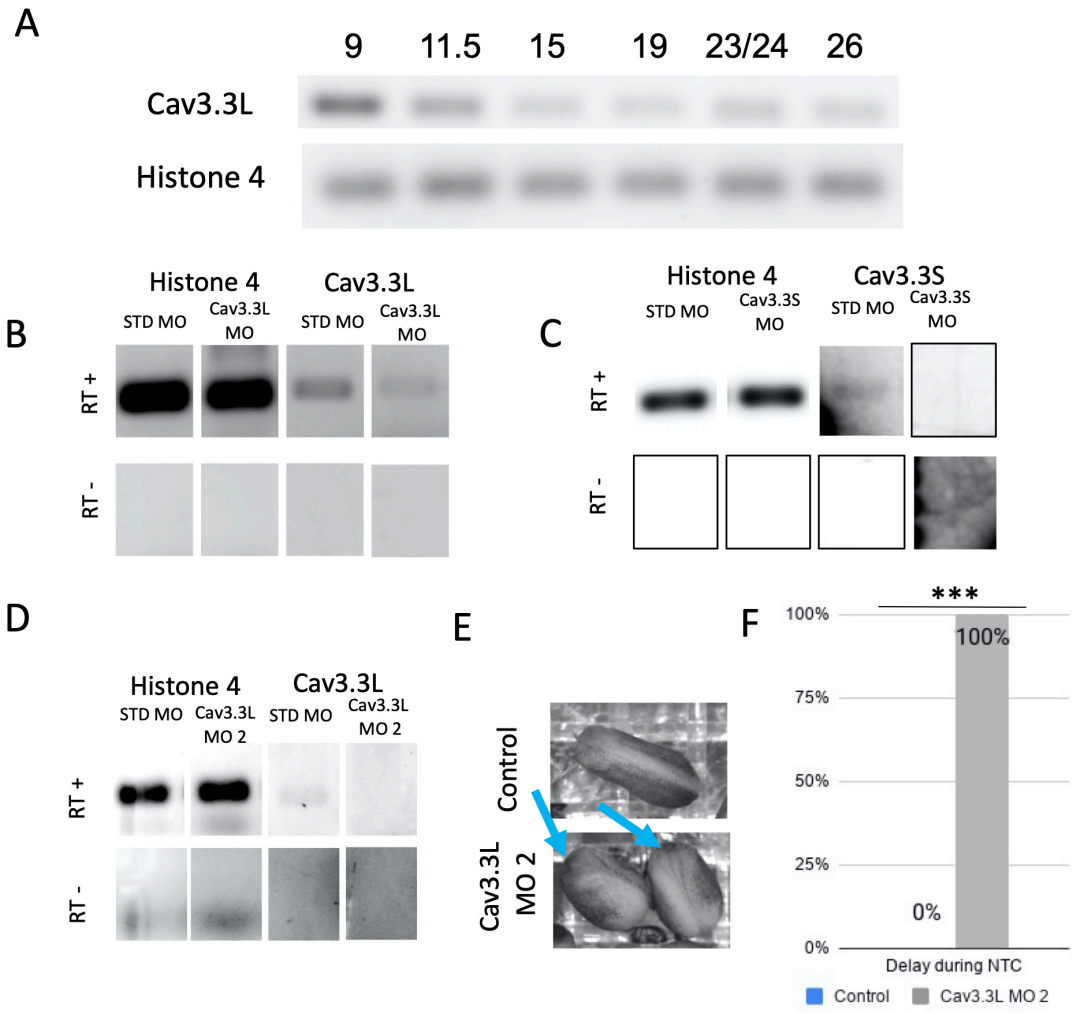


Figure 4. Morpholinos reduce levels of *Cav3.3* transcript **A)** *Cav3.3L* RT-PCR expression across *Xenopus* embryonic late blastula to mid-tailbud (stage 9-26). **B)** RT-PCR of Cav3.3L MO- and Control-injected embryos to assess for knockdown of *Cav3.3L* transcript. **C)** RT-PCR of Cav3.3S MO- and Control-injected embryos to assess for knockdown of *Cav3.3S* transcript. **D)** RT-PCR of Cav3.3L MO 2 and Control embryos to check for knockdown of *Cav3.3L* transcript. **E)** Embryos were injected with 40ng of Cav3.3L MO 2 or control MO at the 1-cell stage. Representative images of

Cav3.3L MO 2 knockdown embryo display neural tube closure delay compared to control embryos. **F)** 100% (10/10) of Cav3.3L MO 2-injected embryos show a delay in NTC compared to 0% (0/7) of control embryos (Fisher exact test, $P < 0.0001$). (* = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$)

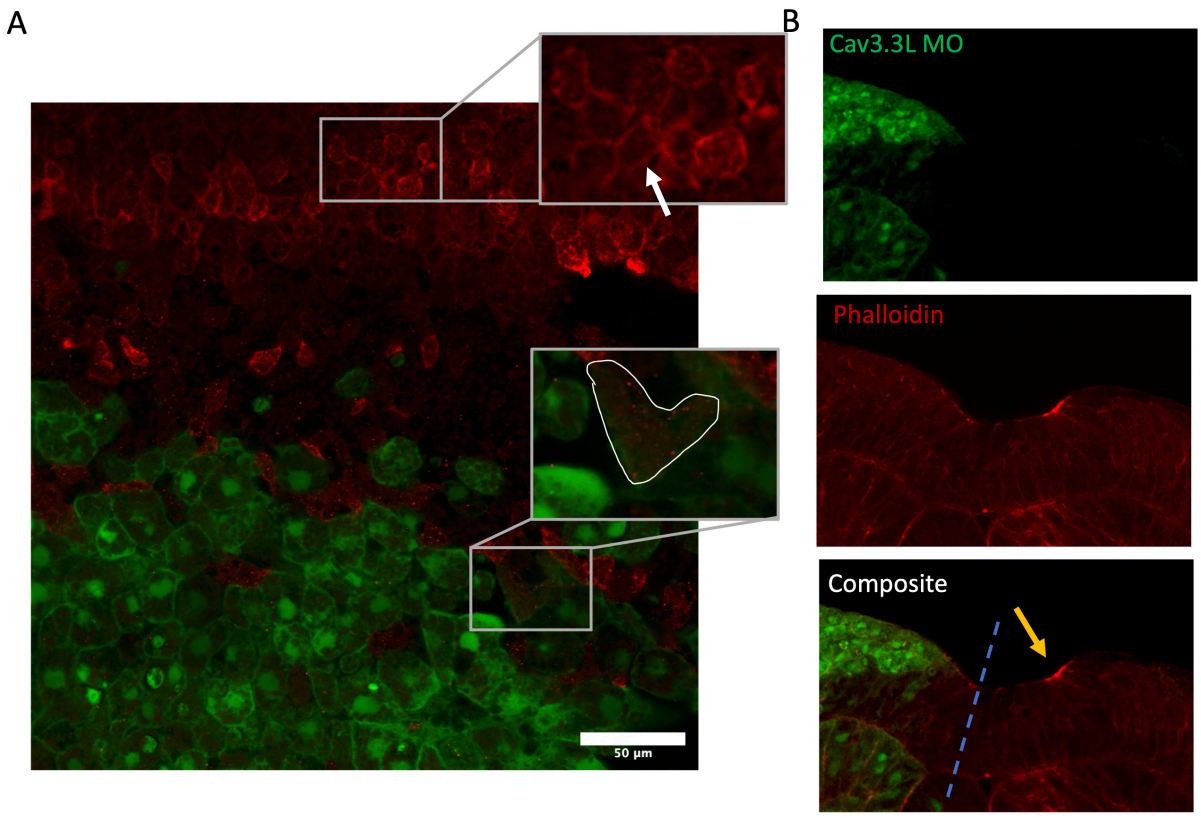


Figure 5. Knockdown of Cav3.3 causes loss of Calpain2-Venus fluorescence and mislocalization of Calpain2L in surrounding cells (white outline) of Calpain2L as well as loss of apical actin accumulation in the neural plate. **A)** Embryos injected at 1-cell stage with Calpain2L-Venus (red) and at 8-cell stage, 1 animal dorsal cell with 10ng Cav3.3S MO (green) Texas Red Dextran was used as a tracer for the MO. Red arrow indicates cells with proper Calpain2L localization to the plasma membrane. **B)** Image of embryo mid-neurula (stage 16/17) injected in 1 animal dorsal cell at 8 cell stage with 10ng Cav3.3L MO with Dextran 488 tracer dye (green). Embryos were stained with Phalloidin after fixation (red). Apical

actin accumulation indicated with yellow arrow. Blue dashed line represents the midline of the embryo. Images are pseudocolored.

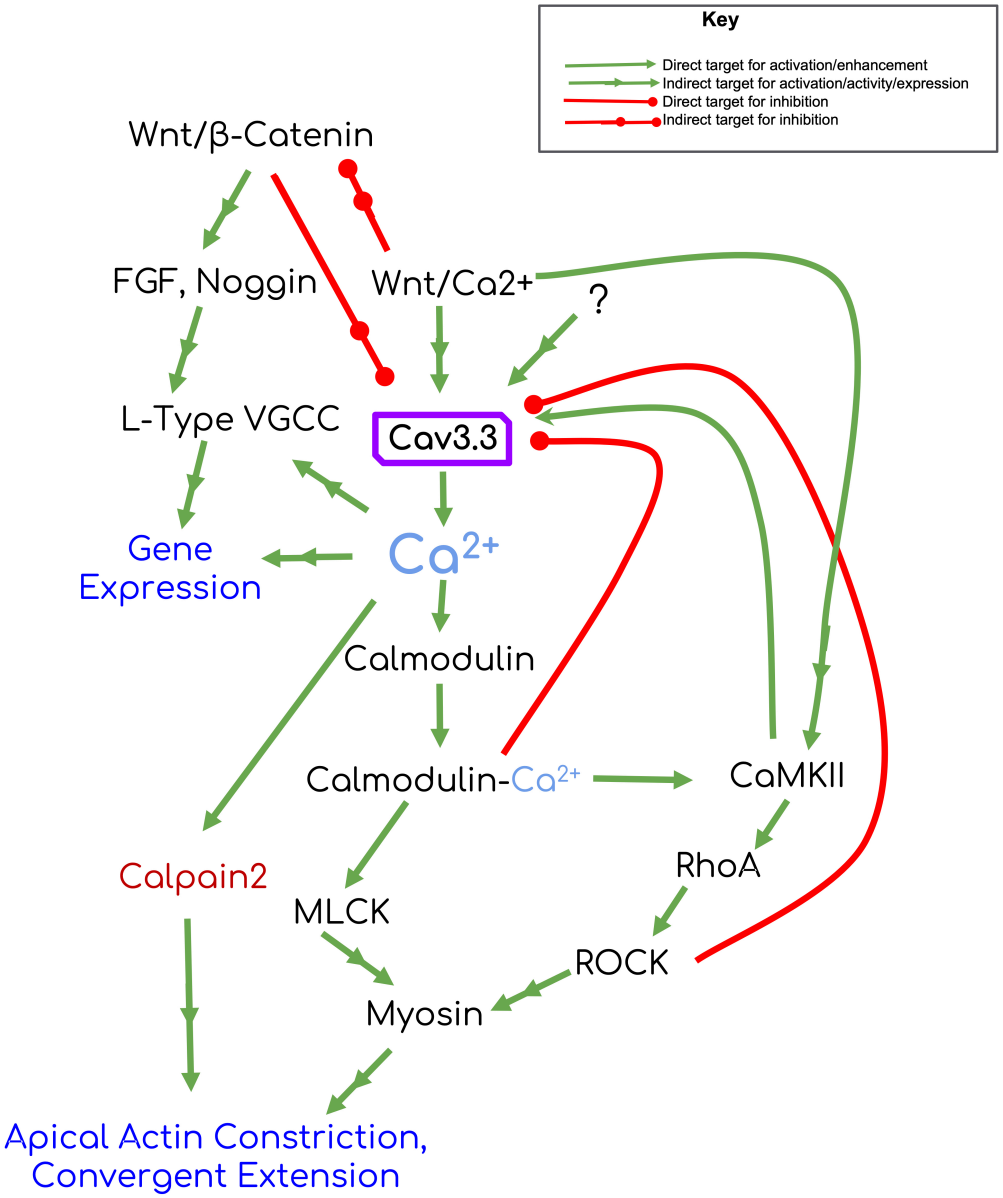


Figure 6. Cav3.3's proposed involvement within the Wnt pathways during gastrulation and neurulation. Single green arrowheads indicate direct target for activation/enhancement and single red circle heads indicate direct target for inhibition. Double arrowheads and double red circle heads

indicate downstream activation/activity/expression or inhibition, respectively.

Table 1. Primers for *Xenopus* experiments

Primer Name	Sequence
Xl Cav3.3L MO	CATGCCTGTGGTGAAGTAGAGAGAA
Xl Cav3.3L MO 2	TCTGGCTGTTGGACAAGCAAAAACA
Xl Cav3.3S MO	GCATACCTGAAGTAGAAAGAAGGGC
Xl Cav3.2 MO 1	TAACATCATCACTTACCATGCTCTC
Xl Cav3.2 MO 2	CCTCATACCTACATGAAAGAGGAGA
Standard Control MO	CCTCTTACCTCAGTTACAATTTATA
Xl Cav3.3L MO 1 Check F	CATTAACCTATCGGCCATCC
Xl Cav3.3L MO 1 Check R	GGTTTCGCAGCAATCCAG
Xl Cav3.3L MO 2 Check F	AGCGACGAAGGCTTTTGTAA
Xl Cav3.3L MO 2 Check R	GTAGTCAAACAGGCCGAAGG
Xl Cav3.3S MO 1 Check F	ACCTATCAGCCATTCGCACT
Xl Cav3.3S MO 1 Check R	TGAAGTTTTCTTCCAAAAAGCA
Xl Histone 4 F	GACGCTGTCACCTACACCGAG
Xl Histone 4 R	CGCCGAAGCCGTAGAGAGTG
Calpain2L with B1 Primer F	AAAAAGCAGGCTCAGAAAAAATGAGCGGGGTCGCCGACAG
Calpain2L with B2 Primer R	AGAAAGCTGGGTTGATCACAGTCAGGGAAAGCC

IV: Teaching and Mentorship

Teaching Experience

Instructor on Record/Teaching Associate

Cell Biology Lab	Winter 2018-2020

Teaching Assistant

Biology Mentoring and Engagement	Spring 2021
Developmental Biology Lab	Winter 2021
Cell Biology Lab	Winter 2014, 2015, 2016, 2017
Introduction to Biology Lab	Fall 2013, 2015, Spring 2015

Mentorship

Graduate Mentor

Graduate Scholars Program	Sept 2019-June 2020

Graduate Mentor/President

Graduate Biology Mentorship Association (GBMA)	Sept 2013 - June 2021

Teaching Philosophy

The teaching philosophies listed below are shaped from my academic experiences as an undergraduate, a teaching assistant, an instructor, and a researcher.

Growth from failure

As the saying goes, "smooth seas never made a skilled sailor." One of the most valuable lessons a student can learn is that failure is not a weakness but a strength. In our current education system, I believe failure is too harshly judged, placing unnecessary expectations upon a student for success as the only metric of learning and intellectual growth. In my laboratory courses, when students are faced with failed results, I explicitly tell them what matters most is that they have learned the method and concepts behind a technique. The purpose is to help students develop the ability to look at a failed experiment objectively, find where it failed, and redo the experiment with proper modifications. Ultimately, this is to prepare them for an environment in which failure and troubleshooting may be a large part of the job, such as research, and to prevent frustration and burnout.

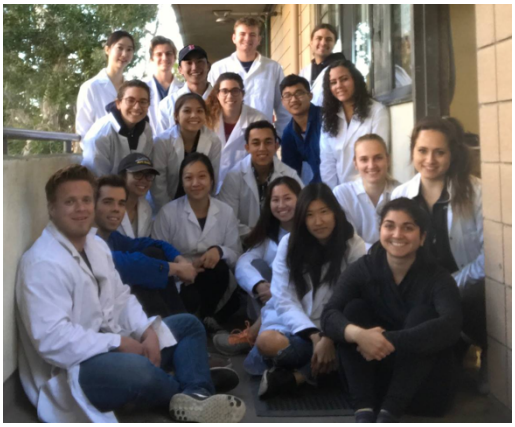
Developing critically-thinking, life-long learners

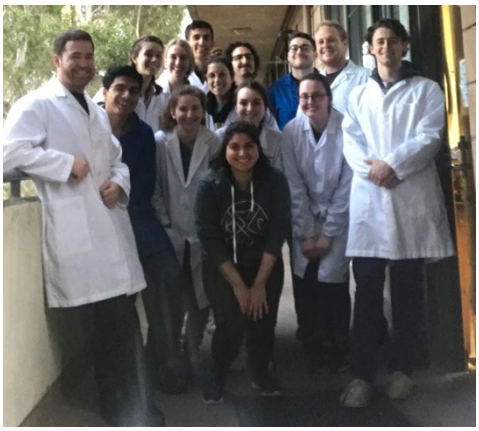
My role as an instructor is to mold students into critically-thinking, life-long learners. I believe, whether in a lecture or laboratory course, these skills must be fostered if students are to succeed in their future careers. First, I promote problem-based learning within my classes by having students develop their own experiments or asking students to solve puzzling conceptual and experimental scientific scenarios. Second, I provide an environment where students are able to learn independently. Typically, when a student asks me a question about a technique, I will respond with a question in order to guide them to an answer. This allows students to process the information they know and come to conclusions themselves, giving them confidence that they have the knowledge and just need to learn how to connect what they know to their current problem. In any academic field, memorization alone is not sufficient for success and we need to promote critical thinking if students are to flourish.

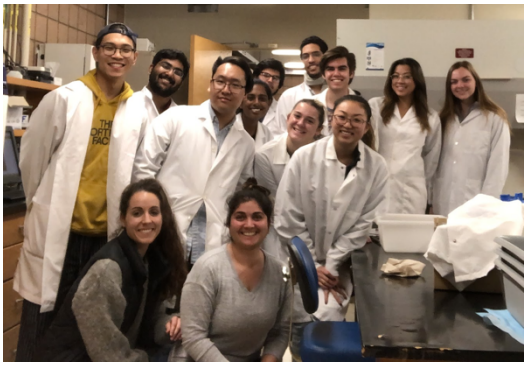
Promoting a safe and comfortable working environment

Lastly, I cultivate an environment where students are comfortable both to approach me for help and gain confidence with their own abilities. Students naturally become stressed in laboratory courses as many are performing experimental techniques for the first time with unfamiliar instruments. Many students have anxiety about performing methods

properly or asking the "wrong" questions when stuck or confused. By creating an environment where I get to know my students, bond with them, and make it explicitly known that I am here to help, my students thrive. I always call them by their names and support them through positive reinforcement of their techniques. Furthermore, I play background music within the lab to create a more relaxed environment. Ultimately, this environment allows students to focus on their learning experience and gain confidence in their abilities, and to worry less about extraneous factors.







References

Abdul-Aziz, N.M., Turmaine, M., Greene, N.D.E., Copp, A.J., 2009. EphrinA-EphA receptor interactions in mouse spinal neurulation: implications for neural fold fusion. Int. J. Dev. Biol. 53, 559–568. https://doi.org/10.1387/ijdb.082777na

Abdul-Wajid, S., Morales-Diaz, H., Khairallah, S.M., Smith, W.C., 2015. T-type Calcium Channel Regulation of Neural Tube Closure and EphrinA/EPHA Expression. Cell Rep. 13, 829–839. https://doi.org/10.1016/j.celrep.2015.09.035

Abdul-Wajid, S., Veeman, M.T., Chiba, S., Turner, T.L., Smith, W.C., 2014. Exploiting the Extraordinary Genetic Polymorphism of Ciona for Developmental Genetics with Whole Genome Sequencing. Genetics 197, 49–59. https://doi.org/10.1534/genetics.114.161778

Anderson, D., Mehaffey, W.H., Iftinca, M., Rehak, R., Engbers, J.D.T., Hameed, S., Zamponi, G.W., Turner, R.W., 2010. Regulation of neuronal activity by Cav3-Kv4 channel signaling complexes. Nat. Neurosci. 13, 333–337. https://doi.org/10.1038/nn.2493

Andreazzoli, M., Gestri, G., Cremisi, F., Casarosa, S., Dawid, I.B., Barsacchi, G., 2003. Xrx1 controls proliferation and neurogenesis in Xenopus anterior neural plate. Development 130, 5143–5155. https://doi.org/10.1242/dev.00665

Arnoult, C., Villaz, M., Florman, H.M., 1998. Pharmacological Properties of the T-Type Ca2+ Current of Mouse Spermatogenic Cells. Mol. Pharmacol. 53, 1104–1111.

Asmara, H., Micu, I., Rizwan, A.P., Sahu, G., Simms, B.A., Zhang, F.-X., Engbers, J.D.T., Stys, P.K., Zamponi, G.W., Turner, R.W., 2017. A T-type channel-calmodulin complex triggers αCaMKII activation. Mol. Brain 10, 37. https://doi.org/10.1186/s13041-017-0317-8

Balashova, O.A., Visina, O., Borodinsky, L.N., 2017. Folate receptor 1 is necessary for neural plate cell apical constriction during Xenopus neural tube formation. Dev. Camb. Engl. 144, 1518–1530. https://doi.org/10.1242/dev.137315

Barriga, E.H., Theveneau, E., 2020. In vivo Neural Crest Cell Migration Is Controlled by "Mixotaxis." Front. Physiol. 11. https://doi.org/10.3389/fphys.2020.586432

Bootman, M.D., 2012. Calcium Signaling. Cold Spring Harb. Perspect. Biol. 4. https://doi.org/10.1101/cshperspect.a011171

Brafman, D., Willert, K., 2017. Wnt/β-catenin signaling during early vertebrate neural development. Dev. Neurobiol. 77, 1239–1259. https://doi.org/10.1002/dneu.22517

Catterall, W.A., 2000. Structure and regulation of voltage-gated Ca2+ channels. Annu. Rev. Cell Dev. Biol. 16, 521–555.

https://doi.org/10.1146/annurev.cellbio.16.1.521
Cazade, M., Bidaud, I., Lory, P., Chemin, J., 2017. Activity-dependent regulation of T-type calcium channels by submembrane calcium ions. eLife 6, e22331. https://doi.org/10.7554/eLife.22331
Chemin, J., Taiakina, V., Monteil, A., Piazza, M., Guan, W., Stephens, R.F., Kitmitto, A., Pang, Z.P., Dolphin, A.C., Perez-Reyes, E., Dieckmann, T., Guillemette, J.G., Spafford, J.D., 2017. Calmodulin regulates Cav3 T-type channels at their gating brake. J. Biol. Chem. 292, 20010–20031. https://doi.org/10.1074/jbc.M117.807925
Christodoulou, N., Skourides, P.A.A., 2015. Cell-Autonomous Ca2+ Flashes Elicit Pulsed Contractions of an Apical Actin Network to Drive Apical Constriction during Neural Tube Closure. Cell Rep. https://doi.org/10.1016/j.celrep.2015.11.017
Cramer, K.S., Miko, I.J., 2016. Eph-ephrin signaling in nervous system development. F1000Research 5. https://doi.org/10.12688/f1000research.7417.1
Créton, R., Speksnijder, J.E., Jaffe, L.F., 1998. Patterns of free calcium in zebrafish embryos 10.
Darken, R.S., Scola, A.M., Rakeman, A.S., Das, G., Mlodzik, M., Wilson, P.A., 2002. The planar polarity gene strabismus regulates convergent extension movements in Xenopus. EMBO J. 21, 976–985. https://doi.org/10.1093/emboj/21.5.976
Ding, Y., Ploper, D., Sosa, E.A., Colozza, G., Moriyama, Y., Benitez, M.D.J., Zhang, K., Merkurjev, D., Robertis, E.M.D., 2017. Spemann organizer transcriptome induction by early beta-catenin, Wnt, Nodal, and Siamois signals in Xenopus laevis. Proc. Natl. Acad. Sci. 114, E3081–E3090. https://doi.org/10.1073/pnas.1700766114
Dolphin, A.C., 2018. Voltage-gated calcium channels: Their discovery, function and importance as drug targets. Brain Neurosci. Adv. 2. https://doi.org/10.1177/2398212818794805
Escuin, S., Vernay, B., Savery, D., Gurniak, C.B., Witke, W., Greene, N.D.E., Copp, A.J., 2015. Rho-kinase-dependent actin turnover and actomyosin disassembly are necessary for mouse spinal neural tube closure. J. Cell Sci. 128, 2468–2481. https://doi.org/10.1242/jcs.164574
Fagotto, F., Winklbauer, R., Rohani, N., 2014. Ephrin-Eph signaling in embryonic tissue separation. Cell Adhes. Migr. 8, 308–326. https://doi.org/10.4161/19336918.2014.970028
Florczak, K., 2021. Wnt/β-Catenin Signalling Inhibits T-Type Calcium Channels in Cardiomyocytes (Thesis). Université d'Ottawa / University of Ottawa. https://doi.org/10.20381/ruor-26205
Fortriede, J.D., Pells, T.J., Chu, S., Chaturvedi, P., Wang, D., Fisher, M.E., James-Zorn, C., Wang, Y., Nenni, M.J., Burns, K.A., Lotay, V.S.,

Ponferrada, V.G., Karimi, K., Zorn, A.M., Vize, P.D., 2020. Xenbase: deep integration of GEO & SRA RNA-seq and ChIP-seq data in a model organism database. Nucleic Acids Res. 48, D776–D782. https://doi.org/10.1093/nar/gkz933

Gu, X., Olson, E., Spitzer, N., 1994. Spontaneous neuronal calcium spikes and waves during early differentiation. J. Neurosci. 14, 6325–6335. https://doi.org/10.1523/JNEUROSCI.14-11-06325.1994

Habas, R., Kato, Y., He, X., 2001. Wnt/Frizzled Activation of Rho Regulates Vertebrate Gastrulation and Requires a Novel Formin Homology Protein Daam1. Cell 107, 843–854. https://doi.org/10.1016/S0092-8674(01)00614-6

Hashimoto, H., Robin, F.B., Sherrard, K.M., Munro, E.M., 2015. Sequential Contraction and Exchange of Apical Junctions Drives Zippering and Neural Tube Closure in a Simple Chordate. Dev. Cell 32, 241–255. https://doi.org/10.1016/j.devcel.2014.12.017

Haws, H.J., McNeil, M.A., Hansen, M.D.H., 2016. Control of cell mechanics by RhoA and calcium fluxes during epithelial scattering. Tissue Barriers 4. https://doi.org/10.1080/21688370.2016.1187326

Hering, S., Zangerl-Plessl, E.-M., Beyl, S., Hohaus, A., Andranovits, S., Timin, E.N., 2018. Calcium channel gating. Pflugers Arch. 470, 1291–1309. https://doi.org/10.1007/s00424-018-2163-7

Holmberg, J., Clarke, D.L., Frisén, J., 2000. Regulation of repulsion versus adhesion by different splice forms of an Eph receptor. Nature 408, 203–206. https://doi.org/10.1038/35041577

Iftinca, M., Hamid, J., Chen, L., Varela, D., Tadayonnejad, R., Altier, C., Turner, R.W., Zamponi, G.W., 2007. Regulation of T-type calcium channels by Rho-associated kinase. Nat. Neurosci. 10, 854–860. https://doi.org/10.1038/nn1921

Jurkovicova-Tarabova, B., Cmarko, L., Rehak, R., Zamponi, G.W., Lacinova, L., Weiss, N., 2019. Identification of a molecular gating determinant within the carboxy terminal region of Cav3.3 T-type channels. Mol. Brain 12, 34. https://doi.org/10.1186/s13041-019-0457-0

Karimi, K., Fortriede, J.D., Lotay, V.S., Burns, K.A., Wang, D.Z., Fisher, M.E., Pells, T.J., James-Zorn, C., Wang, Y., Ponferrada, V.G., Chu, S., Chaturvedi, P., Zorn, A.M., Vize, P.D., 2018. Xenbase: a genomic, epigenomic and transcriptomic model organism database. Nucleic Acids Res. 46, D861–D868. https://doi.org/10.1093/nar/gkx936

Keller, R., Davidson, L.A., Shook, D.R., 2003. How we are shaped: the biomechanics of gastrulation. Differentiation 71, 171–205. https://doi.org/10.1046/j.1432-0436.2003.710301.x

Kim, K., Ossipova, O., Sokol, S.Y., 2015. Neural crest specification by

inhibition of the ROCK/myosin II pathway. Stem Cells. https://doi.org/10.1002/stem.1877
Kinoshita, N., Sasai, N., Misaki, K., Yonemura, S., 2008. Apical Accumulation of Rho in the Neural Plate Is Important for Neural Plate Cell Shape Change and Neural Tube Formation. Mol. Biol. Cell 19, 2289–2299. https://doi.org/10.1091/mbc.E07-12-1286
Klymkowsky, M., Rossi, C.C., Artinger, K.B., 2010. Mechanisms driving neural crest induction and migration in the zebrafish and Xenopus laevis. Cell Adhes. Migr. 4, 595–608. https://doi.org/10.4161/cam.4.4.12962
Kühl, M., Geis, K., Sheldahl, L.C., Pukrop, T., Moon, R.T., Wedlich, D., 2001. Antagonistic regulation of convergent extension movements in Xenopus by Wnt/β-catenin and Wnt/Ca2+ signaling. Mech. Dev. 106, 61–76. https://doi.org/10.1016/S0925-4773(01)00416-6
Kühl, M., Sheldahl, L.C., Park, M., Miller, J.R., Moon, R.T., 2000. The Wnt/Ca2+ pathway: a new vertebrate Wnt signaling pathway takes shape. Trends Genet. 16, 279–283. https://doi.org/10.1016/S0168-9525(00)02028-X
Leclerc, C., Duprat, A.-M., Moreau, M., 1999. Noggin upregulates Fos expression by a calcium-mediated pathway in amphibian embryos. Dev. Growth Differ. 41, 227–238. https://doi.org/10.1046/j.1440-169x.1999.00421.x
Leclerc, C., Lee, M., Webb, S.E., Moreau, M., Miller, A.L., 2003. Calcium transients triggered by planar signals induce the expression of ZIC3 gene during neural induction in Xenopus. Dev. Biol. 261, 381–390. https://doi.org/10.1016/s0012-1606(03)00298-7
Leclerc, C., Moreau, M., Néant, I., 2012. The calcium: an early signal that initiates the formation of the nervous system during embryogenesis. Front. Mol. Neurosci. 5. https://doi.org/10.3389/fnmol.2012.00064
Leclerc, C., Néant, I., Webb, S.E., Miller, A.L., Moreau, M., 2006. Calcium transients and calcium signalling during early neurogenesis in the amphibian embryo Xenopus laevis. Biochim. Biophys. Acta BBA - Mol. Cell Res., 9th European Symposium on Calcium 1763, 1184–1191. https://doi.org/10.1016/j.bbamcr.2006.08.005
Leclerc, C., Webb, S.E., Daguzan, C., Moreau, M., Miller, A.L., 2000. Imaging patterns of calcium transients during neural induction in Xenopus laevis embryos. J. Cell Sci. 113 Pt 19, 3519–29.
Lee, C., Kieserman, E., Gray, R.S., Park, T.J., Wallingford, J., 2008. Whole-Mount Fluorescence Immunocytochemistry on Xenopus Embryos. Cold Spring Harb. Protoc. 2008, pdb.prot4957. https://doi.org/10.1101/pdb.prot4957
Lee, K.W., Moreau, M., Néant, I., Bibonne, A., Leclerc, C., 2009. FGF-

activated calcium channels control neural gene expression in Xenopus. Biochim. Biophys. Acta BBA - Mol. Cell Res., 10th European Symposium on Calcium 1793, 1033–1040. https://doi.org/10.1016/j.bbamcr.2008.12.007
Lee, N., Jeong, S., Kim, K.-C., Kim, J.-A., Park, J.-Y., Kang, H.-W., Perez-Reyes, E., Lee, J.-H., 2017. Ca2+ Regulation of Cav3.3 T-type Ca2+ Channel Is Mediated by Calmodulin. Mol. Pharmacol. 92, 347–357. https://doi.org/10.1124/mol.117.108530
Lewis, B.B., Wester, M.R., Miller, L.E., Nagarkar, M.D., Johnson, M.B., Saha, M.S., 2009. Cloning and characterization of voltage-gated calcium channel alpha1 subunits in Xenopus laevis during development. Dev. Dyn. 238, 2891–2902. https://doi.org/10.1002/dvdy.22102
Li, Xian, Zhou, W., Li, Xinyue, Gao, M., Ji, S., Tian, W., Ji, G., Du, J., Hao, A., 2019. SOX19b regulates the premature neuronal differentiation of neural stem cells through EZH2-mediated histone methylation in neural tube development of zebrafish. Stem Cell Res. Ther. 10, 389. https://doi.org/10.1186/s13287-019-1495-3
Lory, P., Bidaud, I., Chemin, J., 2006. T-type calcium channels in differentiation and proliferation. Cell Calcium, T-type calcium channels: from old physiology to novel functions 40, 135–146. https://doi.org/10.1016/j.ceca.2006.04.017
Markova, O., Lenne, P.-F., 2012. Calcium signaling in developing embryos: Focus on the regulation of cell shape changes and collective movements. Semin. Cell Dev. Biol., RNA Editing & Developmental Cell Behavior 23, 298–307. https://doi.org/10.1016/j.semcdb.2012.03.006
McQuate, A., Latorre-Esteves, E., Barria, A., 2017. A Wnt/Calcium Signaling Cascade Regulates Neuronal Excitability and Trafficking of NMDARs. Cell Rep. 21, 60–69. https://doi.org/10.1016/j.celrep.2017.09.023
Milet, C., Monsoro-Burq, A.H., 2012. Neural crest induction at the neural plate border in vertebrates. Dev. Biol., Neural Crest 366, 22–33. https://doi.org/10.1016/j.ydbio.2012.01.013
Miller, R.K., McCrea, P.D., 2010. Wnt to build a tube: Contributions of Wnt signaling to epithelial tubulogenesis. Dev. Dyn. 239, 77–93. https://doi.org/10.1002/dvdy.22059
Monsoro-Burq, A.-H., Wang, E., Harland, R., 2005. Msx1 and Pax3 Cooperate to Mediate FGF8 and WNT Signals during Xenopus Neural Crest Induction. Dev. Cell 8, 167–178. https://doi.org/10.1016/j.devcel.2004.12.017
Moran, D., 1991. Voltage-dependent -L-type Ca2+ channels participate in regulating neural crest migration and differentiation. Am. J. Anat.

192, 14–22. https://doi.org/10.1002/aja.1001920103
Mulligan, K.A., Cheyette, B.N.R., 2012. Wnt signaling in vertebrate neural development and function. J. Neuroimmune Pharmacol. Off. J. Soc. NeuroImmune Pharmacol. 7, 774–787. https://doi.org/10.1007/s11481-012-9404-x
Murato, Y., Nagatomo, K., Yamaguti, M., Hashimoto, C., 2007. Two alloalleles of Xenopus laevis hairy2 gene—evolution of duplicated gene function from a developmental perspective. Dev. Genes Evol. 217, 665–673. https://doi.org/10.1007/s00427-007-0176-x
Nieuwkoop, P.D., Faber, J., 1994. Normal Table of Xenopus Laevis (Daudin). N. Y. Garland Sci.
Nikolopoulou, E., Galea, G.L., Rolo, A., Greene, N.D.E., Copp, A.J., 2017. Neural tube closure: cellular, molecular and biomechanical mechanisms. Development 144, 552–566. https://doi.org/10.1242/dev.145904
Oelgeschläger, M., Kuroda, H., Reversade, B., De Robertis, E.M., 2003. Chordin Is Required for the Spemann Organizer Transplantation Phenomenon in Xenopus Embryos. Dev. Cell 4, 219–230. https://doi.org/10.1016/S1534-5807(02)00404-5
Omasits, U., Ahrens, C.H., Müller, S., Wollscheid, B., 2014. Protter: interactive protein feature visualization and integration with experimental proteomic data. Bioinformatics 30, 884–886. https://doi.org/10.1093/bioinformatics/btt607
Ossipova, O., Chuykin, I., Chu, C.-W., Sokol, S.Y., 2015a. Vangl2 cooperates with Rab11 and Myosin V to regulate apical constriction during vertebrate gastrulation. Development. https://doi.org/10.1242/dev.111161
Ossipova, O., Kim, K., Sokol, S.Y., 2015b. Planar polarization of Vangl2 in the vertebrate neural plate is controlled by Wnt and Myosin II signaling. Biol. Open. https://doi.org/10.1242/bio.201511676
Papalopulu, N., Kintner, C., 1996. A posteriorising factor, retinoic acid, reveals that anteroposterior patterning controls the timing of neuronal differentiation in Xenopus neuroectoderm. Development 122, 3409–3418. https://doi.org/10.1242/dev.122.11.3409
Parekh, A.B., 2008. Ca2+ microdomains near plasma membrane Ca2+ channels: impact on cell function. J. Physiol. 586, 3043–3054. https://doi.org/10.1113/jphysiol.2008.153460
Park, E., Kim, G.-H., Choi, S.-C., Han, J.-K., 2006. Role of PKA as a negative regulator of PCP signaling pathway during Xenopus gastrulation movements. Dev. Biol. 292, 344–357. https://doi.org/10.1016/j.ydbio.2006.01.011
Ray, H.J., Niswander, L., 2012. Mechanisms of tissue fusion during development. Development 139, 1701–1711.

https://doi.org/10.1242/dev.068338
Rolo, A., Escuin, S., Greene, N.D.E., Copp, A.J., 2018. Rho GTPases in mammalian spinal neural tube closure. Small GTPases 9, 283–289. https://doi.org/10.1080/21541248.2016.1235388
Rolo, A., Skoglund, P., Keller, R., 2009. Morphogenetic movements driving neural tube closure in Xenopus require myosin IIB. Dev. Biol. https://doi.org/10.1016/j.ydbio.2008.12.009
Rosenberg, S.S., Spitzer, N.C., 2011. Calcium Signaling in Neuronal Development. Cold Spring Harb. Perspect. Biol. 3. https://doi.org/10.1101/cshperspect.a004259
Roszko, I., Sawada, A., Solnica-Krezel, L., 2009. Regulation of convergence and extension movements during vertebrate gastrulation by the Wnt/PCP pathway. Semin. Cell Dev. Biol. 20, 986–997. https://doi.org/10.1016/j.semcdb.2009.09.004
Roure, A., Rothbächer, U., Robin, F., Kalmar, E., Ferone, G., Lamy, C., Missero, C., Mueller, F., Lemaire, P., 2007. A Multicassette Gateway Vector Set for High Throughput and Comparative Analyses in Ciona and Vertebrate Embryos. PLoS ONE 2, e916. https://doi.org/10.1371/journal.pone.0000916
Ryan, K., Lu, Z., Meinertzhagen, I.A., 2016. The CNS connectome of a tadpole larva of Ciona intestinalis (L.) highlights sidedness in the brain of a chordate sibling. eLife 5, e16962. https://doi.org/10.7554/eLife.16962
Sadaghiani, B., Thiébaud, C.H., 1987. Neural crest development in the Xenopus laevis embryo, studied by interspecific transplantation and scanning electron microscopy. Dev. Biol. 124, 91–110. https://doi.org/10.1016/0012-1606(87)90463-5
Sagner, A., Briscoe, J., 2019. Establishing neuronal diversity in the spinal cord: a time and a place. Development 146, dev182154. https://doi.org/10.1242/dev.182154
Salas, P., Vinaithirthan, V., Newman-Smith, E., Kourakis, M.J., Smith, W.C., 2018. Photoreceptor specialization and the visuomotor repertoire of the primitive chordate Ciona. J Exp Biol 221. https://doi.org/10.1242/jeb.177972
Schwaller, B., 2010. Cytosolic Ca2+ Buffers. Cold Spring Harb. Perspect. Biol. 2. https://doi.org/10.1101/cshperspect.a004051
Session, A.M., Uno, Y., Kwon, T., Chapman, J.A., Toyoda, A., Takahashi, S., Fukui, A., Hikosaka, A., Suzuki, A., Kondo, M., van Heeringen, S.J., Quigley, I., Heinz, S., Ogino, H., Ochi, H., Hellsten, U., Lyons, J.B., Simakov, O., Putnam, N., Stites, J., Kuroki, Y., Tanaka, T., Michiue, T., Watanabe, M., Bogdanovic, O., Lister, R., Georgiou, G., Paranjpe, S.S., van Kruijsbergen, I., Shu, S., Carlson, J., Kinoshita, T., Ohta, Y., Mawaribuchi, S., Jenkins, J., Grimwood, J., Schmutz,

J., Mitros, T., Mozaffari, S.V., Suzuki, Y., Haramoto, Y., Yamamoto, T.S., Takagi, C., Heald, R., Miller, K., Haudenschild, C., Kitzman, J., Nakayama, T., Izutsu, Y., Robert, J., Fortriede, J., Burns, K., Lotay, V., Karimi, K., Yasuoka, Y., Dichmann, D.S., Flajnik, M.F., Houston, D.W., Shendure, J., DuPasquier, L., Vize, P.D., Zorn, A.M., Ito, M., Marcotte, E.M., Wallingford, J.B., Ito, Y., Asashima, M., Ueno, N., Matsuda, Y., Veenstra, G.J.C., Fujiyama, A., Harland, R.M., Taira, M., Rokhsar, D.S., 2016. Genome evolution in the allotetraploid frog Xenopus laevis. Nature 538, 336–343. https://doi.org/10.1038/nature19840

Shcheglovitov, A., Zhelay, T., Vitko, Y., Osipenko, V., Perez-Reyes, E., Kostyuk, P., Shuba, Y., 2005. Contrasting the effects of nifedipine on subtypes of endogenous and recombinant T-type Ca2+ channels. Biochem. Pharmacol. 69, 841–854. https://doi.org/10.1016/j.bcp.2004.11.024

Sheldahl, L.C., Slusarski, D.C., Pandur, P., Miller, J.R., Kühl, M., Moon, R.T., 2003. Dishevelled activates Ca2+ flux, PKC, and CamKII in vertebrate embryos. J. Cell Biol. 161, 769–777. https://doi.org/10.1083/jcb.200211094

Sive, H.L., Grainger, R.M., Harland, R.M., 2000. Early Development of Xenopus Laevis: A Laboratory Manual. Cold Spring Harbor Laboratory Press.

Skoglund, P., Rolo, A., Chen, X., Gumbiner, B.M., Keller, R., 2008. Convergence and extension at gastrulation require a myosin IIB-dependent cortical actin network. Development. https://doi.org/10.1242/dev.014704

Smith, C.L., Abdallah, S., Wong, Y.Y., Le, P., Harracksingh, A.N., Artinian, L., Tamvacakis, A.N., Rehder, V., Reese, T.S., Senatore, A., 2017. Evolutionary insights into T-type Ca2+ channel structure, function, and ion selectivity from the Trichoplax adhaerens homologue. J. Gen. Physiol. 149, 483–510. https://doi.org/10.1085/jgp.201611683

Smith, M.T., Wood, L.R., 1989. Neonatal Animal Models: Experimental Anencephaly and Similarities to Spina Bifida. J. Am. Coll. Toxicol. 8, 997–1009. https://doi.org/10.3109/10915818909018061

Solnica-Krezel, L., Sepich, D.S., 2012. Gastrulation: Making and Shaping Germ Layers. Annu. Rev. Cell Dev. Biol. 28, 687–717. https://doi.org/10.1146/annurev-cellbio-092910-154043

Sousa, F.G., Matuo, R., Tang, S.W., Rajapakse, V.N., Luna, A., Sander, C., Varma, S., Simon, P.H., Doroshow, J.H., Reinhold, W.C., Pommier, Y., 2015. Alterations of DNA repair genes in the NCI-60 cell lines and their predictive value for anticancer drug activity. DNA Repair Amst 28, 107–15.

https://doi.org/10.1016/j.dnarep.2015.01.011
Stern, C.D., 2005. Neural induction: old problem, new findings, yet more questions. Development 132, 2007–2021. https://doi.org/10.1242/dev.01794
Su, J.B., 2014. Cardioprotective Effects of the If Current Inhibition by Ivabradine During Cardiac Dysfunction. Curr. Pharm. Biotechnol. 14, 1213–1219. https://doi.org/10.2174/1389201015666140515143624
Suzuki, M., Sato, M., Koyama, H., Hara, Y., Hayashi, K., Yasue, N., Imamura, H., Fujimori, T., Nagai, T., Campbell, R.E., Ueno, N., 2017. Distinct intracellular Ca2+ dynamics regulate apical constriction and differentially contribute to neural tube closure. Development 144, 1307–1316. https://doi.org/10.1242/dev.141952
Szabó, A., Mayor, R., 2018. Mechanisms of Neural Crest Migration. Annu. Rev. Genet. 52, 43–63. https://doi.org/10.1146/annurev-genet-120417-031559
Tada, M., Concha, M.L., 2001. Vertebrate gastrulation: Calcium waves orchestrate cell movements. Curr. Biol. 11, R470–R472. https://doi.org/10.1016/S0960-9822(01)00284-6
Theveneau, E., Mayor, R., 2012. Neural crest delamination and migration: from epithelium-to-mesenchyme transition to collective cell migration. Dev. Biol. 366, 34–54. https://doi.org/10.1016/j.ydbio.2011.12.041
Toth, A.B., Shum, A.K., Prakriya, M., 2016. Regulation of neurogenesis by calcium signaling. Cell Calcium 59, 124–134. https://doi.org/10.1016/j.ceca.2016.02.011
Trainor, P.A., 2010. Craniofacial Birth Defects: The Role of Neural Crest Cells in the Etiology and Pathogenesis of Treacher Collins Syndrome and the Potential for Prevention. Am. J. Med. Genet. A. 0, 2984–2994. https://doi.org/10.1002/ajmg.a.33454
Tucker, A.S., Slack, J.M.W., 2004. Independent induction and formation of the dorsal and ventral fins in Xenopus laevis. Dev. Dyn. 230, 461–467. https://doi.org/10.1002/dvdy.20071
Veeman, M.T., Chiba, S., Smith, W.C., 2011. Ciona Genetics. Methods Mol. Biol. Clifton NJ 770, 401–422. https://doi.org/10.1007/978-1-61779-210-6_15
Vitko, I., Bidaud, I., Arias, J.M., Mezghrani, A., Lory, P., Perez-Reyes, E., 2007. The I-II Loop Controls Plasma Membrane Expression and Gating of Cav3.2 T-Type Ca2+ Channels: A Paradigm for Childhood Absence Epilepsy Mutations. J. Neurosci. 27, 322–330. https://doi.org/10.1523/JNEUROSCI.1817-06.2007
Wallingford, J.B., Ewald, A.J., Harland, R.M., Fraser, S.E., 2001. Calcium

signaling during convergent extension in Xenopus. Curr. Biol. 11, 652–661. https://doi.org/10.1016/S0960-9822(01)00201-9
Wallingford, J.B., Harland, R.M., 2002. Neural tube closure requires Dishevelled-dependent convergent extension of the midline. Development 129, 5815–5825. https://doi.org/10.1242/dev.00123
Wallingford, J.B., Harland, R.M., 2001. Xenopus Dishevelled signaling regulates both neural and mesodermal convergent extension: parallel forces elongating the body axis. Development 128, 2581–2592.
Wallingford, J.B., Niswander, L.A., Shaw, G.M., Finnell, R.H., 2013. The Continuing Challenge of Understanding, Preventing, and Treating Neural Tube Defects. Science 339. https://doi.org/10.1126/science.1222002
Wallingford, J.B., Rowning, B.A., Vogell, K.M., Rothbächer, U., Fraser, S.E., Harland, R.M., 2000. Dishevelled controls cell polarity during Xenopus gastrulation. Nature. https://doi.org/10.1038/35011077
Wallingford John B., 2005. Neural tube closure and neural tube defects: Studies in animal models reveal known knowns and known unknowns. Am. J. Med. Genet. C Semin. Med. Genet. 135C, 59–68. https://doi.org/10.1002/ajmg.c.30054
Webb, S.E., Miller, A.L., 2003. Calcium signalling during embryonic development. Nat. Rev. Mol. Cell Biol. 4, 539–551. https://doi.org/10.1038/nrm1149
Weiss, N., Hameed, S., Fernández-Fernández, J.M., Fablet, K., Karmazinova, M., Poillot, C., Proft, J., Chen, L., Bidaud, I., Monteil, A., Huc-Brandt, S., Lacinova, L., Lory, P., Zamponi, G.W., Waard, M.D., 2012. A Cav3.2/Syntaxin-1A Signaling Complex Controls T-type Channel Activity and Low-threshold Exocytosis. J. Biol. Chem. 287, 2810–2818. https://doi.org/10.1074/jbc.M111.290882
Xiang, Z., Thompson, A.D., Brogan, J.T., Schulte, M.L., Melancon, B.J., Mi, D., Lewis, L.M., Zou, B., Yang, L., Morrison, R., Santomango, T., Byers, F., Brewer, K., Aldrich, J.S., Yu, H., Dawson, E.S., Li, M., McManus, O., Jones, C.K., Daniels, J.S., Hopkins, C.R., Xie, X.S., Conn, P.J., Weaver, C.D., Lindsley, C.W., 2011. The discovery and characterization of ML218: A novel, centrally active T-Type calcium channel inhibitor with robust effects in STN neurons and in a rodent model of Parkinson's disease. ACS Chem. Neurosci. https://doi.org/10.1021/cn200090z
Zanardelli, S., Christodoulou, N., Skourides, P.A., 2013. Calpain2 protease: A new member of the Wnt/Ca2+ pathway modulating convergent extension movements in Xenopus. Dev. Biol. https://doi.org/10.1016/j.ydbio.2013.09.017
Zhang, Y., Jiang, X., Snutch, T.P., Tao, J., 2013. Modulation of low-

voltage-activated T-type Ca2+ channels. Biochim. Biophys. Acta BBA - Biomembr., Calcium Channels 1828, 1550–1559. https://doi.org/10.1016/j.bbamem.2012.08.032