STUDY OF CRANIAL NEURAL CREST DEVELOPMENT IN XENOPUS TROPICALIS

by

Aditi Makhija

A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Master in Science in Biological Sciences

Summer 2018

© 2018 Aditi Makhija
All Rights Reserved
STUDY OF CRANIAL NEURAL CREST DEVELOPMENT IN *XENOPUS TROPICALIS*

by

Aditi Makhija

Approved: ____________________________________________
Shuo Wei, Ph.D.
Professor in charge of thesis on behalf of the Advisory Committee

Approved: ____________________________________________
E. Fidelma Boyd, Ph.D.
Chair of the Department of Biological Sciences

Approved: ____________________________________________
George H. Watson, Ph.D.
Dean of the College of Arts and Sciences

Approved: ____________________________________________
Douglas J. Doren, Ph.D.
Interim Vice Provost for Graduate and Professional Education
ACKNOWLEDGMENTS

Simply stated, I could not have done this without my advisor, Dr. Shuo Wei, who accepted me into his lab and helped me overcome my knowledge gaps, always guiding me patiently. I also owe it to my lab members, Mark Perfetto, Chamath Chandrasekera, Congyu Lu, Xiaolu Xu and Andrew Connell. A special thanks to Mark Perfetto for teaching me everything in the lab and answering even my most basic questions. I am extremely grateful to my committee members, Dr. Melinda Duncan and Dr. Jefferey Caplan for their advice and guidance. I would also like to thank Dr. Duncan for her contribution as the Graduate Program Director when I joined the program and for pointing me to my current lab.

I am indebted to the Department of Biological Sciences at the University of Delaware, for accepting me into the program and making me feel welcomed in a new culture. Wolf Hall has been my second home for the past two years. For this, I would also like to thank Ms. Betty Cowgill, without whom I would have been lost in trivialities.

And finally, I’d like to thank my parents, Dr. Atul and Dr. Aruna Makhija and my brother Amodh, for their unending love and support, even from so far away. I wish to extend my gratitude to my friends and family from all walks of life. Their support and encouragement always made me believe in my abilities. Their company made graduate school a personally and professionally enriching experience.
# TABLE OF CONTENTS

**LIST OF TABLES** .............................................................................................................................. vii
**LIST OF FIGURES** .......................................................................................................................... viii
**ABSTRACT** ........................................................................................................................................... x

**Chapter**

1 INTRODUCTION ................................................................................................................................. 1

1.1 Embryonic Development in Vertebrates and *Xenopus Tropicalis* as a Model Organism: .......................................................... 1

  1.1.1 Introduction to Vertebrate Development: .............................................. 1
  1.1.2 Development in *Xenopus*: ............................................................. 2
  1.1.3 *X. Tropicalis* as a Model Organism: ............................................. 3

1.2 Neural Crest Development: .............................................................................................................. 4

  1.2.1 Neural Crest Induction: ................................................................. 5
  1.2.2 Neural Crest Migration and Differentiation: .................................. 6
  1.2.3 Role of *Snai2* in Neural Crest Development: .............................. 9

1.3 Neurocristopathies: ......................................................................................................................... 9

1.4 ADAM13 Regulates ZNF238 During Neural Crest Development: ....... 11

  1.4.1 Role of ADAM13 in Neural Crest Development: ............................ 11
  1.4.2 Role of ZNF238 in Development and a Possible Connection with ADAM13 and Neural Crest: ............................................. 12
  1.4.3 Conjecturing Role of ZNF238 in Neural Crest Development: ....... 14

1.5 Generation of a New Photoconvertible Transgenic Line for the Study of Neural Crest Development: ......................................................... 14

  1.5.1 *Snai2* as a Neural Crest Marker and *Snai2:egfp* Reporter Line: . 14
  1.5.2 Methods of Transgenesis in *Xenopus*: ....................................... 16
    1.5.2.1 DNA Microinjections: .............................................................. 16
    1.5.2.2 Restriction Enzyme-Mediated Integration (REMI): ..... 16
    1.5.2.3 Transposable Elements: .......................................................... 17
REFERENCES ................................................................................................................. 42

Appendix

A PROTOCOL FOR IN SITU HYBRIDIZATION ............................................................ 53
LIST OF TABLES

Table 1: Sequences of antisense morpholino oligonucleotides. They are complementary to respective mRNAs and do not allow translation. ....... 20

Table 2: Sequences of primers used to construct mEos3.2-SV40pA cassette. ............ 23
LIST OF FIGURES

Figure 1 A) The *Xenopus* embryo at stage 12.5. B) ventral view of the embryo at stage 46 (Nieuwkoop and Faber, 1994). ................................................................. 4

Figure 2: Shortly after gastrulation, neural crest is specified at the neural plate border. It then undergoes epithelial to mesenchymal transition, migrates, and forms multiple cell types (a). The hierarchy of gene regulatory network underlying the development of neural crest in (b) (Simões-Costa and Bronner 2015). ................................................................. 5

Figure 3: The cell types arising from the different branches of neural crest (Simões-Costa and Bronner 2013). ................................................................. 8

Figure 4: The Snai2:egfp tadpole at stage 46 in A) dorsal view B) ventral view and C) lateral view. Fluorescence can be observed in craniofacial cartilage, hindbrain, olfactory epithelium and in pigment cells along the length of the embryo; ba-branchial arch, br-brain, nV-trigeminal nerve. ............... 15

Figure 5: ZNF238 is expressed in animal pole of the embryo right after fertilization and at 16 cell stages (A, A', B, B'). It is expressed in dorsolateral part of the embryo at stage 11.5, as denoted by red arrows. DBL: dorsal blastopore lip (C and C'), and in the midline and trigeminal ganglion (D & D'). ......................................................................................... 27

Figure 6: In later stages, ZNF238 is expressed in the midline at stage 15(E and E') in the eye and somites at stage 22-24 (F and F') and in the somites, eye, and the brain in stage 35 (G and G'). ................................................................. 28

Figure 7: Expression of MyoD (A and B) versus expression of ZNF238(A' and B') I obtained. Note the common hypaxial muscle migration pattern (A and A') in late stage embryos. In addition to muscle progenitors, ZNF238 has a strong expression in the eye and the brain. Myo D in situs(A and B) are from (Martin and Harland 2001). ................................................................. 29

Figure 8: At stage 12.5, the expression pattern of *Xenopus tropicalis* ngn1(A) and *Xenopus laevis* ngnr1(B) is similar to *Xenopus tropicalis* ZNF238 pattern I obtained(C). Structures with expression of *Xenopus tropicalis* ngn1 (D and E) and *Xenopus laevis* ngnr1 (G and H) at stage 30 are a subset of *Xenopus tropicalis* ZNF238 at stage 35(F and I). VII, facial epibranchial
placode; IX, glossopharyngeal epibranchial placode; X1, first vagal epibranchial placode; X2, second vagal epibranchial placode; X3, third vagal epibranchial placode; hb, hindbrain; mb, midbrain; nc, notochord; op, olfactory placode; vOT, otic vesicle; tg, trigeminal placode; pg, pineal gland; pP, posterior lateral line placode; pM, middle lateral line placode; pAD, anterodorsal lateral line placode (Nieber, Pieler, and Henningfeld 2009b).

Figure 9: ADAM13 knockdown shows two different patterns. About 40% show expansion(A) and about 60 % show reduction(B) The red asterisk denotes the injected side.

Figure 10: Head cartilage phenotype for embryo injected with control (A) vs ZNF238 MO(B). The red asterisk denotes injected side and the red line segregates the two halves of the embryo. C) The embryos injected with ZNF238 MO show significantly higher head cartilage defect phenotype than those injected with Control MO ( Chi-square test, p<0.0001).

Figure 11: The embryos were stained with alcian blue and observed for head cartilage defect phenotype. The red asterisk denoted the injected side. The head cartilage is smaller and appears undifferentiated in embryos injected with ZNF238 MO (B) versus those injected with control MO.

Figure 12: Stage 17 embryo injected with mEos3.2-N1 plasmid was exposed to UV for 8 minutes. The figure shows the embryo in brightfield before(A) and after UV exposure(A'), in the green channel before(B) and after(B') UV exposure, and in the red channel before (C) and after UV exposure(C'). The embryo has red foci after UV exposure (C') which were not present before(C). Note that there does not seem to a be a change in number of green foci before and after exposure.

Figure 13: Multiple cloning site of mEos3.2-pBluescript. mEos3.2-SV40pA was cloned between NotI and SacII sites. Snai2 is between SalI and NotI as denoted by the blue arrow. The complete Snai2:mEos3.2-SV40pA insert is between the two ISceI sites.
ABSTRACT

The neural crest is a species of multipotent stem cells in vertebrates. It is an ectodermal derivative and is of immense interest to developmental biologists due to its far-reaching migratory capabilities along with the ability to differentiate into multiple cell types such as those of craniofacial skeleton, peripheral ganglia, melanocytes and connective tissue for which it is sometimes also termed as the fourth germ layer. Understanding the development of neural crest is essential as defects during neural crest development can give rise to a variety of disorders such as cleft lip and palate, congenital heart defects, and cancers such as neuroblastoma.

During neural crest induction, ADAM13 (A Disintegrin and Metalloprotease 13) initiates Wnt signaling from the paraxial mesoderm which is essential to the process. Overexpression of ADAM13 showed a downregulation of ZNF238, a muscle, and neural stem cell differentiation factor. In embryos with a ZNF238 knockdown, we also observed reduction in the size of craniofacial structures, suggesting its involvement in CNC development. The first part of this study aims to understand how ZNF238 plays a role in the development of CNC, specifically during induction.

The second part of this thesis discusses the construction of a Snai2:mEos3.2 reporter line as an upgrade to the existing Snai2:egfp line which was previously generated in our lab to facilitate live imaging of neural crest development. mEos3.2 is a photoconvertible protein that emits green fluorescence which changes to red after being exposed to UV light and undergoing an irreversible photoconversion of the chromophore. We plan to target the fluorescent neural crest during migration and track
cell lineage through later stages. Using this technique, we are hopeful that we will discover previously unidentified tissues and cell types with neural crest contribution.
Chapter 1
INTRODUCTION

1.1 Embryonic Development in Vertebrates and Xenopus Tropicalis as a Model Organism:

1.1.1 Introduction to Vertebrate Development:

The fusion of sperm and egg gives rise to the totipotent zygote, a single cell with the capability to form any cell type. This totipotent cell undergoes multiple divisions to form a hollow sphere called the blastula (Hall 1998). The primitive streak generated at the posterior end of the embryo at this stage plays a vital role in the formation of the germ layers (Mikawa et al. 2004). The next and perhaps the most crucial step in the vertebrate development is gastrulation. In all vertebrates, gastrulation involves the formation of the three germ layers (ectoderm, mesoderm, and the endoderm) from the blastula after a complex process of cell migration, ingression and invagination (Gilbert and Barresi 2016). During gastrulation, the ectoderm covers the embryo. Shortly after gastrulation, neural plate is formed at the dorsal ectoderm. At the boundary of the neural plate and non-neural ectoderm (called the neural plate border), the neural crest is specified (Ozair, Kintner, and Brivanlou 2012). At neurulation, the neural plate converges at its edges to form the neural tube and the neural crest cells emerge between the neural tube and the overlying ectoderm (Figure 2a) (Bronner and Simões-Costa 2016). During neurulation, the mesoderm also forms
the somites (muscle precursors) and the heart. From here on, organ specification is different in different vertebrate species (Gilbert and Barresi 2016).

1.1.2 Development in *Xenopus*:

The oocyte has an animal pole and a vegetal pole. Fertilization occurs externally in the animal hemisphere, followed by the first cleavage about 1-1.5 hours later (Heasman 2006). This period also witnesses a cortical rotation which is unique to amphibian embryos. The zygotic cytoplasm rotates by 30 degrees on an axis perpendicular to the animal-vegetal axis. This repositions the sperm entry site in the embryo and establishes the dorsoventral axis (Vincent and Gerhart 1987). The next few cleavage cycles occur in 30-minute intervals up until blastula stage, when zygotic transcription begins and the cycles slow down (Heasman 2006). The blastula has a cavity called the blastocoel surrounded by animal cap cells on the top and the blastocoel floor at the bottom. Prior to gastrulation the animal cap cells thin and spread out to cover the whole embryo, converging at the blastopore (Gilbert and Barresi 2016). This is followed by gastrulation when the germ layers are formed, and the dorsal lip is generated opposite to the repositioned sperm entry site. Directly above this dorsal lip is the Spemann’s Organizer which is required for central nervous system development (Bouwmeester 2001). The blastocoel fades away and is replaced by the archenteron or the primitive gut (Ewald et al. 2004). Shortly after, neurulation occurs where the ectoderm invaginates to form the central nervous system precursor, the neural tube that later forms the brain and spinal cord (Gilbert and Barresi 2016). The ectoderm also gives rise to the epidermis as well as the neural crest cells that will form the peripheral nervous system, craniofacial cartilage, and the pigment cells (Patthey and Gunhaga 2014). The mesoderm gives rise to the notochord, somites and the
precursors of kidney, heart and gut muscles (Burke and Nowicki 2003). The endoderm gives rise to the organs of the digestive system such as the gut, liver and the pancreas and also the epithelium of the digestive and respiratory system (Grapin-Botton and Melton 2000).

1.1.3 *X. Tropicalis* as a Model Organism:

Model organisms are crucial for studying embryogenesis and developmental biology. Of all organisms, *Xenopus* has had a tremendous impact on the field due to its experimental advantages, cost-effectiveness, and relatively close evolutionary relationships to humans (Moody and Sater 2016). The embryos can develop independently of maternal influence, can develop fast and are transparent in tadpole stages, thus making phenotypic observations easier. In addition, *Xenopus* bridges the gap between expensive and low throughput mammalian models and evolutionarily distant zebrafish (Moody and Sater 2016). Historically, major studies in the field of neural crest development have been conducted in chick and quail embryos as they are a good model for explants and transplants. However, gain or loss of function studies are difficult in avian systems in comparison to both *X. laevis* and *X. tropicalis*. In both, injections at two cell stages or later allow for the uninjected cell to serve as an internal control (Mayor and Aybar 2001). An advantage of *X. tropicalis* is that it is diploid, which makes the loss of function studies easier than in the allotetraploid *X. laevis*. Creation of transgenic lines is easier in *X. tropicalis* than *X. laevis* due to its shorter generation time (Hirsch, Zimmerman, and Grainger 2002).

The stages of *Xenopus* development are according to Nieuwkoop and Faber (Nieuwkoop and Faber 1994). Stage 12.5 and stage 46 have been shown here in Figure 1 as they are repeated throughout the course of this thesis. Stage 12.5 is important as
this is when the neural crest induction is completed. The craniofacial phenotypes for the experiments in this thesis were observed at stage 46.

Figure 1 A) The *Xenopus* embryo at stage 12.5. B) ventral view of the embryo at stage 46 (Nieuwkoop and Faber, 1994).

1.2 Neural Crest Development:

Neural Crest cells are a species of stem/progenitor cells which originates from the neural plate border and migrates extensively in the embryonic environment to give rise to multiple cell types. They contribute to a variety of tissues such as the connective and cartilaginous tissues, some neurons and glia, and melanocytes.
Figure 2: Shortly after gastrulation, neural crest is specified at the neural plate border. It then undergoes epithelial to mesenchymal transition, migrates, and forms multiple cell types (a). The hierarchy of gene regulatory network underlying the development of neural crest in (b) (Simões-Costa and Bronner 2015).

1.2.1 Neural Crest Induction:

There are multiple molecular models that describe how induction and specification of neural crest occurs. Of these the most popular is the two-step model. It suggests that the neural crest induction has two steps: neural plate border formation and specification of neural crest cells (Villanueva et al. 2002). The following paragraph explains how it works.

BMP is expressed throughout the ectoderm prior to gastrulation. The dorsal mesoderm secretes BMP antagonists. In addition, a Wnt-dependent mechanism also downregulates BMP signaling (Baker, Beddington, and Harland 1999). This leads to a BMP gradient from dorsal to ventral end of the embryo and results in the formation of the neural plate border along the mediolateral axis. This is of anterior nature, and
posteriorizing signals Wnt, FGF and Retinoic Acid (RA) from the posterior involuting endomesoderm lead to the formation of neural crest cells at the posterior neural plate border (Steventon, Carmona-Fontaine, and Mayor 2005).

Numerous studies in different organisms have revealed different transcription factors and interactions between them that regulate neural crest development. Collectively, these transcription factors and their regulatory networks make a gene regulatory network (GRN). In this GRN model, Wnt, FGF, and BMP signaling pathways first establish the neural plate border between the neural and non-neural ectoderm. They cause expression of neural plate border specifiers Tfap2, Msx1, Zic1, Gbx2, Pax3/7, Dlx5/6, Gata2/3, Foxi1/2, and Hairy2. In Xenopus embryos, induction of neural crest requires Wnt signaling along with intermediate levels of BMP (Steventon and Mayor 2012). Once expressed, these neural plate border specifiers cross-regulate each other to ensure their continued expression, and also regulate expression of neural crest specifiers. The first neural crest specifiers to be expressed are FoxD3, Snai1/2, and Ets1. Many of these neural crest specifier genes are usually involved in cell survival as well.

1.2.2 Neural Crest Migration and Differentiation:

Neural crest specifiers have the key function of activating EMT effector genes that allow neural crest cells to delaminate from the basal lamina, migrate and maintain their undifferentiated state. BMP signaling supports Epithelial to Mesenchymal transition and delamination (Raible and Ragland 2005).

The neural crest cells undergo two stages of migration: group migration where the group of cells migrating together has some cell-cell contact and the second phase where they migrate individually. A very interesting concept during neural crest
migration is contact inhibition of locomotion. The cell or group of cells migrating together encounter another cell or group of cells and migrate away from them. This prevents aggregation of cells during migration. The molecules suspected to be involved in this process are Ephrins, Rac1, RhoA, and cdc42. Ephrins are ligands for the Eph family of receptor tyrosine kinases that can activate Rho GTPases such as RhoA, Rac1, and cdc42 (Mayor and Carmona-Fontaine 2010). Ephrins can also activate RhoA via Wnt-PCP pathway (Tanaka et al. 2003). Together, they alter the cytoskeleton to induce filopodia and lamellipodia for cell migration (Mayor and Carmona-Fontaine 2010).

As neural crest cells migrate and reach their destination, they undergo a mesenchymal to epithelial transition (MET). Based on their axial positions along the embryo, they differentiate and form distinct cell types. The cranial neural crest (CNC) cells develop into bones, cartilage, and connective tissue of the craniofacial and dental tissues. The vagal neural crest gives rise to the cardiac mesenchyme, smooth muscle of the great vessels, and enteric ganglia. The trunk neural crest cells differentiate into a variety of secretory cell types of the adrenal glands and dorsal root ganglia. The sacral neural crest cells give rise to the enteric ganglia. All of them form the pigment cells of the body (Bronner and Simões-Costa 2016) (Figure 3). Wnt signaling induces pigment cells at the expense of glial and neuronal cells from the neural crest; cartilage formation does not require Wnt signaling (Dorsky, Moon, and Raible 1998).

For our study, we have restricted ourselves to CNC. Thus, it is important to understand the development of CNC in particular. The development of craniofacial skeleton from CNC cells is a highly complicated process that involves multiple cell types and multiple types of ossification (Wilkie and Morriss-Kay 2001). The process
of neural crest diversification begins with switching on differentiation circuits which dictate different neural crest lineages via expressing certain transcription factors. These transcription factors are also dependent on the microenvironment of the neural crest cell in question.

Figure 3: The cell types arising from the different branches of neural crest (Simões-Costa and Bronner 2013).
1.2.3 Role of Snai2 in Neural Crest Development:

Snai2 is a neural crest specifier. It is commonly referred to as slug. In Xenopus, it is expressed in pre-migratory and migratory neural crest. It facilitates neural crest migration by repressing E-Cadherin expression (Cano et al. 2000). Heterozygous deletion of Snai2 is correlated with human piebaldism (Sánchez-Martín Manuel et al. 2003) and homozygous deletion is correlated with Waardenburg disease type 2 (Sánchez-Martín et al. 2002). Snai2 is expressed in the pre-migratory and migratory crest in Xenopus. However, it is silenced shortly after migration and is then re-expressed in differentiating neural crest cells (Li and Perfetto et al., manuscript in preparation). Due to its expression pattern and multiple roles in neural crest development, it is used as a neural crest marker in many studies. In Xenopus, Snai2 is required for the expression of other neural crest specifiers such as Sox9, Twist, Ets1, FoxD3 and Sox10 (Lee et al. 2004, LaBonne and Bronner-Fraser 2000; Sasai, Mizuseki, and Sasai 2001; Aybar, Nieto, and Mayor 2003; Linker, Bronner-Fraser, and Mayor 2000; Honoré, Aybar, and Mayor 2003) and is directly regulated by Wnt signaling (Vallin et al. 2001). Snai2 is also expressed in the somitic mesoderm during development (James and Schultheiss 2005).

1.3 Neurocristopathies:

Disorders caused by defects in the development of neural crest are collectively called neurocristopathies. Due to the variety of cell types resulting from neural crest, the neurocristopathies sometimes span distant parts of the body under a singular disorder. However, grouping them under the umbrella of neurocristopathies allows the pathologies with a common etiology to be studied together.
Examples of neurocristopathies include some of the most common birth defects. Congenital heart defects occur one in every 500 births. Hirschsprung’s disease, characterized by the absence of enteric ganglia and a variable length of intestine, affects one in every five thousand births (Etchevers, Amiel, and Lyonnet 2006). Neurocristopathies also include tumors. Neuroblastoma involves the components of the peripheral nervous system and the morphology resembles the undifferentiated neural crest mesenchyme. It is one of the most common childhood cancers (one every seven thousand births) and has the most cases of relapse (Weinstein, Katzenstein, and Cohn 2003).

In general, one-third of birth defects are craniofacial defects that arise due to maldevelopment of the CNC. Of these the most commonly occurring are cleft lip and/or cleft palate (affecting one in a thousand births), which is displayed by a number of congenital diseases (Etchevers, Amiel, and Lyonnet 2006). For example, the Treacher Collins syndrome is characterized by hypoplasia of facial bones and cleft palate (Dixon et al. 2006) and is caused by autosomal dominant mutations in TCOF1 gene. TCOF1 codes for Treacle protein that is hypothesized to play a role in neural crest specification (Werner et al. 2015). The Cleft Lip/Palate-Ectodermal Dysplasia Syndrome (CLEPD1) is characterized by homozygous nonsense mutations in the PVRL1 gene locus that codes for NECTIN1 protein. NECTIN1 is a membrane protein involved in Calcium-independent cell adhesion (Takai Yoshimi et al. 2005).

Not all neurocristopathies have been linked to a specific gene. In most cases, even if a specific gene has been identified, it is involved in other developmental processes as well. Thus, understanding the gene regulatory network of neural crest development is crucial to identifying and studying neurocristopathies.
1.4 ADAM13 Regulates ZNF238 During Neural Crest Development:

The second chapter of this study is based on studying the interaction of ZNF238 with ADAM13 during CNC development. The gene caught our interest as the most downregulated target of ADAM13 overexpression.

1.4.1 Role of ADAM13 in Neural Crest Development:

ADAMs are transmembrane proteins that have a disintegrin domain and a metalloprotease domain. They belong to the metzincin superfamily of metalloproteases. ADAMs have 5 functional domains: 1) A prodomain on the N terminus that keeps the protease inactive and helps with folding and transport through the secretory pathway. 2) A metalloprotease domain that has an active site to bind the Zn$^{+2}$ cation. Of all the ADAMs known, only half are known to have active metalloprotease sites. 3) A disintegrin domain that has a ligand binding site for integrins and thus has a cell to cell interaction function. 4) A cysteine-rich domain and 5) A cytoplasmic domain which has proline-rich binding sites in some ADAMs. ADAMs are known to play a role in controlling cell adhesion and cell migration during embryonic development (Alfandari, McCusker, and Cousin 2009).

In Xenopus, ADAM13 plays a dual role in neural crest development, participating in both induction and migration. During neural crest migration, it cleaves the extracellular domain of Cadherin 11 (Cad-11) to prevent cell-cell adhesion and allows migration via contact inhibition of locomotion. Cad-11 is required during the first phase of migration where the cells migrate in groups via Contact Inhibition of Locomotion (Abbruzzese et al. 2016). Also, its cytoplasmic domain can be cleaved by gamma secretase and can translocate inside the nucleus to regulate gene expression of Calpain-8a, a protease is involved in cell migration (Cousin et al. 2011).
ADAM13 is also expressed in the mesoderm and neural crest cells during gastrula, neurula, and tail bud stages (Alfandari et al. 1997) and is required for CNC induction (Wei et al. 2010). ADAM13 cleaves Ephrin B1/B2 to promote canonical Wnt signaling, which in turn drives expression of neural crest specifier Snai2 (Wei et al. 2010).

ADAM19, a close paralog of ADAM13, non-proteolytically regulates ADAM13 during neural crest specification in a rare example of protease-protease interaction (Li et al. 2018). In the absence of ADAM19, ADAM13 is ubiquitinylated and degraded. ADAM19 prevents ADAM13 degradation and thus allows neural crest to be induced. Both ADAM13 and ADAM19 are required for Wnt signaling during neural crest induction.

1.4.2 Role of ZNF238 in Development and a Possible Connection with ADAM13 and Neural Crest:

ZNF238 is also known as ZBTB18 (updated and most recent gene ID) or Rp58 but is referred to as ZNF238 through the course of this document. It belongs to the POK family of transcriptional repressors which have an N terminal POZ/BTB domain and C terminal Zn finger motifs. The proteins belonging to this family are mostly transcriptional repressors and a majority of them are known to be involved in development and cancer (Okado 2018).

ZNF238 can act as a tumor suppressor (Tatard et al. 2010). In humans, promoter silencing by methylation of ZBTB18 promoter has been implicated in glioblastoma (Fedele et al. 2017). It was found to be absent in mesenchymal population pertaining to malignant glioma and its re-expression in this population impaired migration and tumor forming abilities (Fedele et al. 2017). ZNF238 has also
been shown to play a key role in muscle and neural stem cell differentiation. MyoD, a muscle differentiation factor, promotes expression of ZNF238 in vivo during myogenesis (Yokoyama et al. 2009). Knockdown of ZNF238 prevents the formation of myotubes and myosin heavy chains. In differentiation media, MyoD has been found to bind strongly to the ZNF238 promoter (Yokoyama et al. 2009).

ZNF238 is required for normal development of the brain (Okado et al. 2009). It has also been proposed as a candidate gene in microcephaly and corpus callosum abnormalities and recently in intellectual disabilities (Nagamani et al. 2012; Ballif et al. 2012; Orellana et al. 2009a; Cohen et al. 2017). Chromosomal deletion of ZNF238 in humans is correlated with mental anomalies accompanied by characteristic facial features (Ehmke et al. 2016). During brain development, it was shown to be important for maturation of Glutamatergic and GABAergic neurons and ZNF238 cKO mice exhibit apoptosis of these neurons (Okado 2018). This was also concurrent with an increase in number of progenitor cells in the brain. In addition to differentiation, ZNF238 is also required for neuronal migration via repression of Ngn2 (Okado 2018).

ZNF238 has been well studied in context of neuronal and muscular development but not that of neural crest, especially not in Xenopus. In mouse neuroprogenitors, Rp58 has been shown as a repressor of cell cycle regulatory genes Id1-4 (Clément et al. 2017). In normal brain development, this repression allows for neuronal differentiation to occur (Hirai et al. 2012). Id2 and Id3 genes are also known to be expressed in neural crest development (Martinsen and Bronner-Fraser 1998; Kee and Bronner-Fraser 2005). Overexpression of ADAM13 in Xenopus showed that ZNF238 was repressed in vivo. This negative regulation was also observed upon ADAM13 knockdown (Wang et al. unpublished).
1.4.3 Conjecturing Role of ZNF238 in Neural Crest Development:

ADAM13 regulates ZNF238 expression in vivo and is required for neural crest development, especially induction and migration. ZNF238 represses Id genes that are cell cycle regulators, also involved in neural crest development. Ectopic overexpression of ADAM13 downregulates ZNF238 levels and induces neural crest precursors. This led us to hypothesize that ZNF238 may be involved in neural crest development downstream of ADAM13. We are particularly interested in discerning the role of ZNF238 in CNC development and how it is tied to ADAM13.

1.5 Generation of a New Photoconvertible Transgenic Line for the Study of Neural Crest Development:

One of the major advantages of the Xenopus tropicalis system is the ease of creating transgenic lines. Transgenic animals in which neural crest-specific enhancers/promoters drive expression of fluorescent proteins have been a valuable tool to study CNC development. The reporter lines confer an advantage in tracking cell lineage, especially for highly migratory cell population like the neural crest. Earlier this year, Pax3 and Sox10 transgenic lines were reported for X. laevis (Alkobtawi et al. 2018) but no such lines exist for X. tropicalis. The third chapter of this thesis focuses on the method to create a photoconvertible transgenic line using X. tropicalis.

1.5.1 Snai2 as a Neural Crest Marker and Snai2:egfp Reporter Line:

Most studies on neural crest induction depend upon expression of neural crest markers such as Snai2, Sox 9, FoxD3, which can be examined by in situ hybridization. The entire process is long and labor intensive. It leaves no room for further manipulations since it requires fixing of embryos. As mentioned previously, snai2 is
generally accepted as a neural crest marker. Using the ISceI mediated transgenesis method mentioned above, a Snai2:egfp transgenic reporter line was created (Li and Perfetto et al., manuscript in preparation). In this line, Snai2 promoter drives expression of eGFP. The Snai2:egfp transgenic embryos were found to have peculiar expression pattern in the later stages. At stage 46 (Figure 4), in addition to known derivatives of the neural crest, such as craniofacial tissues and melanocytes, the olfactory epithelium also exhibits snai2 expression.

Figure 4: The Snai2:egfp tadpole at stage 46 in A) dorsal view B) ventral view and C) lateral view. Fluorescence can be observed in craniofacial cartilage, hindbrain, olfactory epithelium and in pigment cells along the length of the embryo; ba-branchial arch, br-brain, nV-trigeminal nerve.
1.5.2 Methods of Transgenesis in *Xenopus*:

For the scope of this thesis, only the following five methods have been discussed. There are other transgenesis methods in the field that have not been mentioned here.

1.5.2.1 DNA Microinjections:

In this method, linearized DNA is directly injected into the embryos after fertilization. Upon integration, the expression persists until adulthood in about 5-10% of the embryos and transgene integration has been shown to occur in these animals.

However, the expression pattern is so mosaic that some individual cells of a certain cell type do not contain the gene at all (Etkin et al. 1984). The mosaicism suggests that the integration either occurs post first cleavage or during first DNA replication. In the latter, integration is suggested to occur randomly through the daughter chromosomal strands. (Etkin et al. 1984) In those embryos that have the integration, the copy number is very low. This makes enhancer-promoter studies difficult (Chesneau et al. 2008).

1.5.2.2 Restriction Enzyme-Mediated Integration (REMI):

In this approach, transgenic embryos are created by DNA integration into sperm nuclei *in vitro* and transplanting them to the eggs. The procedure involves incubating the sperm nuclei with the linearized transgene and the restriction enzyme. Using this method, 30-70% embryos will be transgenic. The exact mechanism of transgene integration is not known (Kroll and Amaya 1996).

The REMI method increases the efficiency of transgenesis as it places the transgene right into the nucleus before the first cleavage occurs. Thus, the
concentration required for injections is much less. Too much DNA will only increase the number of mosaic animals and may even trigger a DNA damage response.

However, the REMI method is technically demanding, and survival rates of the embryos generated using this method is quite low, even lower than that of DNA microinjections (Loeber, Pan, and Pieler 2009). There is also a risk of genome fractionation in this method (Thermes et al. 2002).

1.5.2.3 Transposable Elements:

Transposon mediated insertional mutagenesis which was once widely used in plants and invertebrates has now been adapted to amphibians. This approach involves a transgene flanked by transposable elements and the transposase itself that is responsible for excision and integration. Sleeping beauty and Tol2 are the frequently used transposon systems in Xenopus (Yergeau et al. 2009; Hamlet et al. 2006).

One advantage of this system is that the transposon can now be excised and mobilized into other sites throughout the genome. The disadvantage is that transposons are under a selective pressure as they are often detected and silenced by cellular mechanisms (Houdebine 2007).

1.5.2.4 Bacteriophage Mediated Transgenesis:

The bacteriophage φC31 can insert a plasmid containing attB site into the mammalian genome at a location where the sequence is similar to attP sites. Unlike other systems, the presence of an integrase in phi C31 genome makes the recombination site-specific (Chesneau et al. 2008). Via this approach, integration occurred in 25-35% of the embryos. However, the efficiency of germline transmission for this approach is yet to be determined.
1.5.2.5 IsceI Mediated Transgenesis:

The ISceI mediated transgenesis is a well-established method of tandem genomic insertion in *Xenopus*. It has shown a 30% success rate in *Xenopus tropicalis* (Ogino, McConnell, and Grainger 2006). ISceI is a commercially available meganuclease obtained from the yeast *Saccharomyces cerevisiae* and has an 18bp recognition site. The construct contains these 18bp insertion sites and is first digested and then injected with the enzyme at the one-cell stage. The transgene integration occurs later at the one-cell stage but can occur later as well (Pan et al. 2005). Thus, the founders could have integration on either one side (half transgenics) or both sides (full transgenics) of the embryo in a non-mosaic manner. The mechanism of high efficiency genomic integration of ISceI is still not clear. However, ISceI binding to the recognition sites and the presence of ISceI recognition sequence in the founders suggest that ISceI promotes nuclear localization of the transgene (Thermes et al. 2002). The cleavage followed by a double-stranded break repair which may play a role in the integration of the transgene into the genome (Pan et al. 2005).

This method has many advantages such as limited damage to embryos, high transgene integration and low copy number of integrated genes (Ogino, McConnell, and Grainger 2006). In addition to these advantages, this method has worked for creating our *Snai2:egfp* line previously, hence we have decided to use this method for creation of the *Snai2:mEos3.2* transgenic line.

1.5.3 Motivation for The New Snai2:mEos3.2 Reporter Line:

Since Snai2 is not restricted to the derivatives of neural crest at late stages of the embryo, the *Snai2:egfp* transgenic line is not sufficient to confirm that every green fluorescent cell has neural crest origin. The *Snai2:mEos3.2* is a solution to this
problem. The demarcation between neural crest derivatives and other tissues can be during the migratory stage of neural crest cells as this is when Snai2 expression can be clearly discerned. Exposing green fluorescent cells to UV at this stage will cause them to emit red fluorescent irreversibly. This marked species of cells can then be monitored through development to track the lineage of neural crest cells. Using this method, we will be able to map out previously unidentified derivatives of the neural crest.
Chapter 2
MATERIALS AND METHODS

2.1 Morpholinos:

Antisense morpholinos complementary to ADAM13-3 and ZNF238 were obtained from GeneTools LLC. ZNF238 and ADAM13-3 morpholinos were injected along with Alexa Fluor 555 dextran or FITC dextran tracer dye in one blastomere at the two-cell stage. The other half serves as an internal control. A standard control morpholino was injected in the embryos in a similar fashion as a control for the injection technique itself.

Table 1: Sequences of antisense morpholino oligonucleotides. They are complementary to respective mRNAs and do not allow translation.

<table>
<thead>
<tr>
<th>Morpholino</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADAM13-3</td>
<td>CCCCCGCTCAGTCCG</td>
</tr>
<tr>
<td>ZNF238</td>
<td>TCTGGAAACTCCATAGTCTCAT</td>
</tr>
<tr>
<td>Control</td>
<td>CCTCTTACCTCAGTT</td>
</tr>
</tbody>
</table>

2.2 X. tropicalis Embryos:

Wild-type X. tropicalis adults were obtained from NASCO. Snai2:egfp transgenic frogs were generated as described in Chapter 1. Frogs were primed a day (minimum 16 hours) before the experiment by injecting 20 units of HCG (human chorionic gonadotropin) subcutaneously just behind the posterior line. On the day of the experiment, they were boosted with 200 units of HCG to facilitate natural mating.
Embryos can be obtained 3-4 hours post boosting. The vitelline membrane was removed using 2% cysteine (pH 7.8-8.2), and the embryos were cultured in 0.1X MBS solution for the duration of the experiment. All methods involving live animals were in accordance with the rules of Institutional Animal Care and Use Committees (IACUC) at the University of Delaware.

2.3 Whole Mount In Situ Hybridization:

The plasmid containing zbtb18 was obtained from Dharmacon. The cDNA is cloned between Sp6 and T7 sites. For creating sense probes, the plasmid was linearized using XhoI restriction enzyme, and this linear template was used for in vitro transcription with T7 polymerase. Similarly for antisense probes, plasmid was linearized using SmaI and later transcribed in vitro with Sp6 polymerase. Promega Riboprobe® Combination Systems kit was used to create antisense and sense probes for ZNF238. The probes were added to ISH buffer to make a final concentration of 1026 ng/µl for antisense ZNF238. In situ hybridization was performed according to the protocol in Appendix A (adapted from Sive, Grainger, and Harland 2010). Fresh probes were used every time, and images were taken while staining to ensure robust stain and avoid overstaining. Sense controls were used for staining wherever possible. BM-Purple (Sigma Aldrich) was used as alkaline phosphate substrate.

2.4 Paraffin Embedding and Sectioning:

After in situ hybridization with ZNF238 antisense probe, stage 15 embryos were oriented and embedded in a solution of 3% agarose and 1.67% gelatin (McClelland, Ng, and Bowles 2016). They were oriented so that they can be sectioned perpendicularly to the dorsal-ventral axis. The embedded embryos were processed
routinely and then sectioned into 4-6 µm sections on a microtome. The sections were put on charged slides and examined under a microscope.

2.5 Injections:
Injections were performed using a PLI-100 injector (Harvard Apparatus). Glass needles used for the procedure were created using the World Precision Instruments PUL-1. Embryos were kept in a solution of 6% Ficoll in 0.1X MBS for the duration of injection. After injections, embryos were left in 6% Ficoll for an hour of recovery and then cultured into 0.1X MBS.

2.6 Scoring and Statistics:
Embryos were scored for the reduced head cartilage phenotype as shown in Figure 10. Images of the embryos were taken using Zeiss Axiozoom.v16 epifluorescence microscope.

2.7 Alcian Blue Staining:
Embryos with head cartilage defects were collected at stage 46 and fixed in 4% PFA. It was then stained with Alcian blue as described (Harland 2005).

2.8 Construction of Plasmid mEos3.2-pBluescript:
mEos3.2-N1 was from Michael Davidson (Addgene plasmid # 54525). The construct used to make the Snai2:egfp transgenic line was described in Li and Perfetto, manuscript in preparation. Briefly, the vector backbone of this plasmid is the high copy pBluescript, and the Snai2 promoter and EGFP were cloned between NotI and SacII sites (Figure 13). Since there are no endonuclease sites between EGFP and SV40 polyadenylation signal, I created the mEos3.2 and SV40pA cassette by a PCR with
mEos3.2 Forward primer and SV40 Reverse primer. The original plasmid was then digested with NotI and SacII and mEos3.2-Sv40pA cassette was cloned into the pBluescript backbone using Thermo Scientific™ Rapid DNA Ligation Kit. The plasmid was transformed into Invitrogen™ One Shot™ TOP10 Chemically Competent *E. coli* cells as per manufacturer’s instructions and grown on an LB-ampicillin plate overnight. Multiple clones were picked from this plate and cultured overnight. The plasmid was extracted from these colonies using Thermo Scientific™ GeneJET Plasmid Miniprep Kit. The plasmid obtained was digested with NotI and SacII for confirmation. The plasmid was also sequenced using primers for Sp6 (Reverse) and T7 (Forward).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mEos3.2 Forward</td>
<td>ATAAGCGGGCGCATGAGTGCGATTAAGCCA</td>
</tr>
<tr>
<td>SV40pA Forward</td>
<td>CCGGATCTAGATAACTGACTCGAGCCTCTAGGACTA</td>
</tr>
<tr>
<td>mEos3.2 Reverse</td>
<td>TAGTCCTAGAGGCTCGAGTCAGTTATCTAGA TCCGG</td>
</tr>
<tr>
<td>SV40pA Reverse</td>
<td>ATATCCGCGGGTGAATTGACTACGCGGGG</td>
</tr>
</tbody>
</table>
Chapter 3

EXPLORING THE ROLE OF ZNF238 IN NEURAL CREST DEVELOPMENT

3.1 Background and Rationale:

It has been shown that ADAM13 is required for neural crest induction. It cleaves Ephrin B thus upregulating canonical Wnt pathway which leads to snai2 expression and CNC induction (Wei et al. 2010). To screen for genes that are directly regulated by ADAM13 during neural crest induction, 200 pg of mRNA encoding ADAM13 without a cytoplasmic tail (ADAM13ΔC) was injected in the 2 posterior ventral ectodermal cells of 8 cell stage embryos. This region of the embryo is otherwise predisposed to become the epidermis (Gilbert and Barresi 2016). We removed the cytoplasmic domain as the metalloprotease activity of ADAM13 is required for CNC Induction (Wei et al. 2010). Moreover, ADAM13 regulates transcription of 6121 genes and the cytoplasmic domain itself can regulate the transcription of about a third of these (Cousin et al., 2011).

The embryos were collected at stage 12.5 and their total RNA was extracted and submitted for RNA seq. The data analysis revealed a list of genes that are differentially regulated upon ADAM13ΔC overexpression. Of these, ZNF238 is the most significantly downregulated (logFC=3.4956, P= 2.76 x 10⁻⁷).

ZNF238 codes for ZBTB18 (a.k.a. RP58), which is a transcriptional repressor that binds to E box (Aoki et al. 1998). It plays a key role in differentiation of muscle and neural stem cells, none of which are neural crest derived cells. Thus, its interaction with ADAM13 is intriguing and peculiar. So far, there is no direct evidence
showing that ZNF238 is involved in CNC development, although craniofacial
dysmorphism exhibited by patients with ZNF238 mutations may suggest so. Here, we
investigate its interaction with the developing neural crest.

3.2 Results:

3.2.1 Expression Pattern of ZNF238 at Various Stages of Development:

ZNF238 morpholino (6 pg) was injected in one cell of the embryo at two cell
stage. Embryos were collected at stage 12.5 and fixed for in situ hybridization. I
obtained a cDNA encoding ZNF238 and used it to create sense and antisense probes
for in situ hybridization. Embryos of the following stages were selected: 1 cell, 16
cells, stage 11, stage 12.5, stage 15, stage 22-24 and stage 35. I performed in situ
hybridization for both sense and antisense probes at each of these stages. The staining
pattern of ZNF238 shows expression in the animal pole at 1-cell and 16-cell stage,
suggesting that ZNF238 transcripts are maternally contributed. Thus, ZNF238 may
play a role in early embryonic development (Marlow 2010). At stage 11 it is expressed
in the dorsolateral mesoderm of the embryo which is close to the site of neural crest
induction signal (Figure 5C and C’). At stage 12.5, the expression can be observed in
mediolateral line and the trigeminal placode area. The pattern resembles that of
neuronal differentiation markers X. tropicalis ngn1 and X. laevis ngnr1 at stage 12.5
(Figure 8A, B and C) which are also expressed in trigeminal placode. In stage 15
embryos, it is expressed in two stripes flanking the dorsal midline of the embryo.
(Figure 5D and 5D’). The pattern looks like myoD (see Figure 7 for comparison)
which is expected since MyoD is known to drive expression of ZNF238. MyoD is also
known to be expressed in the mesoderm while spatial and temporal expression of
ZNF238 is not well studied. To analyze the spatial expression of ZNF238, embryos at stage 15 were embedded in paraffin after in situ hybridization and sectioned on a microtome at about 4-6 µm and then observed by bright field imaging. The sections of stage 15 embryos show that ZNF238 is expressed in mesoderm and possibly the endoderm, but not the ectoderm (data not shown).

At later stages in development, ZNF238 expression can be observed in the somitic mesoderm and the head (stage 22-24, and stage 35, Figure 6). At stage 35, the pattern is highly similar to the pattern of hypaxial muscle migration from the somites (Figure 7A and A’). The hypaxial muscle forms the ventral body wall of the embryo.

In the head, the expression is also observed in midbrain, hindbrain, otic vesicle, olfactory placode, the eye, the branchial arches. In addition to MyoD, this pattern also resembles that of neural markers, Ngn1 (X. tropicalis) and ngnr1 (X. laevis) (Figure 8D-F). Ngn1 and Ngnr1 are important for neuronal induction and commitment of progenitors to neuronal cell fate (Nieber, Pieler, and Henningfeld 2009a).
Figure 5: ZNF238 is expressed in animal pole of the embryo right after fertilization and at 16 cell stages (A, A', B, B'). It is expressed in dorsolateral part of the embryo at stage 11.5, as denoted by red arrows. DBL: dorsal blastopore lip (C and C'), and in the midline and trigeminal ganglion (D & D').
Figure 6: In later stages, ZNF238 is expressed in the midline at stage 15 (E and E') in the eye and somites at stage 22-24 (F and F') and in the somites, eye, and the brain in stage 35 (G and G')
Figure 7: Expression of MyoD (A and B) versus expression of ZNF238(A' and B') I obtained. Note the common hypaxial muscle migration pattern (A and A') in late stage embryos. In addition to muscle progenitors, ZNF238 has a strong expression in the eye and the brain. Myo D in situ (A and B) are from (Martin and Harland 2001).
Figure 8: At stage 12.5, the expression pattern of *Xenopus tropicalis* ngn1(A) and *Xenopus laevis* ngnr1(B) is similar to *Xenopus tropicalis* ZNF238 pattern I obtained(C). Structures with expression of *Xenopus tropicalis* ngn1 (D and E) and *Xenopus laevis* ngnr1 (G and H) at stage 30 are a subset of *Xenopus tropicalis* ZNF238 at stage 35(F and I). VII, facial epibranchial placode; IX, glossopharyngeal epibranchial placode; X1, first vagal epibranchial placode; X2, second vagal epibranchial placode; X3, third vagal epibranchial placode; hb, hindbrain; mb, midbrain; nc, notochord; op, olfactory placode; vOT, otic vesicle; tg, trigeminal placode; pg, pineal gland; pP, posterior lateral line placode; pM, middle lateral line placode; pAD, anterodorsal lateral line placode. A, B, D, E, G and H are from (Nieber, Pieler, and Henningfeld 2009b).

3.2.2 ADAM13 Knockdown Shows a Change in The Expression Pattern of ZNF238:

ADAM13-3 morpholino (6ng/µl) was injected in one cell of two-cell stage embryos. Embryos were collected and fixed at stage 12.5 and *in situ* hybridization with ZNF238 antisense probes was carried out. The embryos exhibit a reduction (60%
of the embryos) or expansion (40% of the embryos) as shown in figure 9. To observe whether there is a change in temporal expression, a solution of Benzyl Alcohol to Benzyl Benzoate in a 3:1 ratio was used to clear in situ hybridized embryos. In these embryos, the staining pattern in mesoderm and endoderm are easily observable. However, there does not seem to be conclusive change just by observation, sectioning of these embryos may reveal more information about changes in expression pattern inside the embryo. The change in overall expression of ZNF238 upon ADAM13 knockdown can also be confirmed by a quantitative RT-PCR.

Figure 9: ADAM13 knockdown shows two different patterns. About 40% show expansion(A) and about 60 % show reduction(B) The red asterisk denotes the injected side.

3.2.3 Knockdown of ZNF238 Shows Defects:

ZNF238 morpholino (6 pg) was injected in one blastomere of WT (wild type) embryos at two-cell stage. Embryos were scored for head cartilage defect phenotype in stage 46 and analyzed with chi-square test. A significant reduction on the injected side of the embryo was observed in comparison to the control (Figure 10, p<0.00001). Some of the embryos also exhibited a gut reduction phenotype (Figure 10B). A few
embryos also exhibited a twitching movement at late tail bud and tadpole stages. (data not shown)

Embryos were collected at stage 46 and stained with Alcian blue to confirm cartilage reduction phenotype. (Figure 11).

![Image of embryos with Alcian blue staining]

Figure 10: Head cartilage phenotype for embryo injected with control (A) vs ZNF238 MO(B). The red asterisk denotes injected side and the red line segregates the two halves of the embryo. C) The embryos injected with ZNF238 MO show significantly higher head cartilage defect phenotype than those injected with Control MO (Chi-square test, p<0.0001).
Figure 11: The embryos were stained with alcian blue and observed for head cartilage defect phenotype. The red asterisk denoted the injected side. The head cartilage is smaller and appears undifferentiated in embryos injected with ZNF238 MO (B) versus those injected with control MO.

3.3 Discussion:

Many neurocristopathies also overlap with defects of the central nervous system (CNS). Although disorders arising solely due to CNS defects are studied separately, molecular origins of the two kinds of disorders could be common in certain cases (Shahar and Shinawi 2003). ZNF238 may be one example of these common origin molecules. In various studies with humans and mice, ZNF238 deletions and mutations typically manifest as CNS defects such as hypoplasia of the CNS, microcephaly (Xiang et al. 2012a), intellectual disabilities (Cohen et al. 2017) and corpus callosum abnormalities (Orellana et al. 2009b).

Previous studies in our lab found that ADAM13, which is critical for neural crest induction, downregulates ZNF238, which is important for the central nervous system and muscular development (Wang et al., unpublished data). It has been
confirmed in mice that RP58 is required for neuronal differentiation and brain expansion (Xiang et al. 2012b). However, in our knockdown embryos, we have not yet identified any neurological defects that might suggest phenotypes similar to those observed in mice. One reason for that could be that ZNF238 is not yet expressed in the brain at this stage according to studies in mice. In mice, it is not expressed in the brain before E13 stage which is corresponding to much later stages than stage 46 (Ohtaka-Maruyama et al. 2007).

The spatial-temporal expression pattern of ZNF238 is consistent with its role in neuronal differentiation in the brain, but it also indicates its involvement in neural crest development, since there is an expression in the face as well. For instance, we saw that knockdown of ZNF238 causes a significant head cartilage defect in Xenopus embryos. Recently, craniofacial dysmorphism has been observed in a human patient with 1q44 deletion. This chromosomal region includes ZNF238 as well as AKT (Cohen et al. 2017). Since CNS defects, in this case, are accompanied with craniofacial defect phenotype, one might expect that genes involved in neural crest development and genes involved in CNS development may have interactions during development. However, the CNS precursor, the neural tube separates from the neural crest at neurulation. This suggests the possibility of crosstalk between the two even after this separation occurs.

ZNF238 is also directly regulated by MyoD (Yokoyama et al. 2009). Knockdown of ZNF238 impaired differentiation of muscle progenitors and they were unable to form multinucleated myotubes or Myosin heavy chains which are otherwise characteristic of the skeletal muscle in mice (Yokoyama et al. 2009). Thus, we expected to see some muscular defects in the knockdown embryos. Although some
tadpoles did exhibit twitching movements in late tailbud and tadpole stage, it is not clear if they were caused by defects in muscular development. Analysis of muscle composition at various developmental stages after ZNF238 knockdown will provide a clearer picture of how muscular development is regulated by ZNF238.

From stage 35 onwards, we can see that ZNF238 can also be observed in the eye and the face, even though there is no ZNF238 expression in migrating CNC arches typically observed in stage 22-24 (Le Douarin and Kalcheim 1999). One explanation for this observation could be that ZNF238 may be expressed in differentiating craniofacial muscles downstream of MyoD. It has already been shown in chick embryos that MyoD (a muscle differentiation factor) and Myf5 (a skeletal muscle specific gene) are expressed in differentiating craniofacial muscles and is also paralleled by Myosin heavy chain expression (Noden et al., 1999). Previous studies have also observed that the myoblast population and the neural crest population migrate simultaneously into the branchial arches. The neural crest cells surround the craniofacial muscles and infiltrate into them later (Noden 1991; McClearn and Noden 1988). Thus, another possibility is that ZNF238 is expressed in the neural crest cells themselves. However, since the molecular interaction between the two mesenchymal populations is not exactly clear, more studies are needed to establish the role of ZNF238 in the craniofacial region.

3.4 Conclusions and Future Directions:

ZNF238 was found to act downstream of ADAM13 and is repressed by it, but the exact mechanism could not be concluded. To further explore the possible role of ZNF238 in neural crest development, observe neural crest markers at stage 12.5 after knocking down ZNF238 could be observed. This would tell if ZNF238 plays a role in
neural crest induction. However, the morpholino is not enough, it must be verified with rescue experiments with ZNF238 mRNA as well. Additionally, Western Blot will be required to compare ZNF238 (Rp58) protein levels in control, knockdown and rescued embryos.
Chapter 4

GENERATION OF A NEW PHOTOCONVERTIBLE REPORTER TRANSGENIC LINE

4.1 Background and Rationale:

Understanding of CNC development is critical due to its significant involvement in neurocristopathies. However, our knowledge of CNC biology remains limited as genes involved at every step of CNC development form a highly complex network, and this network imparts a highly dynamic nature to the CNC during embryonic development. This calls on a tool that could make real-time in vivo monitoring of CNC development possible. The Snai2:egfp transgenic line addresses this issue by expressing eGFP downstream of neural crest marker snai2 promoter. Snai2 is a well-established neural crest marker that is expressed in migratory and premigratory neural crest population in Xenopus. Using this line, we have observed eGFP expression in neural crest tissues consistent with the expression pattern from the Snai2 in situ hybridized embryos (Li and Perfetto et al., manuscript in preparation). However, Snai2 is also expressed in mesodermal derivatives such as somites post-migration. Thus, the Snai2:egfp does not answer the question on the exclusivity of fluorescent cells as a neural crest derivative. This could be greatly improved by marking derivatives of Snai2 expressing cells in the embryo as a neural crest or non-neural crest derived. One way is to mark Snai2:egfp fluorescent cells in pre-migratory stages as the Snai2 expression is exclusive to neural crest in this stage. For this method, we could use a photoconvertible protein downstream of Snai2 which would
undergo irreversible photoconversion upon UV exposure and express red fluorescence (Nowotschin and Hadjantonakis 2009). The neural crest cells with red fluorescence can be monitored during development and this would help us in tracing their derivatives. As a tool, its scope can be extended to the entire field of CNC study.

4.2 Results:

4.2.1 Photoconversion of mEos3.2 is Possible in Non-Transparent Xenopus Embryos:

Even though *X. tropicalis* tadpoles are transparent, the embryos at premigratory and migratory stages (18-26) are not transparent. Our study would involve exposing embryos between these stages to UV and monitoring fluorescent cells post-conversion. To see whether this is possible, we conducted a pilot study where we injected the plasmid mEos3.2-N1 (10 pg/ml) in one cell of two cell stage embryos. The embryos were cultured till stage 17 and then exposed to UV for different lengths of time to also figure out an optimum exposure time. We observed that the embryos exposed for 8 minutes had red fluorescent foci along with green foci (Figure 12). The same embryos when exposed up to 10 minutes did not show either green or red fluorescence (data not shown), suggesting that photobleaching of the fluorophore can occur upon long durations of exposure. We did not check for recovery of fluorescence after photobleaching and embryos were cultured for another day and then sacrificed. This recovery factor and optimal exposure time will need to be evaluated for the transgenic line as well.
Figure 12: Stage 17 embryo injected with mEos3.2-N1 plasmid was exposed to UV for 8 minutes. The figure shows the embryo in brightfield before(A) and after UV exposure(A’), in the green channel before(B) and after(B’) UV exposure, and in the red channel before (C) and after UV exposure(C’). The embryo has red foci after UV exposure (C’) which were not present before(C). Note that there does not seem to be a change in number of green foci before and after exposure.

4.2.2 Construction of Plasmid with Snai2:mEos3.2 Insert:

The most common method used for transgenesis in X. tropicalis is ISceI mediated transgenesis. To create a new transgenic line using this method, a plasmid was constructed that has a Snai2:mEos3.2 insert in the region between the ISceI sites. This was confirmed by sequencing and digestion. This plasmid can be digested and injected with ISceI meganuclease in wild-type X. tropicalis embryos immediately after fertilization. The map of this plasmid is presented in Figure 13.
Figure 13: Multiple cloning site of mEos3.2-pBluescript. mEos3.2-SV40pA was cloned between NotI and SacII sites. Snai2 is between SalI and NotI as denoted by the blue arrow. The complete Snai2:mEos3.2-SV40pA insert is between the two ISceI sites.

4.3 Discussion:

The new plasmid has mEos3.2 instead of egfp in the pBlueScript plasmid. Our plan is to inject this plasmid along with ISceI in one cell stage wild-type X. tropicalis embryos.

Photoconvertible transgenic lines are not a novel concept to developmental biology. One example is the transgenic X. laevis line Xla.Tg(CAG: KikGR)la code (Nowotschin and Hadjantonakis 2009) with the KikGR photoconvertible protein under a CAG promoter which is ubiquitously expressed. In contrast, our Snai2:mEos3.2 transgenic line will be a reporter line wherein the snai2 promoter will drive expression of mEos3.2. The idea is to target neural crest during migratory stages (18-26). The green fluorescent neural crest cells at this stage will be selectively exposed to UV radiation. The resulting red fluorescent neural crest cells will be monitored throughout development. In this manner, we will be able to identify previously unknown derivatives of neural crest cells via lineage tracing.

Potential limitations to this experiment include damage to embryos due to UV exposure. Although it is well known that vegetal exposure of one cell stage embryos to
UV irradiation will cause problems with axis formation, germ cell count and cell movements during gastrulation (Malacinski, Brothers, and Chung 1977). Toxicology studies on *Xenopus laevis* have observed that UV exposure can cause pigmentation defects, reduced swimming activity and reduced growth (Bruggeman, Bantle, and Goad 1998). However, no such studies have been performed on irradiation of *X. tropicalis* embryos at post gastrulation stages. Therefore, a pilot study on this transgenic line would be to observe the possible defects that can occur due to UV irradiation in later stages. We also do not know if UV will be able to penetrate the embryo and cause photoconversion in cells that are not superficial on the surface of the embryo. Thus, all these factors will need to be considered for successful lineage tracing studies on the *Snai2:mEos3.2* transgenic line.

4.4 Conclusions and Future Directions:

Wrapping up, I built a construct that is ready to be used for generation of *Snai2:mEos3.2* transgenic line. The next step is to create the founders of *Snai2:mEos3.2* reporter line using ISceI mediated transgenesis. Once founders of the transgenic line are obtained, insertion sites will be determined via full genome sequencing. RT-PCR of the flanking genes will also be necessary to ensure their transcription is not compromised.

For the lineage tracing studies, exposure time will have to be optimized for the embryos of *Snai2:mEos3.2* transgenic line. Effects/damages due to UV exposure must be considered both qualitatively and quantitatively during this study. The efficiency of photoconversion might also be a concern and should not differ too much within embryos. A protocol is available for *Xenopus* where EosFP was photoconverted using a xenon arc based fluorescent microscope (Chernet, Adams, and Levin 2012).
REFERENCES


Truncating Variants in ZBTB18 Cause Intellectual Disability with Variable Features.”


Sánchez-Martín, Manuel, Arancha Rodríguez-García, Jesús Pérez-Losada, Ana Sagrera, Andrew P. Read, and Isidro Sánchez-García. 2002. “SLUG (SNAI2)


Appendix A

PROTOCOL FOR IN SITU HYBRIDIZATION

1) Fix embryos with 4% PFA overnight at 4 °C or for 2 hours at Room Temperature.

2) Wash three times with 1X PTW

3) Transfer the embryos to methanol by a gradient wash.
   a) Wash in 25% methanol/75% PTW.
   b) Wash in 50% methanol/50% PTW.
   c) Wash in 75% methanol/50% PTW.
   d) Put in 100% methanol. The embryos are stored overnight at -20 °C. They could also be stored for a long time.

4) Bleach the embryos. This could be done before or after staining
   a) Make fresh bleaching solution with 8.5 ml water, 250 µl 20X SSC, 500 µl formalin, 750 µl 30 % H₂O₂. Vortex
   b) Put embryos in bleaching solution. Put in white light for 30-60 minutes. Keep observing and remove when they are white.
   c) Wash 3 times in 4 % PFA
   d) Fix overnight in 4% PFA at 4°C.

5) Next day, wash 3 times in PTW.

6) Treat with proteinase K solution (1µl proteinase K stock, 1ml H₂O, 30 µl Tris at pH 8.0) for 2 minutes for stage 12.5 embryos.

7) Wash with 4% PFA. Fix at room temperature in 4% PFA for 20 minutes.

8) Discard PFA. Wash 3 times with PTW.
9) Prewarm In Situ Hybridization buffer, PTW and probes at 65°C in a water bath.
10) Incubate embryos in 500 µl of 1:1 mix of PTW and ISH buffer in the shaking water bath for 1 hour at 65°C.
11) Remove the above solution. Now add 500 µl of ISH buffer. Incubate for 1 hour in shaking water bath at 65°C.
12) Add probes. Incubate at 58°C overnight (minimum of 8 hours).

Day 2:
13) Remove probes. They can be stored for future usage. Prewarm 2X SSC+0.1% CHAPS and 0.2X SSC+ 0.1% CHAPS at 65°C in a shaking water bath.
   a) Wash 2-3 times with 2X SSC+0.1% CHAPS for 15 minutes each at 65°C
   b) Wash 2-3 times with 0.2X SSC+0.1% CHAPS for 15 minutes each at 65°C
14) Make fresh MAB (Maleic Acid Buffer with 5.8g MAB, 4.383 g NaCl, neutralize with NaOH to get pH of 7.5). Wash with MAB twice for 15 minutes each at Room temperature.
15) Warm lamb serum and 10 % BMB to 37°C. Prepare blocking solution(7 ml MAB, 2 ml 10% BMB, and 1 ml lamb serum). Put embryos in 500 µl blocking solution per vial for 2 hours at room temperature.
16) Add anti DIG antibody to the blocking solution. Put embryos in the antibody and incubate overnight at 4°C.

Day 3:
17) Discard the antibody. Perform end to end washing with MAB (6 washes, 30 min each)
18) Add 500 µl of BM purple per vial. Keep tubes in dark.
19) Keep observing throughout staining.
20) After staining, Wash 2-3 times in 4% PFA. Fix embryos in 4% PFA overnight at 4 °C

Day 4

21) Wash with PTW and take images. Transfer embryos to methanol using the gradient method in step 3) for long term storage. Transfer back to PTW whenever imaging.
Appendix B

INSTITUTIONAL APPROVALS
**Title of Protocol(s):** *Xenopus tropicalis* as a model for cell signaling and development

**AUP Number(s):** 1307-2016-AP  (4 digits only)

**Principal Investigator:** Shuo Wei, Ph.D.

**Common Name:** Western clawed frog

**Genus Species:** *Xenopus tropicalis*

**Pain Category:** *(please mark one)*

<table>
<thead>
<tr>
<th>USDA PAIN CATEGORY: <em>(Note change of categories from previous form)</em></th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>□ B</td>
<td>Breeding or holding where NO research is conducted</td>
</tr>
<tr>
<td>□ C</td>
<td>Procedure involving momentary or no pain or distress</td>
</tr>
<tr>
<td>□ D</td>
<td>Procedure where pain or distress is alleviated by appropriate means (analgesics, tranquilizers, euthanasia etc.)</td>
</tr>
<tr>
<td>□ E</td>
<td>Procedure where pain or distress cannot be alleviated, as this would adversely affect the procedures, results or interpretation</td>
</tr>
</tbody>
</table>

IACUC approval of major changes to an animal protocol must be reviewed and approved prior to initiating the work.

Official Use Only

IACUC Approval Signature: [Signature]

Date of Approval: 10/12/16
## Principal Investigator Assurance

1. I agree to abide by all applicable federal, state, and local laws and regulations, and UD policies and procedures.

2. I understand that deviations from an approved protocol or violations of applicable policies, guidelines, or laws could result in immediate suspension of the protocol and may be reportable to the Office of Laboratory Animal Welfare (OLAW).

3. I understand that the Attending Veterinarian or his/her designee must be consulted in the planning of any research or procedural changes that may cause more than momentary or slight pain or distress to the animals.

4. I declare that all experiments involving live animals will be performed under my supervision or that of another qualified scientist listed on this AUP. All listed personnel will be trained and certified in the proper humane methods of animal care and use prior to conducting experimentation.

5. I understand that emergency veterinary care will be administered to animals showing evidence of discomfort, ailment, or illness.

6. I declare that the information provided in this application is accurate to the best of my knowledge. If this project is funded by an extramural source, I certify that this application accurately reflects all currently planned procedures involving animals described in the proposal to the funding agency.

7. I assure that any modifications to the protocol will be submitted to the UD-IACUC and I understand that they must be approved by the IACUC prior to initiation of such changes.

8. I understand that the approval of this project is for a maximum of one year from the date of UD-IACUC approval and that I must re-apply to continue the project beyond that period.

9. I understand that any unanticipated adverse events, morbidity, or mortality must be reported to the UD-IACUC immediately.

10. I assure that the experimental design has been developed with consideration of the three Rs: reduction, refinement, and replacement, to reduce animal pain and/or distress and the number of animals used in the laboratory.

11. I assure that the proposed research does not unnecessarily duplicate previous experiments. *(Teaching Protocols Exempt)*

12. I understand that by signing, I agree to these assurances.

**Signature of Principal Investigator**  
**Date**  

---

**Rev 10/2014**

---

58
SIGNATURE(S) OF ALL PERSONS BEING ADDED TO THIS PROTOCOL

I certify that I have read this protocol, accept my responsibility and will perform only the procedures that have been approved by the IACUC.

<table>
<thead>
<tr>
<th>Name</th>
<th>Signature</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Huaping Xie</td>
<td></td>
</tr>
<tr>
<td>2. Jian Wang</td>
<td></td>
</tr>
<tr>
<td>3. Mark Perfetto</td>
<td></td>
</tr>
<tr>
<td>4. Congyu Lu</td>
<td></td>
</tr>
<tr>
<td>5. Aditi Makhija</td>
<td></td>
</tr>
<tr>
<td>6. Pathirenehelage Chandrasekera</td>
<td></td>
</tr>
<tr>
<td>7. Melinda Duncan</td>
<td></td>
</tr>
<tr>
<td>8. Yan Wang</td>
<td></td>
</tr>
<tr>
<td>9. Christopher Materna</td>
<td></td>
</tr>
<tr>
<td>10. Click here to enter text.</td>
<td></td>
</tr>
<tr>
<td>11. Click here to enter text.</td>
<td></td>
</tr>
<tr>
<td>12. Click here to enter text.</td>
<td></td>
</tr>
<tr>
<td>13. Click here to enter text.</td>
<td></td>
</tr>
<tr>
<td>14. Click here to enter text.</td>
<td></td>
</tr>
<tr>
<td>15. Click here to enter text.</td>
<td></td>
</tr>
</tbody>
</table>
Please complete the table below and state whether the personnel have received animal facility training. (Include specific details of type of work and the number of years’ experience with proposed species. Indicate status: Post-Doc, Ph.D., Graduate, Undergraduate, Student, Technician, etc. If no experience, list who will train.)

<table>
<thead>
<tr>
<th>Name</th>
<th>Home Phone Number</th>
<th>Office Phone Number</th>
<th>Received Animal Facility Training</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Huaping Xie</td>
<td>319-383-9983</td>
<td>831-6094</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>x</td>
</tr>
</tbody>
</table>

Status: Post-Doc (Wei Lab)

Qualifications: 1 year experience working with *X. tropicalis* and 8 years working with other aquatic vertebrates (such as zebrafish)

Responsibilities: Carry out experiments using frogs, participate in frog husbandry, and train other lab personnel

<table>
<thead>
<tr>
<th>Name</th>
<th>Home Phone Number</th>
<th>Office Phone Number</th>
<th>Received Animal Facility Training</th>
</tr>
</thead>
<tbody>
<tr>
<td>2. Jian Wang</td>
<td>304-276-1938</td>
<td>831-6094</td>
<td>x</td>
</tr>
</tbody>
</table>

Status: Post-Doc (Wei Lab)

Qualifications: 5 years experience working with other aquatic vertebrates (such as rainbow trout). Will be trained by Shuo Wei, Huaping Xie and Mark Perfetto.

Responsibilities: Carry out experiments using frogs, and participate in frog husbandry

<table>
<thead>
<tr>
<th>Name</th>
<th>Home Phone Number</th>
<th>Office Phone Number</th>
<th>Received Animal Facility Training</th>
</tr>
</thead>
<tbody>
<tr>
<td>3. Mark Perfetto</td>
<td>301-928-0303</td>
<td>831-6265</td>
<td>x</td>
</tr>
</tbody>
</table>

Rev 10/2014

# 1307-2016-AP
Status: Graduate Student (Wei Lab)

Qualifications: 4 years experience working with *X. tropicalis*.

Responsibilities: Carry out experiments using frogs, participate in frog husbandry, and train other lab personnel.

| 4. Congyu Lu | 302-897-5839 | 831-6265 | Click here to enter text. | x |

Status: Graduate Student (Wei Lab)

Qualifications: No experience. Will be trained by Shuo Wei, Huaping Xie and Mark Perfetto.

Responsibilities: Carry out experiments using frogs, and participate in frog husbandry.

| 5. Aditi Makhija | 302-510-2689 | 831-6265 | Click here to enter text. | x |

Status: Graduate Student (Wei Lab)

Qualifications: No experience. Will be trained by Shuo Wei, Huaping Xie and Mark Perfetto.

Responsibilities: Carry out experiments using frogs, and participate in frog husbandry.

| 6. Pathirennelahage Chandrasekera | 254-723-2799 | 831-6265 | Click here to enter text. | x |
Status: Graduate Student (Wei Lab)

Qualifications: No experience. Will be trained by Shuo Wei, Huaping Xie and Mark Perfetto.

Responsibilities: Carry out experiments using frogs, and participate in frog husbandry

<table>
<thead>
<tr>
<th>7. Melinda Duncan</th>
<th>302-593-5074</th>
<th>831-0533</th>
<th>x</th>
<th>Click here to enter text.</th>
</tr>
</thead>
</table>

Status: Professor

Qualifications: 30 years experience working with vertebrate model organisms including mice, rats, rabbits, chickens, fish, and frogs

Responsibilities: Supervise experiments using frogs

<table>
<thead>
<tr>
<th>8. Yan Wang</th>
<th>302-897-3712</th>
<th>831-0497</th>
<th>x</th>
<th>Click here to enter text.</th>
</tr>
</thead>
</table>

Status: Lab manager (Duncan Lab)

Qualifications: Will be trained by Shuo Wei, Huaping Xie and Mark Perfetto.

Responsibilities: Carry out experiments using frogs

<table>
<thead>
<tr>
<th>9. Christopher Materna</th>
<th>978-866-1211</th>
<th>Click here to enter text.</th>
<th>Click here to enter text.</th>
<th>x</th>
<th></th>
</tr>
</thead>
</table>
Status: Graduate Student (Duncan Lab)

Qualifications: No experience. Will be trained by Shuo Wei, Huaping Xie and Mark Perfetto.

Responsibilities: Carry out experiments using frogs

## Project Personnel Deletions:

<table>
<thead>
<tr>
<th>Name</th>
<th>Effective Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Click here to enter text.</td>
<td>Click here to enter text.</td>
</tr>
<tr>
<td>2. Click here to enter text.</td>
<td>Click here to enter text.</td>
</tr>
<tr>
<td>3. Click here to enter text.</td>
<td>Click here to enter text.</td>
</tr>
<tr>
<td>4. Click here to enter text.</td>
<td>Click here to enter text.</td>
</tr>
<tr>
<td>5. Click here to enter text.</td>
<td>Click here to enter text.</td>
</tr>
</tbody>
</table>