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Epithelial membrane protein 3 (Emp3b) is a novel regulator of sympathetic nervous system development in zebrafish.

Jessica M. White
Virginia Commonwealth University

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A thesis submitted in partial fulfillment of the requirements for degree of Master of Science at Virginia Commonwealth University.

by

Jessica M. White,

B.S. Biology, VCU 2019

Advisor: Erich W. Damm, Ph.D.

Assistant Professor, Department of Biology

Virginia Commonwealth University

Richmond, VA

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List of Abbreviations

AGM	Aorta gonad mesonephros
ALK	Ana-plastic lymphoma kinase
anti-HU	Antibody targeting RNA-binding proteins in neurons
ascl1a	*achaete-scute family bHLH transcription factor 1a*
bp	Base pair
BCIP	5-bromo-4-chloro-3-indolyphosphate
BMP	Bone morphogenetic protein
cDNA	Complementary DNA
CRISPR	Clustered regularly interspaced short palindromic repeats
DA	Dorsal aorta
dbh	*dopamine beta hydroxylase*
DEPC	Diethyl pyrocarbonate
DI	Deionized
DNA	Deoxyribonucleic acid
dNTP	Dideoxyribonucleotide triphosphate
dpf	Days post fertilization
DRG	Dorsal root ganglia
E3	Embryo medium
E.coli	*Escherichia coli*
ednrab	*endothelin receptor ab*
EDTA	Ethylenediaminetetraacetic acid
Egf	Epithelial growth factor
Egfr	Epithelial growth factor receptor
Emp3	Epithelial membrane protein 3
emp3b	*epithelial membrane protein 3b*
EMT	Epithelial to mesenchymal transition
erbb3b	*receptor tyrosine-protein kinase erbB3b*

FGF	fibroblast growth factor
fli-1	Friend leukemia integration transcription factor
foxd3	*forkhead box D3*
gata2	*gata binding protein 2*
gata3	*gata binding protein 3*
g	grams
GFP	Green fluorescent protein
sgRNA	Single Guide RNA
GS	Goat serum
HCC	Hepatocellular carcinoma
hpf	Hours post fertilization
HSC	Hematopoietic stem cells
iPSC	Induced pluripotent stem cells
ISH	*In situ* hybridization
mRNA	Messenger RNA
$MgCl_2$	Magnesium chloride
mL	Milliliters
MO	Morpholino antisense oligonucleotide
N.A.	Numerical aperture
NaCl	Sodium chloride
NBT	4-Nitro blue tetrazolium chloride
NC	Neural crest
NCC	Neural crest cells
ng	Nanogram
NO	Notochord
Notch	Type-1 transmembrane protein
NPB	Neural plate boarder
NRG	Neuregulin

NT	Neural tube
PBS	Phosphate-buffered saline
PBT	PBS with 0.1% Tween-20
PBTx	PBS with 0.1% Triton X
PCR	Polymerase chain reaction
PCV	Post cardinal vein
PFA	Paraformaldehyde
phox2a	*paired like homeobox 2A*
phox2b	*paired like homeobox 2Bb*
PI	Principal Investigator
RNA	Ribonucleic acid
RPM	Rotations per minute
SA	Sympathoadrenal
SCG	Superior cervical ganglion
sgRNA	Single guide RNA
SNS	Sympathetic nervous system
SSC	Saline-sodium citrate
Sox10	*sry-box transcription factor 10* (NC marker)
TAE	Tris-Acetate-EDTA
Taq	*Thermus aquaticus*
th	*tyrosine hydroxylase*
Tg	Transgenic
µL	microliters
UTR	Untranslated region
V	Volts
Wnt	Lipid-modified ligands of the Wnt signaling pathway
WT	Wildtype

Abstract

EPITHELIAL MEMBRANE PROTEIN 3 (EMP3B) IS A NOVEL REGULATOR OF SYMPATHETIC NERVOUS SYSTEM DEVELOPMENT IN ZEBRAFISH.

By Jessica M. White, B.S.

A thesis submitted in partial fulfillment of the requirements for degree of Master of Science at Virginia Commonwealth University.

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Director: Erich W. Damm, Ph.D.
Assistant Professor, Department of Biology

Notable progress has been made in understanding the development of the neural crest (NC). Neural crest cells (NCCs) can be divided into 4-5 subpopulations. A major lineage among trunk NC derivatives are sympathoadrenal (SA) cells, which give rise to the sympathetic nervous system (SNS). Trunk NCCs follow a ventromedial migration pathway where signals derived from the dorsal aorta instruct migrating NCCs to a SA cell fate. Defects in NC development can result in syndromes known as neurocristopathies. Neuroblastoma is one of the most common forms of pediatric cancer affecting nearly 800 infants each year in the United States (Takita *et al.*, 2021). This neurocristopathy is characterized by extracranial solid tumors caused by malignancy of the SNS, specifically SA precursor cells. In high-risk neuroblastoma, *EMP3* is highly methylated, correlating with low levels of *EMP3* mRNA and protein expression (Wang *et al*., 2017) suggesting a potential tumor suppressor function. However, other studies have shown that the overexpression of *EMP3* promotes proliferation and migration but suppresses cell adhesion in urinary cancer (Wang *et al.*, 2017). EMP3 signaling has been studied in the context of cancer, but its role in embryonic development has not been described. Here I examine the role of *emp3b* during SNS

development in zebrafish. Overall, my work suggests when *emp3b* is knocked down, SA progenitor cells do not differentiate into the SNS. Through further investigation I found that normal NC migration is disrupted in Emp3b morphants. In conclusion this study demonstrates a potential mechanism by which *emp3b* regulates SNS development and NC migration through *erbb3b* regulation. EMP3 signaling is highly complex and misunderstood. Therefore, additional investigation is required to clarify the factors that regulate *EMP3* expression and activity to understand its involvement in NC migration and SNS development.

Introduction

Neural Crest

The neural crest (NC) is a transient embryonic population that is sometimes considered to be the 4th germ layer because of the diversity of tissue types that it contributes to. It is composed of transient, migratory cells that originate from the neural plate borders (NPB) on either side of the neural plate (Le Douarin *et al.*, 2004). NPB genes activate expression of NC regulatory genes, such as *SOX10*, through specific and complex regulatory interactions between the neural and non-neural ectoderm (Rocha *et al.*, 2020).

After neurulation, NCCs undergo an epithelial-to-mesenchymal transition (EMT). Epithelial cells are pre-migratory NCCs that are characterized by apically localized adherens junctions and a basal lamina (Taneyhill, 2008). Migratory NCCs have mesenchymal characteristics that facilitate increased spreading by exhibiting a flattened morphology with filopodia and lamellipodia. The EMT process induces changes in cell–cell adhesion and polarity; that permits the cytoskeleton to remodel and gain migratory and invasive properties enhancements (Roche, 2018). During EMT, these cells experience dynamic changes in cadherin expression at different developmental stages. This is considered the "cadherin switch", where NCCs expressing E-cadherin switch to expressing N-cadherin on the cell surface which allows for the re-setting of the intracellular polarity (Rocha *et al.*, 2020). E-cadherin is essential for contact inhibition of locomotion (CIL) by controlling protrusions. CIL is a process during migration where cells change their direction upon contact with another cell (Mayor and Carmona-Fontaine, 2010). In contrast, N-cadherin is expressed in migratory NCCs, where it plays a critical role in promoting migration. Cadherins are cell surface expressed cell-cell adhesion proteins that are important for NCC specification because they

segregate NCCs from other cells in the neural tube, while influencing specific interactions between NCC sub-populations during migration and differentiation (Taneyhill, 2008).

Once EMT takes place NCCs migrate along stereotypical pathways in the head and trunk to embryonic tissues in specific locations. These migratory pathways are highly conserved among vertebrates. The fate of NCCs occurs along the regionalization of the neural crest. NCCs can be divided into 4-5 subpopulations; cranial, cardiac, vagal, trunk and sometimes sacral, depending on their origin along the anterior-posterior axis of the embryo. The cranial NCCs that invade the surrounding cranial mesenchyme give rise to chondrocytes, osteocytes, cranial sensory ganglia, pigment cells, connective tissue, Schwann cells, and satellite cells (Rocha *et al.,* 2020). The vagal and cardiac NCCs are an overlapping subpopulation that generate cardiomyocytes, outflow tract and bulbus arteriosus, enteric neurons, and glial cells (Rocha *et al.,* 2020). Trunk NCCs follow a ventromedial migration pathway to form sympathoadrenal progenitor cells that will generate sympathetic neurons, adrenal chromaffin cells, and Schwann cells. Trunk NCCs migrating on this pathway will also generate the dorsal root ganglia (DRG). Another subset of trunk NCCs follow the dorsolateral migration path to form pigment cells, including melanophores, xanthophores, iridophores and cyanophores (Vega-Lopez, 2017).

Sympathetic Nervous System

In zebrafish, streams of ventrally migrating trunk NCCs follow evolutionarily conserved medially and laterally restricted pathways (Rocha *et al.,* 2020). These cells can be identified by NC markers such as *SOX10* and *EDNRA*, where their expression is regulated by WNT, BMP, FGF, and NOTCH signaling pathways (Ji *et al.,* 2019). The trunk NC begins to migrate at 18 hpf. During migration these NCCs pass by a multitude of cell types such as the neural tube (NT), notochord (NO), and dorsal aorta (DA) that express different signaling molecules. These cells populate the

region adjacent to the DA between 24 hpf and 36 hpf, where signaling molecules may be selectively expressed and sympathoadrenal (SA) progenitor cells may exhibit differential responsiveness. Only NCCs following the ventromedial pathway give rise to SA cells, which are the precursor cells of sympathetic neurons, associated Schwann cells and chromaffin cells of the adrenal medulla (Morrison *et al*., 2016).

During migration, SA progenitor cells express ERBB3 receptors on the outer membrane to facilitate the recognition of the ligand Neuregulin-1 (NRG-1) and other growth factors that are secreted from endothelial cells in the DA. NRG-1 is a multifunctional protein and part of the epidermal growth factor family of receptor tyrosine kinase protein ligands that can signal in an autocrine or paracrine manner (Teo *et al.,* 2016). NRG-1 expression is known to play a fundamental role in SA progenitor cell migration to its target site as well as its origin. Migrating trunk NCCs that lack components of the NRG-1 pathway are unable recognize their target location or exhibit serve underdevelopment of the sympathetic ganglion chain, while the migration of cranial NC-derived enteric neurons seems normal (Britsch *et al.,* 1998; Morrison *et al*., 2016). This is a crucial pathway involved with trunk NC migration that in turn controls sympathetic neuron development.

Sympathetic neurons of the SNS are responsible for mediating the fight or flight response in organisms through catecholaminergic neurotransmitters in response to external stressors. In parallel, chromaffin cells are secretory endocrine cells that directly deposit catecholamines and endorphins into circulation once stress is stimulated (Morrison *et al.,* 2016). Migrating SA progenitor cells that derive from trunk NCCs receive BMP signaling cues from the DA to induce their differentiation into sympathetic neurons and chromaffin cells. BMP-induced SNS development results in the activation of various transcription factors. These include the paired

homeodomain factors *PHOX2A* and *PHOX2B*, which are essential for the survival of sympathetic neuroblasts; the zinc finger factors *GATA2/3* that are responsible for regulating the genes that encode the enzymes required for norepinephrine synthesis, and *ASCL1* (*zash-1a*) that regulates SNS proliferation (Morikawa *et al.*, 2009).

During SNS development, two populations of sympathetic ganglion neurons differentiate at different times. At 48 hpf the rostral population that is composed of the superior cervical ganglia (SCG), which is the largest and most anterior sympathetic ganglion, differentiates first. This involves SA progenitor cells expressing *PHOX2B* acquiring the noradrenaline (NA) neurotransmitter phenotype (Morrison *et al.* 2016). This shift in cellular functionality is marked by the presence of NA and the expression of NA associated genes such as *tyrosine hydroxylase (TH)*, and *dopamine-β-hydroxylase (DBH)* (Teo *et al.*, 2016). The expression of *TH* is the main indicator of fully formed and differentiated sympathetic neurons because it is a required enzyme involved in the conversion of amino acid L-tyrosine to NA. Neurons expressing *DBH* also marks SNS differentiation because it is required for the enzymatic conversion of dopamine to NA, although it is also associated with dopaminergic neurons in the central nervous system (Morikawa *et al.*, 2009). This is considered a key point in SNS development as these genes participate in the expression and maintenance of sympathetic neuron function. Then, at 5 dpf the more caudal trunk sympathetic neurons develop as a row of clustered or single neurons adjacent to the DA.

When NC Development Goes Wrong

Because of the diversity of cell types that originate from the NC, defects in NC development can result in syndromes known as neurocristopathies. This happens when defects in NCC specification and migration occur. The effect may vary depending on the location of the dysfunction (Cibi *et al.*, 2019). Some neurocristopathies include Cleft palate; where the tissues of the mouth or lip do

not form properly during development, Treacher Collins syndrome; which affects the way the bones and tissues of the face develop resulting in distinctive craniofacial abnormalities, Familial Dysautonomia; where the lack of certain nerve survival and development cause poor growth, frequent lung infection, and decreased pain and temperature perception, Neuroblastoma; characterized by lumps or swelling with no pain, weight loss, and problems breathing, and Melanoma; when the skin develops a tumor derived from melanocytes (Xi and Lui, 2021).

Since there is a wide distribution of tissues that the NC contributes to, neurocristopathy phenotypes are often complex and involve multiple organ systems. Although most neurocristopathies follow a Mendelian pattern of inheritance, the large number of genes involved in migration and differentiation of the NC makes determining the manner of causation difficult and distinct (Cibi *et al.,* 2019). The DA and other surrounding tissues provide signals that dictate trunk NCC differentiation and final location in the embryo (Rogers *et al*., 2012). Understanding the genes and signaling networks that are involved is crucial to understanding NCC development and can be used to explore NC developmental mechanisms that could contribute to the development of neurocristopathies.

Neuroblastoma

Neuroblastoma is one of the most common forms of pediatric cancer affecting nearly 800 infants each year in the United States (Takita, 2021). It is characterized by extracranial solid tumors caused by malignancy of the sympathetic nervous system (SNS), specifically sympathoadrenal precursor cells. The dysregulation of cell proliferation and survival of the sympathetic ganglia contribute to neuroblastoma (Morrison *et al.,* 2016). Tumors can arise anywhere along the SNS, primarily from the adrenal medulla or paraspinal ganglia. Early-stage neuroblastoma is treatable with a low likelihood of complications. Conversely, later-stage, aggressive tumors often infiltrate and

surround critical nerves, vessels, and local organ structures such as the regional lymph nodes and bone marrow by means of the hematopoietic system, with no opportunity to resect the tumor at the time of diagnosis (Maris, 2010). Mutations resulting in oncogene activation, such as the tyrosine kinase domain of the *ana-plastic lymphoma kinase* (*ALK*) oncogene, and loss-of-function mutations in the homeobox gene *PHOX2B*, which encodes a DNA-binding protein that regulates gene expression and cell differentiation, has been suggested to be the genetic cause of neuroblastoma (Maris, 2010).

The study of cell communication is fundamental when examining the molecular basis of disease. Receptor proteins that are embedded in the cell membrane are involved in the molecular connection that triggers the ongoing dynamic chemistry occurring inside a cell in response to the external environment (Alberts *et al.*, 2002). Cells display a set of receptors that enable responses to a corresponding set of signaling molecules produced by other cells. Cells have evolved a multitude of signaling mechanisms, where signaling molecules work in combinations to regulate the behavior of the cell to relay crucial biological information (Alberts *et al.*, 2002). For an example, growth factors interact with transmembrane receptors that regulate gene expression; adhesion molecules can convey tension-generated forces that direct cells to remain or pivot direction of movement; and developmentally regulated receptors that guide the path of a migrating cell (Taneyhill, 2008). Inappropriate induction of cell signaling can result in systemic issues including adverse effects on neighboring tissues. This suggests that cell lineages that derive from a common tissue, such as the trunk NCC, may be linked to or contribute to the development of another NC derived tissue (Rodger *et al.*, 2012). The signals developing cells receive are contingent on proximity to the DA or other neighboring cells, such as glial and Schwann cells that provide regulatory signals. Identifying all regulators of SNS development is essential to improve

the understanding of NC development and how changes in NC development can lead to neurocristopathies (Bronner and LeDouarin 2012).

Epithelial membrane protein 3

Epithelial membrane protein 3b (*emp3b*) was identified by our laboratory as a potential novel regulator of zebrafish NC development through a microarray subtractive profiling screen strategy, where it was found to be the gene with the most significant increase in expression in migratory NCCs when compared with pre-migratory NCCs. Human epithelial membrane protein 3 (*EMP3*) is the ortholog of the *emp3a* and *emp3b* paralogs in zebrafish. This protein has a 4-pass transmembrane structure with a conserved claudin domain. *EMP3* is a member of the peripheral myelin protein 22-kDa (*PMP22*) gene family according to its structural homology. This group of proteins has been demonstrated to be involved in the regulation of cell survival, cell proliferation, membrane organization, signal transduction processes, and cell-to-cell interactions (Fumoto *et al.*, 2008; Martija and Pusch, 2021). EMP3 has been demonstrated to crosstalk with integrins and growth factor receptors such as EGFR (Wang *et al.,* 2014). Recently, reduced expression of *ERBB2*, a member of the *EGFR* family, was seen when *EMP3* was knocked down in human bladder cancer cells. There also appeared to be a reduction in *EMP3* expression when *ERBB2* was knocked out. Interestingly, when *EMP3* was overexpressed, there was an observed increase in *ERBB2* expression (Martija and Pusch, 2021). This suggests that Emp3 regulates gene transcription, most likely mediated by its transmembrane domains.

EMP3 has been implicated as a tumor suppressor. In high-risk neuroblastoma and glioma, the 19q13 chromosomal region containing the *EMP3* gene is highly methylated, correlating with low levels of *EMP3* mRNA and protein expression (Wang *et al*., 2017). This phenotype has been associated with a very poor clinical prognosis for neuroblastoma and suggests that EMP3 could

function as a tumor suppressor. Tumor suppressors regulate several cellular processes including the inhibition of uncontrolled cell proliferation, and the regulation of cell survival. Loss of tumor suppressor regulatory function due to loss of expression or deleterious mutation can result in an overgrowth of cells leading to tumor development (Cooper, 2000). In some cancers, EMP3 has been seen to promote malignancy. *EMP3* expression levels were observed to be significantly higher in hepatocellular carcinoma (HCC) tissue compared with healthy tissue and this was negatively correlated with the ability of tumor cells to differentiate. Following knockdown of *EMP3,* the growth of HCC was significantly suppressed, and the cells were arrested at the G1 phase of the cell cycle (Hsieh *et al*., 2015). Similarly, in breast cancer, the levels of *EMP3* expression in the carcinomas present in primary breast tissues were higher than in healthy breast tissue. They demonstrated this claim by repressing the expression of *EMP3*, using a microRNA that is typically downregulated in breast carcinomas. Although the tissues exhibited clear growth, metastatic activity was inhibited when *EMP3* was suppressed. This suggests *EMP3* may function as an oncogene (Wang et al., 2017). It appears that the role of EMP3 in cancer is highly context dependent.

Zebrafish

The unique embryonic cell type called the NC arose early in vertebrate evolution and has remained conserved in all vertebrates (Bronner *et al.,* 2012). Because of this conservation, researchers can study NC development in a variety of vertebrate model organisms with the likelihood that the findings will be representative across the vertebrate subphylum. NC development has been very well characterized in the zebrafish embryo, which has resulted in the discovery of molecular mechanisms that underlie neural crest specification, differentiation, and migration. For this reason, I have chosen the zebrafish embryo as a model system to explore the role of *emp3b* in NC

development. There are several advantages to using zebrafish as a model organism. The zebrafish genome is fully sequenced and revised regularly and 84% of genes known to be associated with human disease have a zebrafish homolog (Howe *et al.*, 2013). They produce nearly transparent embryos allowing for live imaging of the development of all organ systems. Zebrafish embryos are externally fertilized allowing for collection of large numbers of embryos and forward and reverse genetic techniques are highly advanced. In comparison to mice, zebrafish are small with relatively inexpensive husbandry (Teame *et al.*, 2019 and Gore *et al.*, 2018).

Outline and Objectives

Experimental Approach

To examine the role of Emp3b in SNS development, I will knock down expression of this protein using an antisense morpholino oligonucleotide (MO) that binds to either the start codon of *emp3b* mRNA or binds to a specific sequence that straddles the boundary between exon 2 and intron 2 of the *emp3b* pre-mRNA transcript to prevent the translation or spliceosome processing respectively. Embryos will be injected with a concentration of 1 ng/nl, at the 1-cell stage to ensure incorporation in embryonic cells through yolk streaming. Embryos will also be injected with sgRNAs and Cas9 protein targeting four locations within the *emp3b* gene to generate mutant embryos for analysis. The strains of zebrafish that will be injected are wild-type AB/WIK and *Tg(sox10:eGFP),* and *Tg(fli1:eGFP)* transgenic lines, where *sox10* expressing NCCs are labeled with green fluorescent protein (GFP) and *fli1* expressing endothelial cells of vascular network at labelled with GFP.

In situ hybridization (ISH) will be used to qualify differences in expression of NC and SNS marker genes in uninjected embryos, Emp3bMO injected embryos and *emp3b* crispant embryos at the 24 hpf, 36 hpf, and 48 hpf developmental stages. Fluorescent immunohistochemistry will be used to

examine sympathetic ganglion formation, localization of SNS marker proteins, and cell death in uninjected embryos and Emp3bMO injected embryos at 24 hpf, 48 hpf, and 5-7 dpf developmental stages .

NC and SNS Development

Preliminary experiments from our laboratory have shown that Emp3b is required for development of hematopoietic stem cells (HSCs). This is likely because Emp3b is regulating normal development of NC derived SNS precursor cells, which is a prerequisite for HSC development (Damm and Clements, 2017). However, no developmental role has been determined for this gene. Here, I show that Emp3b knockdown results in defects in the development cranial and trunk NC derivatives including jaw cartilage and melanophores suggesting that Emp3b could be required for normal development of NC derivatives. My work also shows that knockdown of Emp3b results in defects in SNS development as indicated by reduced expression of *th* and *dbh*, which are NC-derived neuronal markers. Furthermore, I observed the same phenotype in the *emp3b* crispants, confirming the requirement of Emp3b in SNS development. Additionally, I show that knockdown of Emp3b results in the reduction of NC expression of a receptor tyrosine kinase called Erbb3b that is a member of the Egfr family, which has previously been implicated as being important for SNS development (Morrison *et al.* 2016). My work improves our understanding of the role of Emp3b in developmental processes.

Methods

Zebrafish Husbandry

AB/WIK wild type, *Tg(fli1:EGFP)* (Lawson and Weinstein, 2002), and *Tg(-7.2sox10:EGFP)* (Hoffman *et al.*, 2007) adult zebrafish were kept in 6 liter tanks on an Aquaneering zebrafish

housing system. The automated system continuously circulates and filters water and monitors and maintains temperature, pH, and conductivity to keep them within the optimal parameters. In addition, the fish circadian rhythm is maintained by an automatic system that turns the lights on at 9am and off at 11pm every day. Fish were fed once daily with Gemma Micro, a complete zebrafish diet. Over the age of three months, the zebrafish are eligible for spawning. Embryos were obtained through natural mating. The afternoon before the day of spawning, adult zebrafish were separated by sex and placed in their respective chambers in the crossing tank. After breeding, the eggs were collected in 10 cm^2 petri dishes. They were raised in 1X E3 (Westerfield, 2000) at 28^{o}C. Adult zebrafish were kept under approved IACUC protocol AD10002202.

Fluorescent Immunocytochemistry

A maximum of 20 embryos fixed in 4% paraformaldehyde (PFA) were rinsed once with 1X PBTx (49.75 mL of 1X PBS + 250 µL 20% TritonX-100), and then washed 3 times in 1X PBTx for 5 minutes with gentle agitation at room temperature. Embryos were incubated in Proteinase K solution (0.5 µL of Proteinase K + 1mL of DEPC water) for 35 minutes at 37^o C and then washed once with 5% goat serum (GS) blocking solution (250 µL of GS + 4.5 mL of 1X PBTx). Embryos were incubated in ultrapure water 3 times for 1 hour each. Primary antibody (anti-HU Thermofisher #A21271 and *th* Abcam #GR3204690-9) diluted in blocking solution to a concentration of 1:500 for anti-HU or 1:250 for TH was added and incubated overnight at room temperature with gentle agitation. The next day, the embryos were washed 3 times with 1X PBTx for 5 minutes with gentle agitation. The secondary antibody (anti-HU: 594 invitrogen #A11005 and TH: 488 invitrogen #A11008), based on the host that generated the primary antibody, diluted in blocking solution to a concentration of 1:1000 for TH or 1:750 for anti-HU was added. The Eppendorf tubes were covered in foil and incubated overnight at 4^o C. The next day, the embryos

were washed with PBTx once for 5 minutes and then 6 times for 30 minutes each. They were stored in 50% glycerol/PBS solution at -20^{o}C before imaging. Embryos were imaged on either a Nikon C2 confocal microscope or a Lecia M205 FCA model organism Thunder Imager.

RNA *in situ* Hybridization Probe Synthesis

Antisense RNA probes were generated for *th, dbh, erbb3b, phox2b* and *ascl1a*. 2-5 µg of circular plasmid DNA (dependent on the concentration of DNA) was linearized by restriction enzyme digest (Table 2). Digests were set up as 5 µL of 10x CutSmart Buffer, 0.5 µL of a specific restriction enzyme, specific volume of template and DEPC water up to a total volume of 50 µL. The reaction was incubated overnight at 37^{o}C followed by a cleanup using a DNA purification kit (Qiagen QIEX II). *In vitro* transcription reactions were carried out by mixing 6 µL of linearized plasmid DNA, 2 µL 10X digoxigenin RNA labeling mix, 2 µL of 10X transcription buffer, 8 µL of DEPC water, and 2 µL of RNA polymerase. This reaction was incubated for 2 hours at 37^{o}C followed by the addition of 0.5 µL DNase1. This mixture was incubated for another 20 minutes at 37^{o}C to degrade the DNA template. To purify the probe, Illustra ProbeQuant G-50 Micro Columns were used according to the manufacturer instructions.

Bacterial Transformation

To propagate plasmid DNA, *Escherichia coli* (*E. coli*) was used to transform the plasmids. Top 10 competent cells (Invitrogen #C404004) were stored in a -80^{o} C freezer and thawed on ice for 10 minutes. 1 µL of concentrated plasmid DNA was added to the competent cell tubes, flicked to mix, and placed on ice for 30 minutes. Tubes were heat shocked at 42^{o} C for 30 seconds and then placed on ice for 5 minutes. 950 µL of S.O.C. Outgrowth medium (New England Biolabs #B9020S) was added, and tubes were shaken at 250 rpm for 1 hour. Then, 200 µL of that solution was added to

Luria Bertani (LB) agar plates warmed to 37° C. Plates were incubated overnight at 37°C. The following day, single colonies of bacteria from the plate were used to inoculate 5 mL LB broth cultures containing 0.2% ampicillin. Culture was shaken at 250 rpm overnight. The following day, plasmid was purified from the cultured bacteria using the GeneJET plasmid miniprep kit (Thermo Scientific #K0503) according to manufacturer instructions. The concentration of the purified plasmid was measured by a NanoDrop and stored at -20° C.

CRISPR-cas9 Mutagenesis

Single guide RNAs (sgRNAs) targeting the *emp3b* gene were designed using the CHOPCHOP online algorithm (https://chopchop.cbu.uib.no) (Table 1). sgRNAs (Synthego) were designed to target exons 2, 3 and 4. 250 pg of each sgRNA (total 1 µg) and 1 µg of Cas9 protein (NEB) was injected into embryos at the single cell stage. Genomic DNA was extracted from a subset of injected embryos using lysis buffer (80mM Tris pH 8.3, 200mM NaCl, 0.5% SDS, 5mM EDTA, 1 mg/ml Proteinase K). PCR was performed using primers that flanked the sgRNA binding sites and PCR products were sent for DNA sequencing (Eurofins Genomics) to confirm mutagenesis. The remaining crispant embryos were fixed with 4% PFA and used in downstream experiments.

Confocal Microscopy

The processed embryos stored in 50% glycerol/PBS solution at -20°C were allowed to reach room temperature before imaging. Samples were mounted on slides and imaged using the 20X objective (N.A. = 0.75) with a 488 nm wavelength laser or a 561 nm wavelength laser on a Nikon C2 laser scanning confocal microscope.

Gel Electrophoresis

1% agarose gels were used (1 g of agarose and 100 mL of TAE) combined and microwaved for 3 minutes at 50% power. 5 μL of ethidium bromide was added to the mixture being casted with an 8 well comb. 1 μL of each DNA sample, 1 μL of 6X gel loading dye purple (New England Biolabs B7024S), and 4 μL of DEPC water were combined and loaded into the gel. GeneRuler DNA ladder mix (#SM0331) was used to size the sample bands. Electrophoresis was performed at 140V for 25 minutes and the BioRad Gel Doc Go imaging system was used for visualization.

***In situ* Hybridization (ISH)**

To visualize the location of gene expression we performed in ISH on embryos at 24 hpf, 36 hpf, and 48 hpf. Embryos stored in 100% methanol (MeOH) at -20^{o} C were re-hydrated gradually using a graded re-hydration series (75% MeOH, 50% MeOH, 25% MeOH for 5 minutes each, then 1X PBT 3 times for 5 minutes). Once the embryos were rehydrated proteinase K (PK) digestion was performed using a PK treatment solution (10 μg/mL proteinase K diluted in 1 X PBT) for 10-30 minutes depending on the age of the embryos. Followed by the refixing of the PK treated embryos in 4% PFA for 20 minutes. The refixed embryos were washed twice in 1 X PBT for 5 minutes each. To increase binding susceptibility the embryos were incubated in hybridization solution (hyb) (50% formamide + 5X SSC + 500 μg/mL torula tRNA + 50 μg/mL heparin + 0.1% tween-20 + 9nM citric acid (pH 6-6.5)) for a minimum of 1 hour at 67^{o} C. Then, the hyb solution was replaced with 1:50 dilution of (*th, dbh, erbb3b, phox2b, ascl1a, emp3b, crestin, ednrab*) RNA probe diluted in hyb and incubated at 67^{o}C overnight. The next day, stringency washes were performed on the embryos (Table 3). To help prevent nonspecific binding of the anti-digoxigenin antibody, the embryos were blocked in blocking solution (2% heat-inactivated goat serum + 2 mg/mL BSA in PBT) for a minimum of 1 hour at room temperature. Then, the blocking solution

was replaced with an anti-digoxigenin-alkaline phosphatase fab antibody solution (1:5000 in block solution). The embryos were incubated overnight at 4^{o}C. The following day, embryos were washed with 1 X PBT for 10 minutes 5 times, then 3 times with alkaline phosphate buffer (AP buffer) (100 Mm Tris 9.5 + 50mM $MgCl_2$ + 100mM NaCl + 0.1% Tween) for 5 minutes. Next, the appropriate amount of developing solution ((AP buffer + Nitro blue tetrazolium chloride (NBT)/5-bromo-4-chloro-3-indolyl-phosphate (BCIP)) was added. The embryos were transferred to wells of a 12-well plate. The developing time varied depending on the probe. Once desired development was reached the reaction was stopped by aspirating the developing solution and adding 100% MeOH to the wells. The stained embryos were transferred to 1.5mL Eppendorf tubes and stored at -20^{o} C.

To image the processed embryos stored in 100% methanol (MeOH) at -20^{o} C, they were re-hydrated gradually using a graded re-hydration series (75% MeOH, 50% MeOH, 25% MeOH for 5 minutes each, then 1X PBT 3 times for 5 minutes). Samples were mounted on slides and imaged using a Lecia M205 FCA model organism Thunder Imager.

Morpholino Injections

Broad *emp3b* knockdown was achieved through microinjecting the embryo yolk at the 1 cell stage with 1 ng of *emp3b* splice blocking antisense morpholino oligonucleotide (MO) or 2.5 ng of *emp3b* translation blocking MO. Universal *erbb3b* knockdown was achieved through microinjecting the embryo yolk at the 1 cell stage with 3 ng *erbb3b* splice blocking MO. After injection, embryos were dechorionated, raised to the desired age, and fixed overnight with 4% PFA in 1X PBS at 4^{o} C. Once the embryos were fixed, they were placed in 100% methanol or PBTx for further analysis. Morpholino sequences are listed in Table 4.

Polymerase Chain Reaction (PCR) and Reverse Transcription PCR (RT-PCR)

Total RNA was isolated from embryos using Trizol extraction. RNA was extracted according to the manufacturer protocol. Invitrogen Superscript IV first-strand synthesis system (ref. #18091050) was used to generate cDNA from the extracted RNA for use in PCR. The reaction (5 μg DNase treated mRNA + 1 μL oligo(dT) primer (50 μL) + 1 μL 10 mM dNTP mix, up to 10 μL DEPC H_2O) was assembled in a 0.5 ml PCR tube. It was incubated for 5 minutes at 65^oC and placed on ice for 1 minute. Then, 10 μL of the RT master mix was added to the tube. The tube was incubated in a thermocycler for 50 minutes at 50^o C, followed by 5 minutes at 85^oC. 1 μL of RNaseH was added to the tube and was incubated for 20 minutes at 37^oC to degrade the remaining RNA template. The cDNA was used for PCR. 10 μL of 2X Platinum SuperFi II PCR Master Mix (Platinum Taq HiFi polymerase, magnesium, dNTPs, and buffer), 1 μL of both forward and reverse primers (Table 1) 1 μL of cDNA, and 7 μL of DEPC water were combined. PCR was carried out and followed by purification using QIAquick PCR Purification Kit (ref. #28106).

Alcian Blue Staining

To determine if there are developmental defects in cranial and trunk NC derivatives, Alcian Blue staining was used to visualize the development of cartilage in the embryos. 5 dpf embryos were fixed with 2% PFA for 2 hours at room temperature. The fixed embryos were dehydrated in 50% EtOH/PBS for 10 minutes at room temperature. The dehydrated embryos were incubated in 0.02% Alcian Blue in PBS overnight at room temperature. The next day, the embryos were bleached with 3% H_2O_2/2% KOH for 20 minutes. The beached embryos were washed with 20% glycerol/0.25% KOH for 30 minutes at room temperature, followed by 50% glycerol/0.25% KOH for 2 hours at room temperature. The stained embryos were stored in 50% glycerol/0.1% KOH at 4^oC.

Post-fixation Pigment Bleaching

Zebrafish embryos start to develop melanocytes at 24 hpf, therefore embryos beyond that age require pigment bleaching to ensure proper visualization of the tissues of interest. Embryos stored in 100% methanol (MeOH) at -20^{o} C were re-hydrated gradually using a graded re-hydration series (75% MeOH, 50% MeOH, 25% MeOH for 5 minutes each, then 1X PBT 3 times for 5 minutes). The 1X PBT was replaced with the bleaching solution (3% H_2O_2 + 0.5% KOH in 1X PBT), then the tubes were laid on their side to avoid mixing the embryos with the generated bubbles and incubated for a minimum of 10 minutes at room temperature. Once the desired results were achieved, the embryos were washed with 1X PBT 3 times for 10 minutes each. After the washes were complete the embryos were ready for further processing.

Statistics

Statistical analysis was performed using GraphPad Prism. All data analysis used the non-parametric Mann-Whitney U-test at the 99% confidence level. All experiments were repeated with a minimum of three biological replicates.

Results

Emp3b is expressed in migrating neural crest cells

Previous work from our lab has shown that *emp3b* is expressed in NCCs but has low level expression in other tissues such as endothelial cells in 24 hpf zebrafish embryo (**Figure 1A-A'**). To confirm expression of *emp3b*, I generated an *emp3b*-specific ISH probe and examined its expression in 24 hpf zebrafish embryos. I found that *emp3b* expression corresponded with the location of trunk neural crest cells migrating on the ventromedial pathway with *emp3b* expression visible between the somites, neural tube, notochord, and axial vasculature (**Figure 1B, C**). To confirm this localization, I examined the expression of the NC marker *ednrab* in sibling embryos

(**Figure 1D**). Like *emp3b*, *ednrab* was expressed in NC cells migrating along the ventromedial pathway with expression present between the somites, neural tube, notochord, and axial vasculature. (**Figure 1C-D**). These results show that the expression of *emp3b* and *ednrab* overlap in migrating NC cells confirming the transcriptional profiling data and demonstrate that *emp3b* is expressed in migrating NCCs.

Emp3b is required for development of cranial and trunk neural crest tissues

To investigate the role of the Emp3b in NC development, I performed MO knockdown experiments using a splice-blocking MO that targeted the second exon/intron splice junction (**Figure 2A**). To confirm an effective knockdown of the Emp3b, I extracted RNA from both MO injected and uninjected embryos and performed RT-PCR for *emp3b*. The goal was to determine if the MO altered the splicing of the *emp3b* pre-mRNA. This would appear as a change in the size of the amplified DNA when compared to the amplified DNA from uninjected embryos. My results show two bands, one higher band corresponding to the expected size of the full length *emp3b* amplicon (498 bp) and a second lower band with an approximate size of 290 bp (**Figure 2B**). This lower band corresponds to the predicted size of 297 bp if exon 2 was lost from the transcript. DNA sequencing confirmed that the lower band corresponded to a morpholino induced splice variant of the *emp3b* transcript that lacked exon 2.

To determine if Emp3b is required for the development of NC derivative tissues, I examined the development of the jaw and certobranchial cartilage, which are derivatives of the cranial NC, in Emp3b morphant embryos by Alcian-blue cartilage staining at 5 dpf. I observed that jaw development appeared unaffected in Emp3b morphants, which showed Meckel's, palatoquadrate, ceratohyal and basihyal cartilage similar to uninjected control embryos (**Figure 3A-B**). However, the certobranchial cartilage, that forms the skeletal component of the branchial arches, appeared

to be fused together when compared with uninjected control embryos (**Figure 3A-B**). In addition to defects in cranial NC derivatives, I observed that melanophores, which derive from the trunk NC, exhibited aberrant morphology in Emp3b morphants compared to the uninjected embryos (**Figure 3C-D**). The melanophores on the head of Emp3b morphants exhibited finer cellular processes, appeared vacuolated and to contain less melanin compared with control embryos (**Figure 3C-D**). Together, these data show that Emp3b is required for the normal development of NC-derived tissues. Importantly, the NC appeared to be normally specified in Emp3b morphants as NCCs were present and expressed EGFP in *Tg(-7.2sox10:EGFP)* transgenic embryos in a migratory pattern similar to uninjected control embryos (**Figure 4A-B**) Additionally, the NC marker *ednrab* was expressed in migrating NCCs in Emp3bMO injected embryos (**Figure 4C-D**). Together, these data indicate that despite the defects observed in NC derived tissue development, the NC appears to be specified and migratory in *emp3b* morphant embryos.

Emp3b is required for development of the sympathetic nervous system

Since the epigenetic silencing of *emp3b* gene expression has been implicated in poor prognosis neuroblastoma and neuroblastoma originates from defective differentiation of sympathoadrenal precursor cells, I wanted to determine if Emp3b is required for normal development of the SNS. The earliest sympathetic ganglion to develop in zebrafish is the SCG (Morrison *et al.*, 2016). Th and Dbh are key enzymes required for the synthesis of catecholamines and are expressed by NC-derived SNS neurons. These genes can also serve as a marker of overt SNS neuronal specification, where they can be visualized at 48hpf in the SCG by ISH. To determine if SNS development was disrupted when *emp3b* is knocked down, I examined *th* and *dbh* gene expression in the SCG of uninjected and *emp3b* morphant embryos at 48hpf by ISH. I found that both *th* and *dbh* expression was strongly reduced in *emp3b* morphant embryos. 72% of morphant embryos (n = 79) exhibited

reduced *th* expression while 70% of morphant embryos (n = 102) exhibited reduced expression of *dbh.* (**Figure 5A-F**). In comparison only 4% (n = 48) and 4% (n = 56) of uninjected embryos exhibited reduced *th* and *dbh* expression respectively (**Figure 5A-F**). Importantly, the development of *th* and *dbh* expressing neuroectoderm derived neurons of the brain and hindbrain were unaffected in *emp3b* morphants (**Figure 5A-E'**) indicating that Emp3b is specifically required for development of neurons derived from the NC. I further confirmed that the reduction in *th* expression resulted in a reduction of Th protein expression using fluorescent immunocytochemistry. I found that Th expression in the SCG of 48 hpf morphant embryos was strongly reduced as shown by reduced fluorescence in the SCG of *emp3b* morphant embryos compared with control embryos (**Figure 6A-B**). To confirm the specificity of the *emp3b* morpholino, two sgRNAs targeting locations in exons 3 and 4 of the *emp3b* gene were co-injected with Cas9 protein at the single cell stage and ISH for *th* and *dbh* expression was performed on the resulting crispant embryos at 48 hpf (**Figure 7A-F**). Similar to *emp3b* morphant embryos, *emp3b* crispant embryos also showed a strong reduction in *th* and *dbh* expression in the SCG (**Figure 7A-F**). 89% of crispant embryos (n = 64) exhibited reduced *th* expression while 81% of crispant embryos (n = 78) exhibited reduced expression of *dbh* (**Figure 7A-F**). In comparison 22% (n = 58) and 21% (n = 113) of uninjected embryos exhibited reduced *th* and *dbh* expression respectively (**Figure 7A-F**). The effectiveness of the injected sgRNA was confirmed by DNA sequencing of PCR products corresponding to regions targeted by the sgRNA. DNA sequencing results confirmed successful mutagenesis at the sgRNA target sites as indicated by reduced electropherogram signal beyond the sgRNA target sequence (**Figure 7G-J**). This loss of signal is consistent with the indel mosaicism of crispant embryos.

To determine if Emp3b is required for the development of other NC-derived neuronal populations, I assessed the development of DRG sensory neurons. DRG sensory neurons are the second population of NC-derived neurons to differentiate within the trunk of the embryo at approximately 5 dpf (Morrison *et al.,* 2016). I examined the development of the DRG at 5 dpf by fluorescent immunocytochemistry using the pan-neuronal antibody anti-Hu in Emp3b morphant *Tg(fli1:eGFP)* embryos, which express eGFP in all vascular endothelial cells (Figure 8). I examined the somite segments located along the yolk extension and found that on average 15.5 +/- 1.5 (n = 15) segments contained DRG clusters in uninjected embryos (**Figure 8A,C**). In contrast, I observed that ganglion clusters were missing from several segments in morphant embryos (**Figure 8A-C**). On average I found that morphant embryos had significantly fewer segments with DRG clusters; 10.2 +/- 3.3 segments ($p < 0.0001$) (**Figure 8A-C**). These data suggest that Emp3b is required for the normal development of multiple populations of NC-derived neurons.

Emp3b and trunk neural crest migration

The population of migrating trunk NCCs that generates the neuronal populations such as those of the SNS and the DRG follow the ventromedial migration pathway in which the cells migrate ventrally to the DA, between the somites, neural tube, and notochord where they receive signaling cues such as BMP from the DA (Morrison *et al.,* 2016). Defects in NC migration could result in a failure of NC cells to correctly differentiate into neurons because these cells would not be exposed to requisite differentiation signals. I examined NC migration in Emp3b morphant *Tg(-7.2sox10:EGFP)* embryos (**Figure 9A-C**). *Sox10* is a robust marker of migrating NCCs and therefore all migrating NCCs express eGFP in this transgenic line. In each somite segment along the yolk extension, the NC migrates as streams of cells from dorsal positions to ventral positions

near the DA. I found that in uninjected embryos 7.8 +/- NC streams (n = 32) reach the DA by 24 hpf while on average only 1.4 +/- 1.7 NC streams (n = 32) reach the DA in *emp3b* morphants (**Figure 9A-C**). This was a significant reduction ($p < 0.0001$) in the number of NCC streams that had reached the DA by 24 hpf in *emp3b* morphants compared with uninjected embryos (**Figure 9A-C**). Rather than migrating to their normal positions near the DA, the streams of cells accumulated dorsal of the DA at the level of the notochord (**Figure 9B**). This data suggests that normal NC migration to positions near the DA is disrupted in Emp3b morphants.

Emp3b is not required for survival of neural crest cells

A potential explanation for why NCCs are not successfully migrating to the DA could be that the cells are undergoing cell death before they reach that position. To determine whether *emp3b* morphants experienced increased cell death in the NCC population, I examined *emp3b* morphant *Tg(-7.2sox10:EGFP)* embryos for the presence of cleaved caspase-3, a marker of programmed cell death, in NCCs by fluorescent immunocytochemistry. While it was challenging to optimize the Cleaved caspase-3 antibody for this experiment, I never observed colocalization between cleaved caspase-3 staining and eGFP expressing NCC streams in uninjected embryos and extremely rarely in *emp3b* morphant embryos (Avg. 0.64 +/- 2.0 streams per embryo, $p = 0.2273$) (**Figure 10A-C**). These data suggest that the migrating NCC population is not undergoing a preferential amount of cell death.

Emp3b is required for *erbb3b* expression in neural crest cells

Previous studies have shown that the EGF family receptor ERBB3 is required for normal migration of sympathoadrenal precursor cells. ERBB3 mutant embryos exhibit defective NC migration and sympathoadrenal precursor cells accumulate in dorsal positions around the notochord, failing to reach the DA (Britsch *et al.,* 1998; Crone and Lee, 2002; Murphy *et al.,* 2002). This phenotype is

highly similar to what I observed in *emp3b* morphant embryos (**Figure 9**). Emp3 signaling has previously been shown to support the expression of receptor tyrosine kinase genes, including EGF family receptors (Martija and Pusch, 2021). Zebrafish have two paralogs of *ERBB3, erbb3a* and *erbb3b*. During the stages of embryonic development where the NC is migrating, *erbb3b* is strongly expressed in the migrating NC, while *erbb3a* expression is excluded from the NC (**Figure 11A**). To determine if *erbb3b* expression is modulated by knockdown of Emp3b, I examined the expression of *erbb3b* in Emp3b morphant embryos by ISH. I found that in *emp3b* morphants, *erbb3b* expression was strongly reduced in migrating streams of NCCs at 24 hpf. The average number of *erbb3b* expressing NC streams in *emp3b* morphants was 1.8 +/- 2.0 compared with 8.5 +/- 2.3 in uninjected controls (**Figure 11A-B, D**). This was a highly significantly reduction in Emp3b morphants ($p < 0.0001$) (**Figure 11A-B, D**). I also found that *erbb3b* was strongly reduced in *emp3b* crispant embryos (**Figure 11C**). Crucially, I did not observe a change in the expression pattern of *crestin* (pan-NC marker) in *emp3b* morphant sibling embryos (**Figure 11E**), indicating that NCCs were still present in the embryo trunk; although, expression of *erbb3b* in migrating NC streams was affected. These data suggest that Emp3b could be acting through the regulation of *erbb3b* expression in the control of SNS development.

To determine if Erbb3b signaling is required for development of the SNS, embryos were injected with a splice-blocking morpholino targeting *erbb3b* mRNA and I examined the expression of key marker genes of SNS development by ISH. I found that both *th* and *dbh* expression was strongly reduced in *emp3b* morphant embryos. 100% of morphant embryos exhibited reduced *th* ($n = 28$) and *dbh* ($n = 32$) expression in the SCG at 48 hpf in the morphant embryos compared with the control embryos (**Figure 12A-E**). These results suggest that Erbb3b is required for normal SNS

development and that Emp3b could be regulating SNS development by controlling the expression of *erbb3b.*

Discussion

The protein Emp3 has been shown to be involved in the control of cell proliferation and cell-cell interactions (Martija and Pusch 2021) and has been associated with both the inhibition of cancer development and the progression of cancer. In humans, *EMP3* is located in the 19q13 chromosomal region, which is a transcriptionally repressed region in neuroblastoma suggesting it could function as a tumor suppressor in this cell type (Alaminos *et al.,* 2005). However, these studies did not identify the mechanism in which Emp3 carries out its function. Also, no developmental function has been ascribed for Emp3, specifically in the context of progenitor cells derived from the NC. The ability of the SNS to work properly is involved with other systems and essential to sustaining life. My work has aimed to understand the role of Emp3 in NC and SNS development.

My work has revealed that the zebrafish homolog, Emp3b, plays an important role in NC development, including cranial and trunk NC derivatives seen in Emp3b morphants. I found that *emp3b* is expressed in migrating NCCs, as indicated by a similar gene expression to the NC marker *ednrab*. When I examined the expression of *ednrab* there was no reduction in expression in Emp3b morphants. This shows the NC is still intact when Emp3b is knocked down. Interestingly, I found that the number of migrating NCC streams that reach the level of the DA by 24hpf was reduced in Emp3b morphants. My data suggests that NCC migration requires functional Emp3b activity.

One milestone during SNS development is the cellular acquisition of the noradrenaline phenotype as indicated by the initiation of *th* and *dbh* expression (Morrison *et al.,* 2016). Expression of these

genes is an indication of overt SNS neuronal differentiation. I found there to be a significant reduction of *th* and *dbh* gene expression, which points to a reduction in SNS neuronal development in Emp3b morphants. My work determined that when Emp3b is knocked down, SA progenitor cells do not differentiate into SNS neurons.

Based on my work, I concluded that NCC migration is likely regulated by Emp3b through the expression of *erbb3b*. This was indicated by the reduction of *errb3b* expression in Emp3b morphants. As expected, I saw reduced expression of *th* and *dbh* in Erbb3b morphants which suggests a reduction of SNS neurons. *Erbb3b* expression is found in trunk NCCs as they emerge from the neural tube and continues throughout the migration of the NCCs in the embryo (Britsch *et al.,* 1998). Trunk NCCs migrates to their final location in the embryo by ventromedial pathway migration (some pigment precursors and sympathoadrenal precursors) or dorsolateral pathway migration (predominantly pigment cell precursors) (Morrison *et al.,* 2016). The ventromedial migration pathway requires the cells to pass by the DA which releases signaling molecules such as BMP, NRGs and EGFs (Huber, 2016). Once trunk NCCs are near the DA, Erbb3b can bind to neuregulin-1 (*NRG-1*) which is an EGF-like growth and differentiation factor. The neuregulin signaling system is required during NCC migration to the mesenchyme lateral of the DA (Murphy *et al.,* 2002). *NRG-1* has been observed to be expressed at the origin of NCCs, along the migratory route and target site of SA progenitor cells (Britsch *et al.,* 1998). Previously, *emp3b* has been demonstrated to crosstalk with EGF signaling (Wang *et al.,* 2017). Here I identify a potential novel function for Emp3b in NC migration and SNS development through *erbb3b* regulation.

Embryonic catecholamine biosynthesis occurs in the SCG which requires *erbb3b* expression for normal development. Catecholamines are known to be essential for fetal development (Britsch *et al.,* 1998). *Th* and *dbh* are genes that encode key enzymes in catecholamine biosynthesis (Morrison

et al., 2016), therefore, a reduction of *th* and *dbh* expression can point to defects in trunk NC migration. For successful trunk NC derivative development, the precursor cells need to be in the right place at the right time to avoid missing crucial developmental signals that guide them to their final location in the embryo and trigger differentiation programs. The trunk NC depends on NRG-1 and Erbb3 signaling system to migrate to the mesenchyme lateral of the DA where they develop the SNS (Britsch *et al.,* 1998). Any lapse in this signaling cascade may cause the downregulation or upregulation of membrane receptors or the cells may not reach crucial structures in the embryo resulting in flawed NC development.

Phox2b is one of the numerous transcription factors that turns on prior to sympathetic neuronal differentiation of SA progenitor cells (Huber, 2006). A study on the role of Phox2b in SNS development showed significant reduction of *th* and *dbh* expression in Phox2b morphant embryos (Pei *et al.*, 2013). This is similar to the phenotype I observed when I examined the expression of *th* and *dbh* in Emp3b morphant embryos. The similarity observed in both morphants suggests the expression of *phox2b* may be involved in a mechanism by which Emp3b could direct SNS development. *Phox2b* is co-expressed with the transcription factor *ascl1a*. It was found that *th* and *dbh* expression was unaffected in *ascl1a* morphants (Pei *et al.*, 2013). This suggests the mechanism Emp3b uses to regulate SNS development may involve regulating NC migration.

Since I had an idea of the mechanism in which Emp3b regulated SNS development, I wanted to examine cell behavior. I assayed the NCCs for indications of cell death in *emp3b* morphants by looking for co-localization of NCCs in *Tg(-7.2sox10:EGFP)* embryos with cleaved caspase-3 antibody staining. I experienced challenges while optimizing the cleaved caspase-3 antibody staining, however from the data retrieved I never observed eGFP and cleaved caspase-3 antibody staining co-localization indicating that NCCs were not undergoing apoptosis. I did however

observe increased cell death in other tissues of *emp3b* morphant embryos. Therefore, it is possible that cells along the NC migration pathway may be experiencing cell death, thus influencing their migration pattern. A study that investigated the behavior of NCCs during neural development found that NCCs respond rapidly to dying cells (Zhu *et al.*, 2019). Additionally, NCCs have an unexpected phagocytic function that clears cellular debris left behind from cell death. Dead cells release *Il-1β* which stimulates an immune response that is regulated by the amount of cytokines released such as *Il-1β* (Zhu *et al.*, 2019). Overactivation of this function may have implications in their migration pattern. Inappropriate inactivation of cell signaling in one instance can result in systemic issues including adverse effects on their derivatives and neighboring systems. As previously discussed, NCCs constantly receive signaling cues from neighboring cells (Morrison *et al.*, 2016). Therefore, the death of neighboring cells could prevent NCCs from receiving crucial signals which may result in NCC migration defects in *emp3b* morphants.

To strengthen the results showing that Emp3b affects NC migration and SNS development through *erbb3b* regulation, timelapse confocal imaging of what is happening to the NCCs along the NC migration pathway using *Tg(-7.2sox10:EGFP)* could provide a better understanding of the mechanism including whether stereotypical NC migration mechanisms such as contact inhibition of locomotion are affected in *emp3b* morphants. It is also important to establish a stable *emp3b* mutant line to facilitate confirmation of *the emp3b* morphant results. Transcriptomic analysis to examine differences in gene expression between uninjected, *erbb3b* morphants and *erbb3b* mutant embryos before (18 hpf) and after (24 hpf) NC migration is initiated could help inform which genes are being upregulated or downregulated at each critical time point. Depending on the results, this kind of experiment could help determine whether Emp3b regulates NC migration, differentiation of NC derivatives or both.

My work may have implications for understanding human diseases such as neuroblastoma. The expression of *EMP3* is epigenetically silenced in high grade neuroblastoma with poor prognosis (Alaminos *et al.,* 2005) demonstrating a potential tumor suppressor function. Conversely, previous studies have shown that the overexpression of *EMP3* promotes proliferation and migration but suppresses cell adhesion in urinary cancer (Wang et al., 2017). Although *EMP3* is normally expressed in blood cells and neurons (Martija and Pusch, 2021), its role in embryonic development has not been described. My work has identified a developmental role for Emp3b in the control of NC and SNS development. Here I add Erbb3b to the growing list of crosstalk membrane proteins for Emp3b. My data suggests that the cooperation between these receptors is required for normal integrated intracellular signaling that modulates cell to cell interactions during the development of one of the most important vertebrate embryonic tissues, the NC. Identifying crucial regulators of normal NC and SNS development will lead us in the right direction when choosing a target to develop a drug or treatment to correct defects in these tissues. The extent to which *EMP3* is mutated or expressed gives researchers insight on the likelihood of patient survival regarding certain cancers (Alaminos *et al.,* 2005). Furthermore, researchers and clinicians should strive to continue to utilize epigenetics as an indicator of prognosis and need for more or less intensive therapy. Future studies should continue to characterize the functional role of Emp3 in developmental and disease processes.

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Figures and Tables

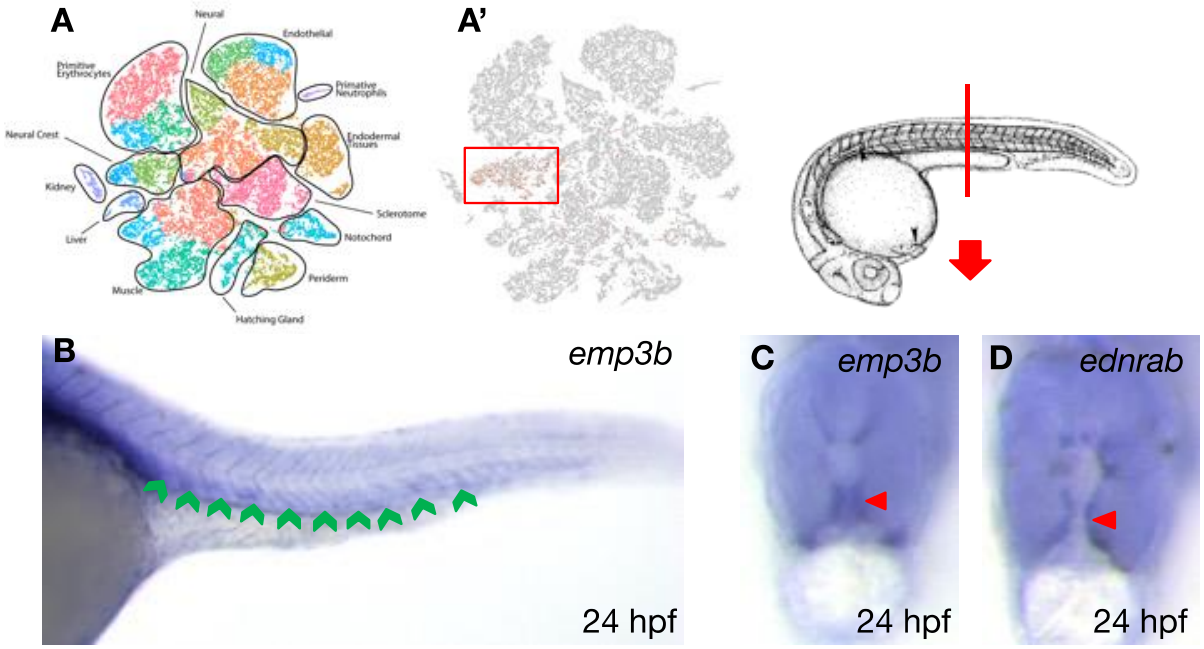

Figure 1: *emp3b* is expressed in migrating NCCs. scRNA sequencing data represented as a tsne plot showing expression of *emp3b* in the NC population (red box) at 24 hpf (**A, A'**), ISH for *emp3b* in laterally mounted (**B**) and embryo cross section (**B**) and *ednrab* in cross section (**D**). Green arrows indicates *emp3b* expression in NCCs that have migrated to the DA, red arrows indicate expression in SNS precursor NC. Zebrafish embryo *camera lucida* drawing adapted from Kimmel *et al.*, 1995.

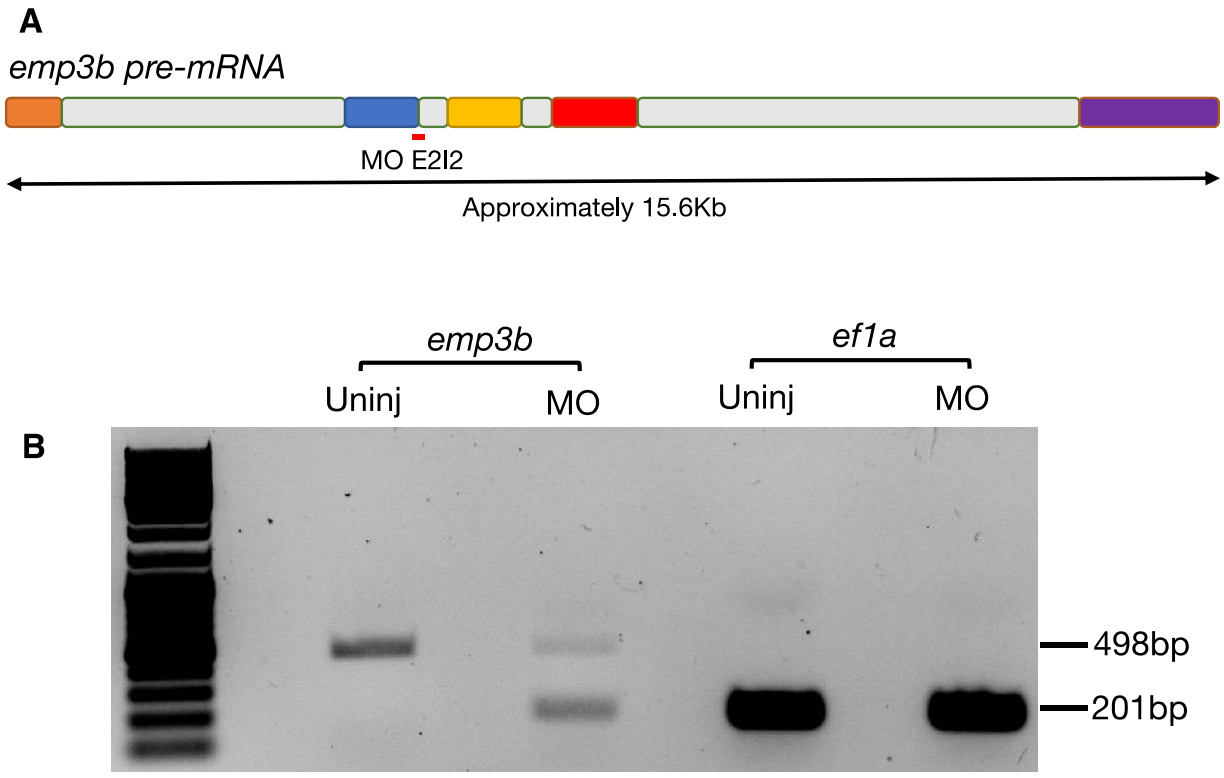

Figure 2: *emp3b* morpholino disrupts *emp3b* pre-mRNA splicing. Schematic representation of *emp3b* pre-mRNA with morpholino binding site spanning exon 2 and intron 2 boundary indicated (**A**), RT-PCR showing aberrant splicing of *emp3b* pre-mRNA splicing in Emp3bMO injected embryos, *ef1a* is used as a loading control, expected amplicon sizes are indicated (**B**).

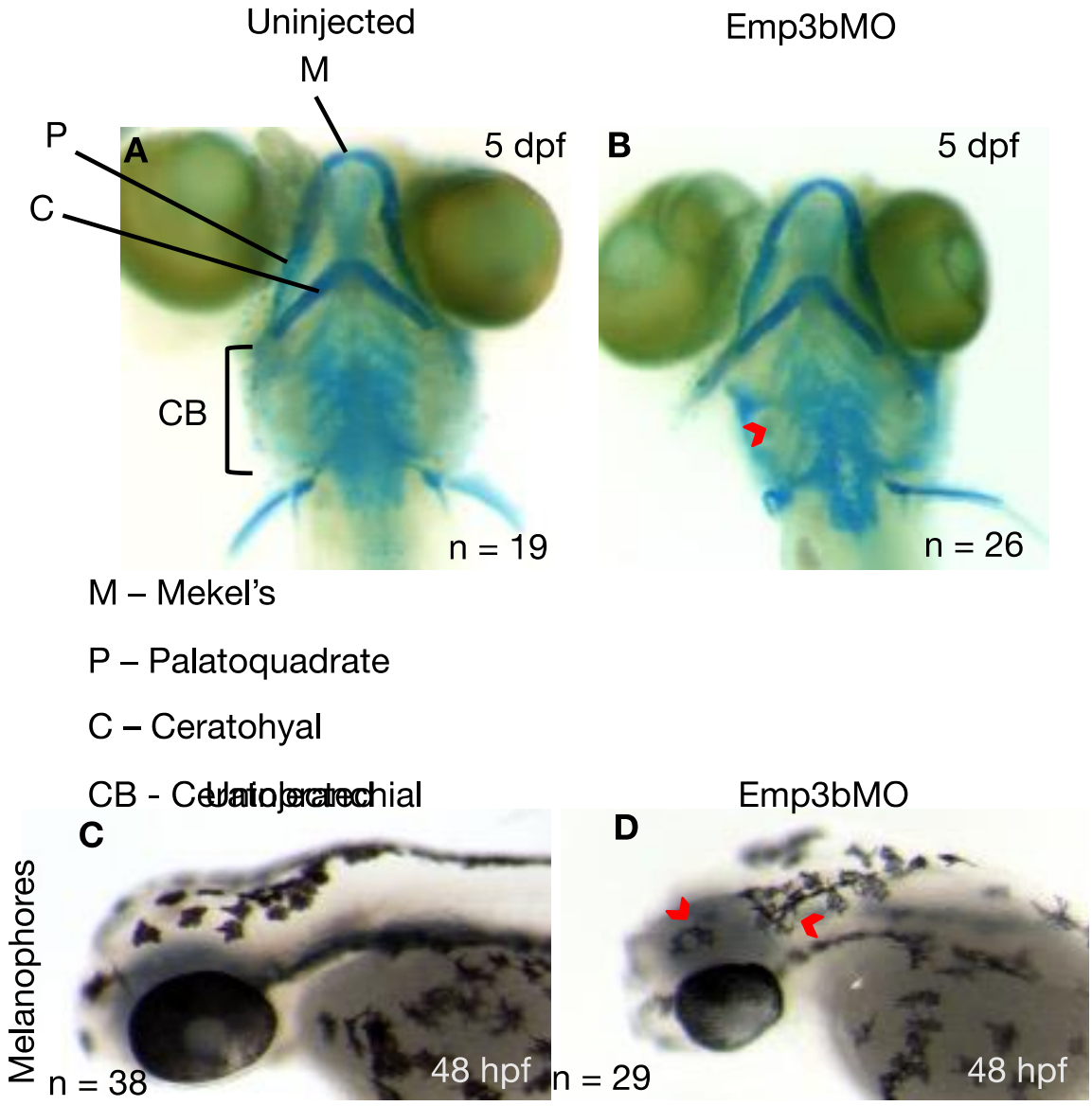

Figure 3: Cranial and trunk NC development is disrupted in *emp3b* morphants. Alcian blue staining of cranial cartilage at 5 dpf in uninjected (**A**) and Emp3b MO injected (**B**) embryos. Red arrow indicates aberrant certobranchial cartilage fusion in morphant embryos. Widefield microscopy images of cranial melanophores in 48 hpf zebrafish embryos. Red arrow indicates abnormal morphology and reduced melanin in Emp3b morphant melanophores. N indicates the total number of embryos examined for each condition.

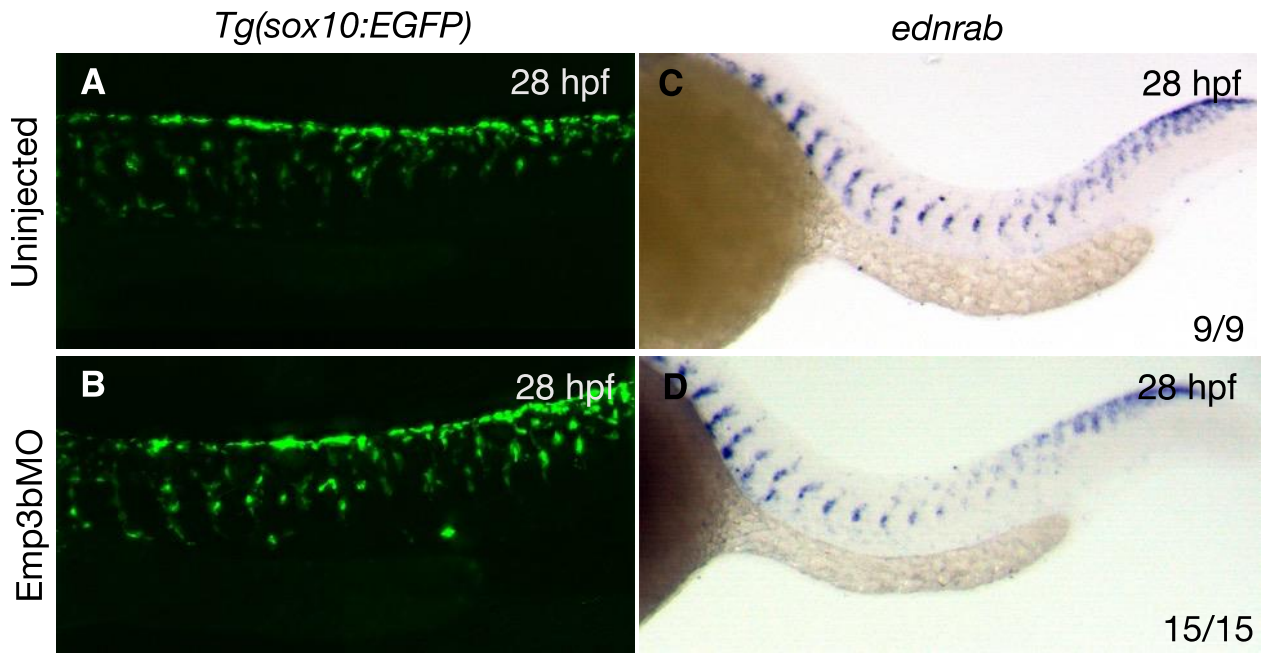

Figure 4: NC specification is not affected in *emp3b* morphants. Migrating NCCs labelled by EGFP in uninjected *sox10:EGFP* transgenic embryos (**A**) and in *emp3b* morphant transgenic embryos (**B**) at 28 hpf. ISH for the NC marker *ednrab* in uninjected (**C**) and emp3b MO injected (**D**) embryos at 28hpf.

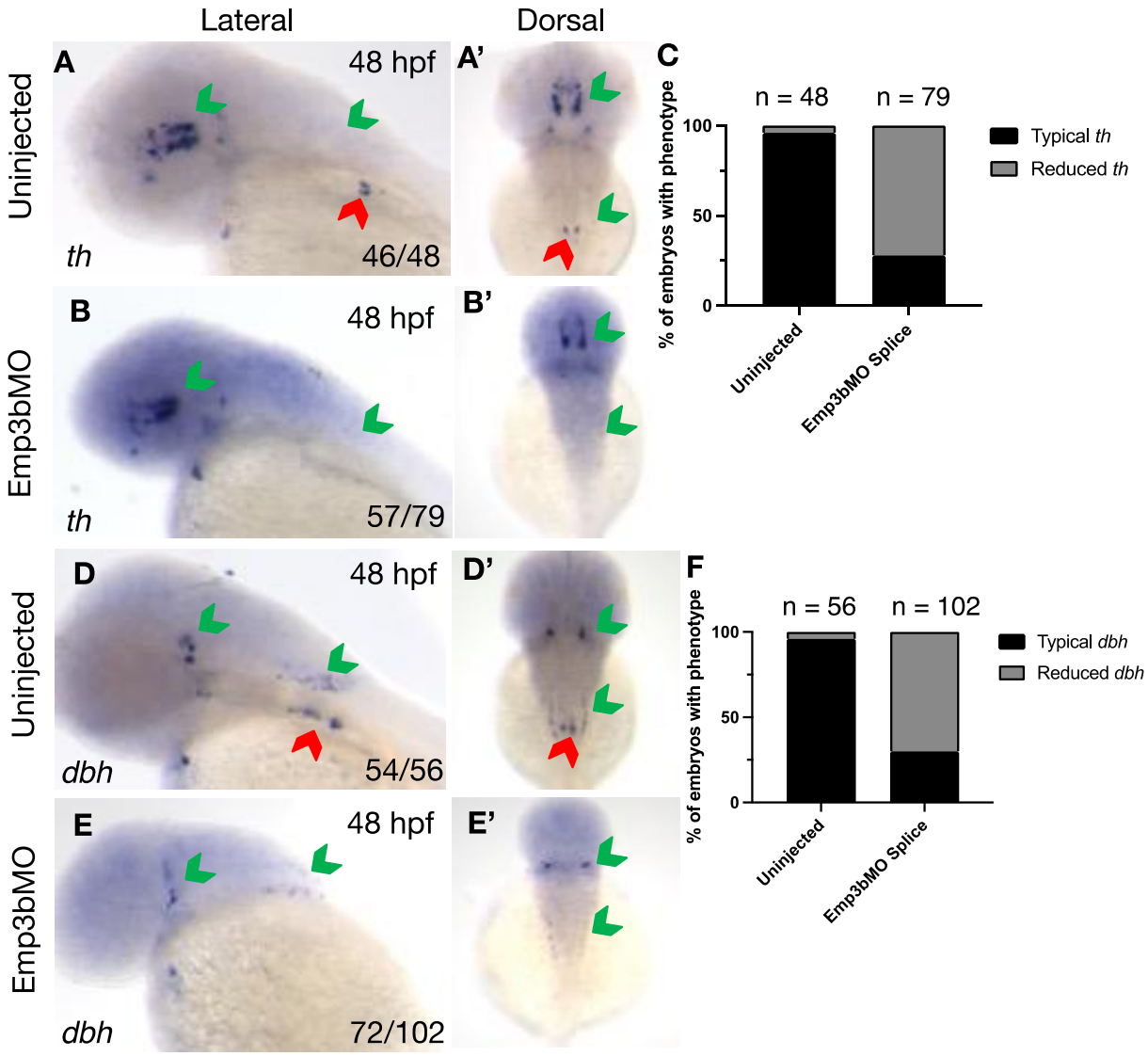

Figure 5: Emp3b is required for SNS development. Lateral (**A, B, D, E**) and dorsal (**A', B', D', E'**) views of ISH for *th* and *dbh* in uninjected (**A, A', D, D'**), Emp3b MO injected (**B, B', E, E'**) at 48hpf. Red arrows indicate *th* or *dbh* expression in the superior cervical ganglion, green arrows indicate *th* or *dbh* expression in hindbrain dopaminergic neurons. Numbers in bottom right corner indicate the number of individual embryos from the total represented by the image. Phenotypic distribution of *th* (**C**) and *dbh* (**F**) in uninjected and embryos injected with an *emp3b* splice blocking morpholino. N indicates the total number of embryos examined for each condition.

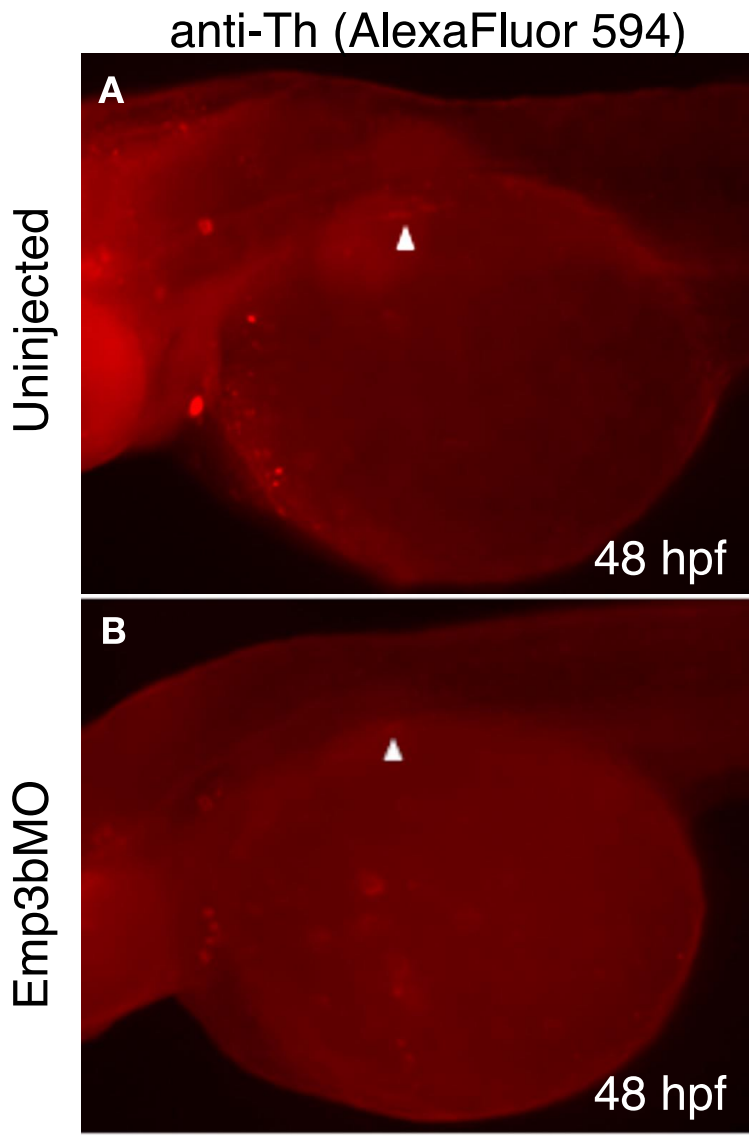

Figure 6: NC-derived neurons are reduced in *emp3b* morphants.
Fluorescent immunohistochemistry for Tyrosine Hydroxylase in the SCG at 48 hpf of uninjected (**A**) and *emp3b* morphant embryos (**B**) at 48 hpf. White arrow indicates location of the SCG.

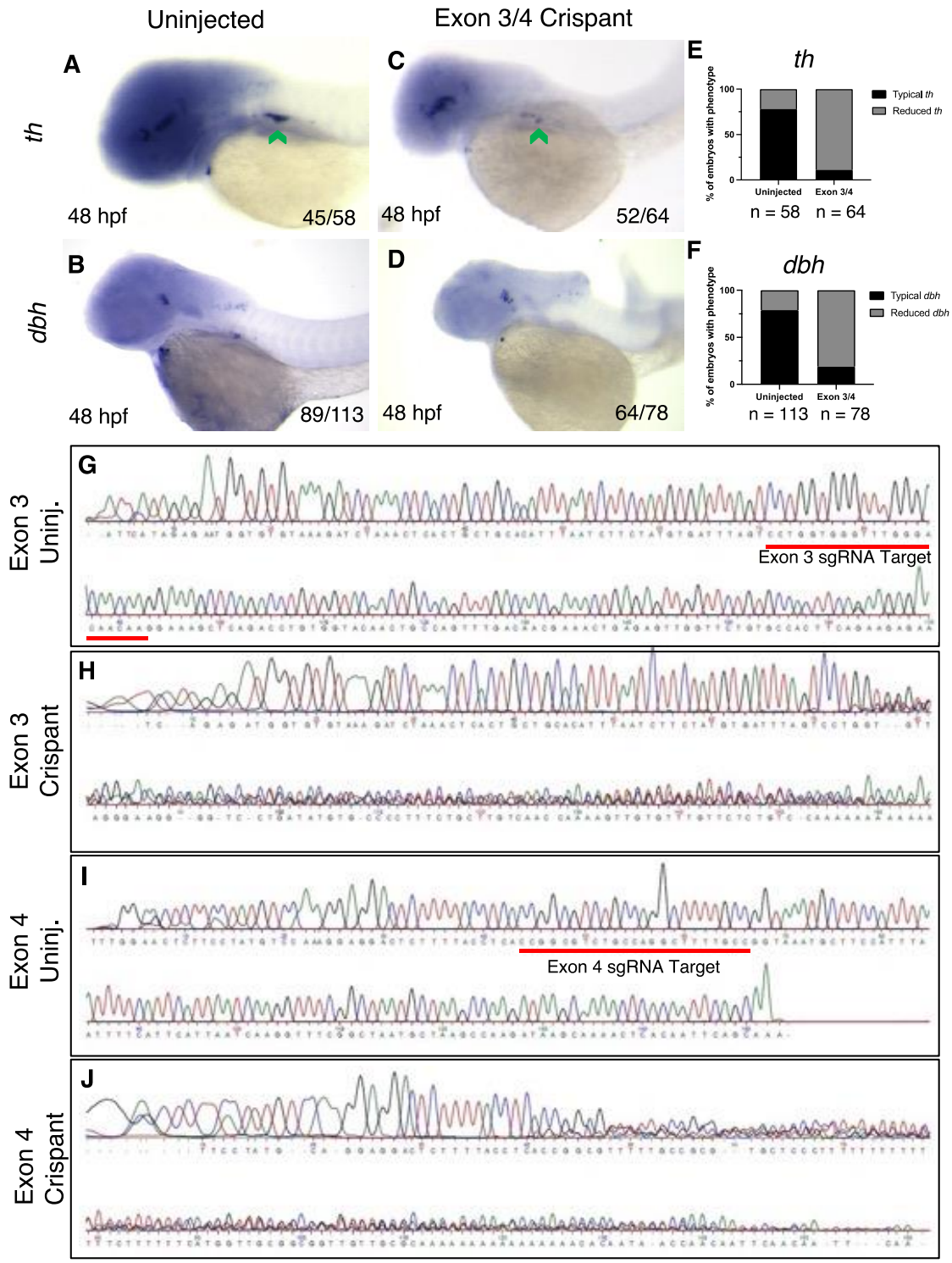

Figure 7: *emp3b* crispants exhibit defective SNS development. ISH for *th* (**A, C**) and *dbh* (**B, D**) in uninjected (**A, B**) and embryos injected with two sgRNAs targeting locations in exons 3 and 4 (**C, D, G, I**). Green arrow indicates location of *th* or *dbh* SCG expression. Quantification of ISH phenotype distribution for *th* and *dbh* (**E-F**). Electropherograms for exon 3 and 4 amplicons from uninjected (**G, I**) and crispant embryos injected with two sgRNAs targeting locations in exons 3 and 4 (**H, J**). Location of sgRNA target is indicated by red line. Numbers in bottom right corner indicate the number of individual embryos from the total represented by the image. N indicates the total number of embryos examined for each condition.

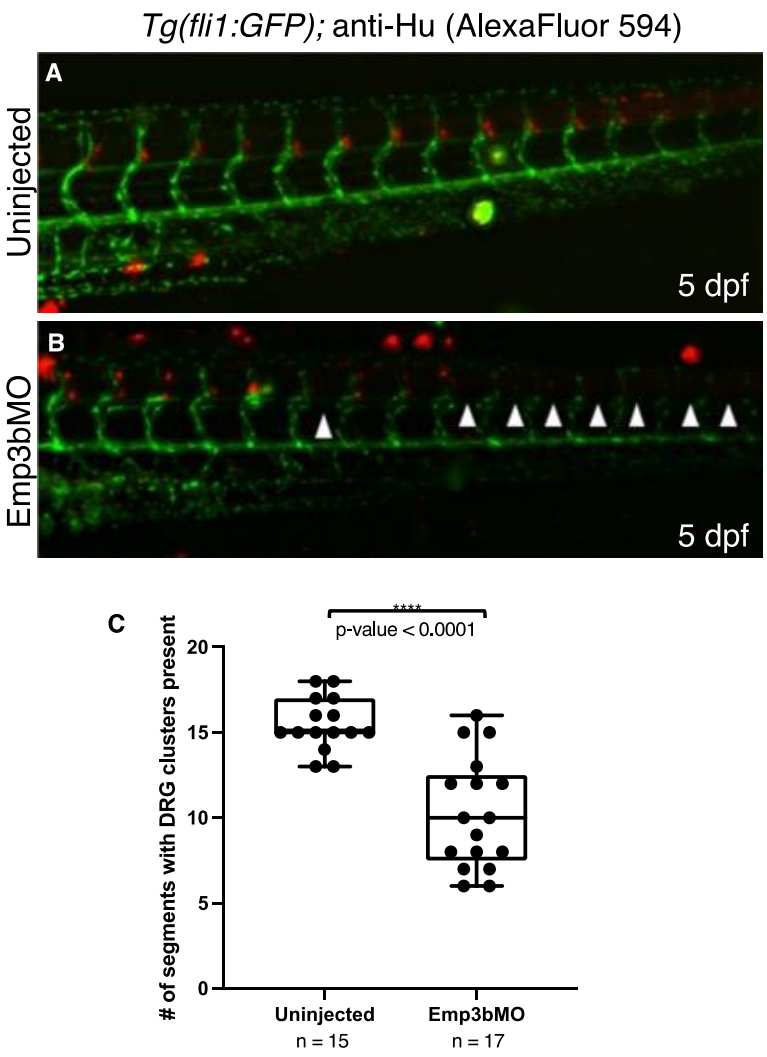

Figure 8: DRG sensory neuron formation is disrupted in *emp3b* morphants. Immunodetection of DRG by the pan-neuronal antibody anti-Hu in uninjected (**A**) and *emp3b* morphant *Tg(fli1:eGFP)* embryos (**B**), which express eGFP in all vascular endothelial cells. DRG sensory neurons in their respective segments are visible at 5 dpf. White arrows indicate segments with missing ganglion clusters. Quantification of the number of somite segments containing DRG in uninjected and *emp3b* morphant embryos. N indicates the total number of embryos examined for each condition. Asterisk indicates highly significant Mann-Whitney U Test result at the 99% confidence level, p value < 0.0001.

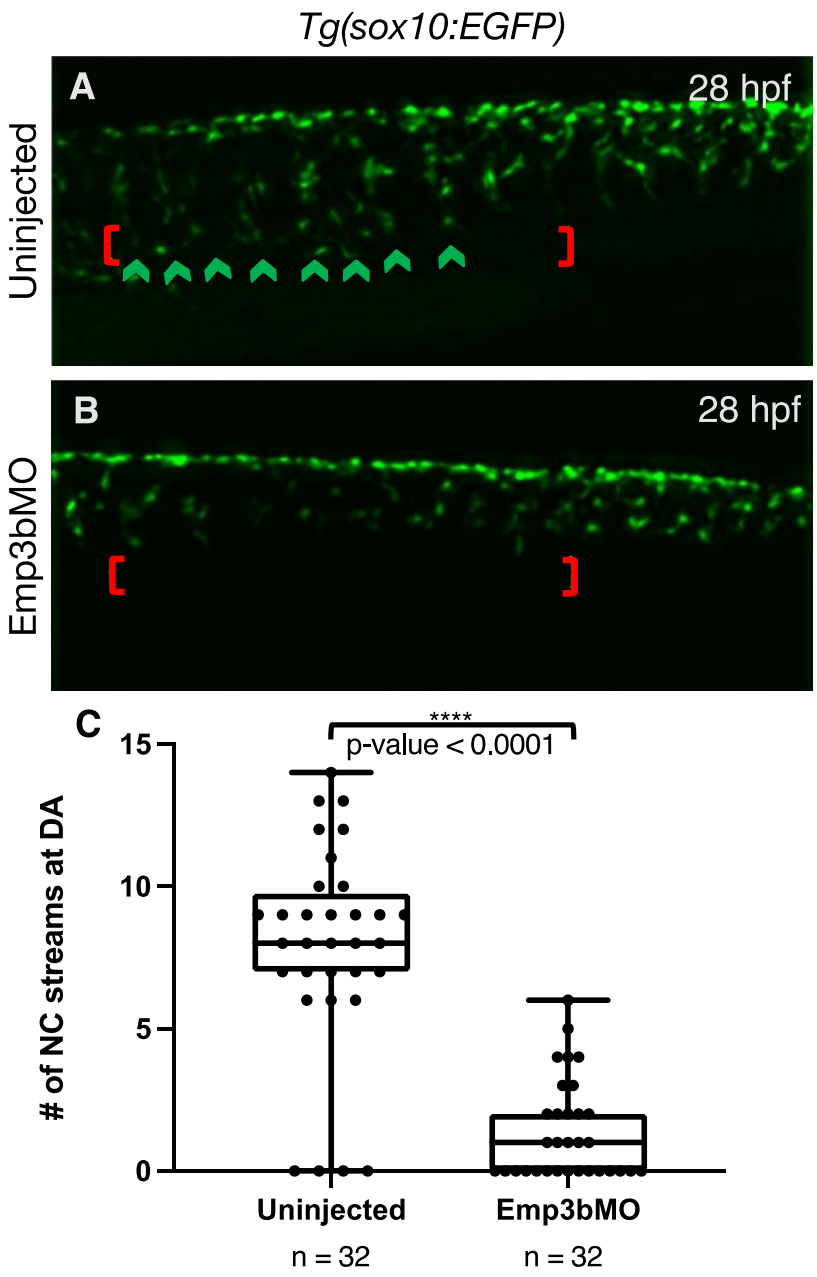

Figure 9: NC migration to the DA is disrupted in *emp3b* morphants. Uninjected (**A**) and *emp3b* morpholino injected (**B**) *sox10:EGFP* transgenic embryos, segmented NC migratory streams are visible at 28hpf. Yellow brackets indicate the position of the embryonic dorsal aorta (DA). Quantification of the number of NC streams that reach the DA in uninjected and *emp3b* morphant *sox10:EGFP* transgenic embryos (**C**). N indicates the total number of embryos examined for each condition. Asterisks indicate highly significant Mann-Whitney U Test result at the 99% confidence level, p value < 0.0001.

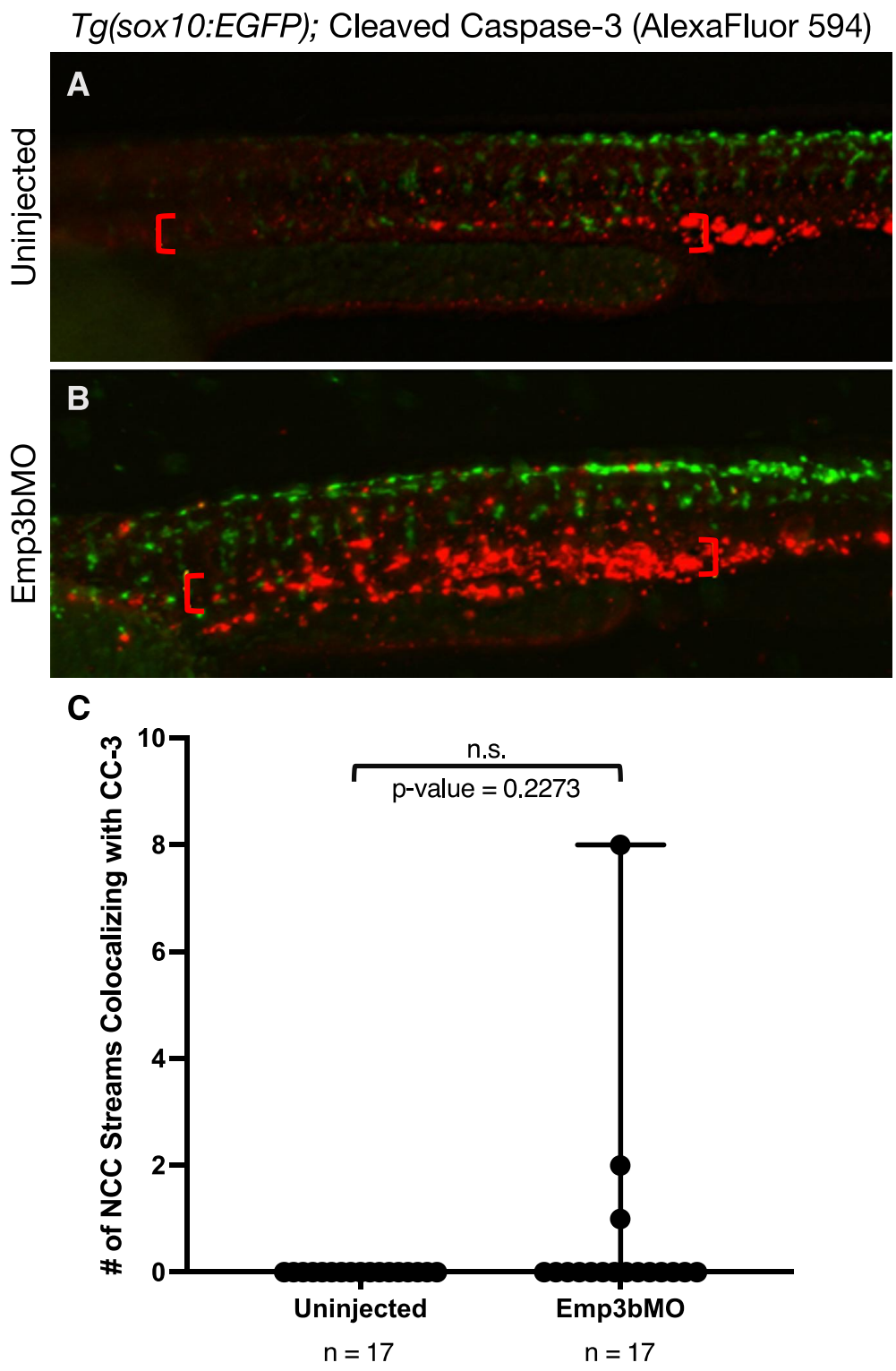

Figure 10: Migrating NCCs are not undergoing cell death. Uninjected (**A**) and *emp3b* morpholino injected (**B**) *Tg(-7.2sox10:EGFP)* transgenic embryos stained with an antibody against Cleaved caspase-3. Segmented NC migratory streams are visible at 28 hpf. Yellow brackets indicate the position of the embryonic dorsal aorta (DA). Quantification of the number of NCC streams that colocalize with anti-cleaved caspase-3 (**C**). N.S. indicates non-significant Mann Whitney U Test result at the 99% confidence level, p value = 0.2273.

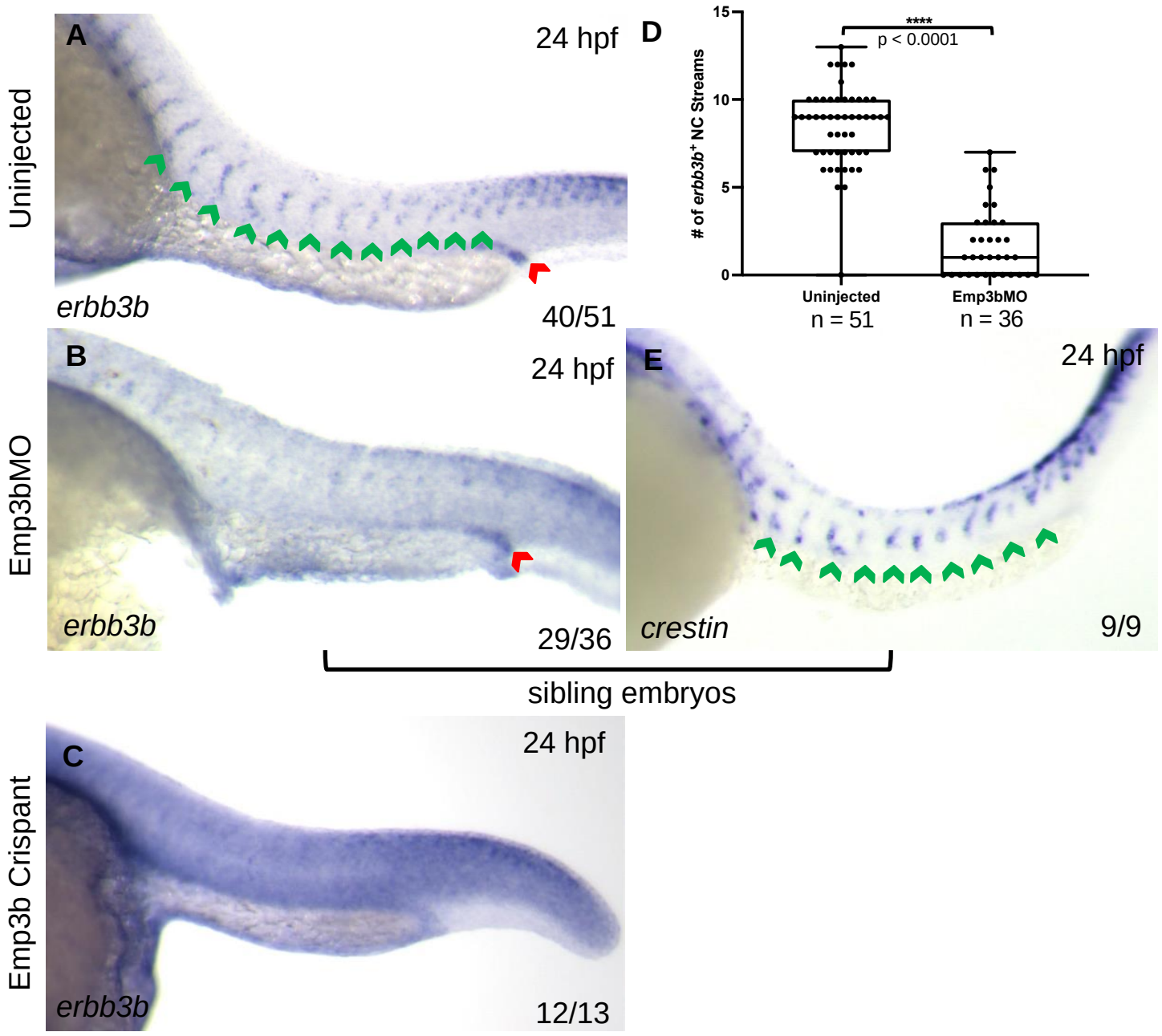

Figure 11: *emp3b* is required for expression of *erbb3b* in migrating neural crest cells. ISH for expression of *erbb3b* in uninjected (**A**), *emp3b* morpholino injected (**B**) and *emp3b* crispant (**C**) embryos. Green arrows indicates expression of *erbb3b* in migrating trunk NCCs, red arrow indicates *erbb3b* expression in the proctodeum. Quantification of neural crest streams expressing *erbb3b* in migrating NCCs (**D**). ISH for the NCC marker *crestin* in *emp3b* morphant embryos (**E**). Green arrows indicates *crestin* expression in streams of migrating trunk NCCs. *Emp3b* morphant embryos stained for *erbb3b* and *crestin* are sibling embryos injected with morpholino at the same time. Numbers in bottom right corner indicate the number of individual embryos from the total represented by the image. N indicates the total number of embryos examined for each condition. Asterisks indicate highly significant Mann-Whitney U Test result at the 99% confidence level, p value < 0.0001.

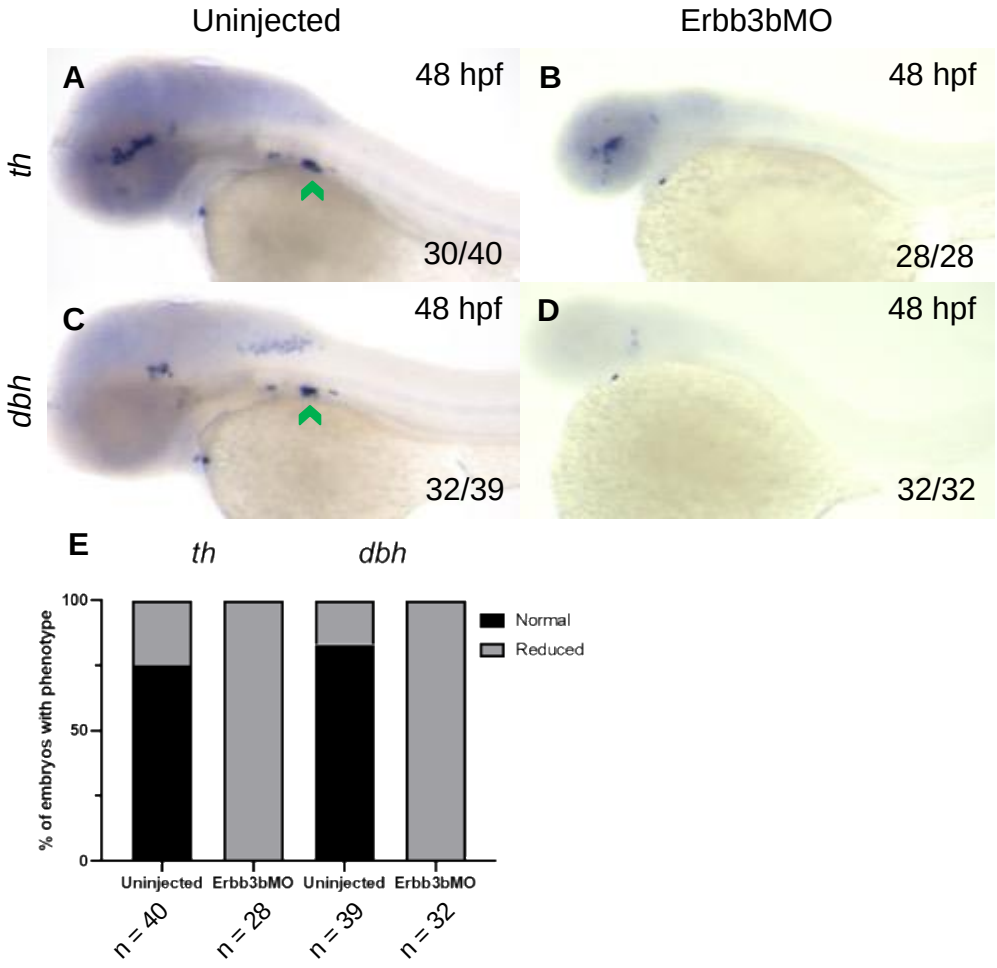

Figure 12: *erbb3b* is required for SNS development. ISH images for *th* (A-B) and *dbh* (C-D) in uninjected (A, C) and *erbb3b* morpholino injected (B, D) embryos at 48 hpf. Green arrow indicates expression of *th* or *dbh* in the SCG. Quantified phenotype distribution of *th* and *dbh* expression in uninjected and *erbb3b* morpholino injected embryos (E). Numbers in bottom right corner indicate the number of individual embryos from the total represented by the image. N indicates the total number of embryos examined for each condition.

Table 1. List of Custom PCR Primers

Plasmid	Annealing Temperature (ºC)	Sequence (5'-3')
emp3b	60ºC	F: ATGGCGTCTCTTCTGATTTT R: TCATTCCCTTTTGCGCAGAT
emp3b CRISPR Exon 3	60ºC	F:TTTTCATCAAACAGTCCCTCCT R:TCTCTTCTGAAGTGGCACAGAA
emp3b CRISPR Exon 4	60ºC	F: TGTTCTCCTCCATTTCATTCCT R: TGCTGAATTGTGAGTTTTGCTT

Table 2. *In situ* Hybridization Probe List

Plasmid	Restriction Enzyme	Polymerase
th	Xbal	SP6
dbh	Not1	SP6
erbb3b	Not1	SP6
phox2b	Not1	SP6
ascl1a	BamH1	T7
crestin	Not1	SP6
ednrab	Not1	SP6

Table 3. *In situ* Hybridization Stringency Washes

Washes	Duration (minutes)
75% Hyb /25% 2X SSC	15
50% Hyb/ 50% 2X SSC	15
25% Hyb/ 75% 2X SSC	15
100% 2X SSC	15
100% 0.2X SSC	30 x 2
75% 0.2X SSC/ 25% 2X PBT*	5
50% 0.2X SSC/ 50% 2X PBT*	5
25% 0.2X SSC/ 75% 2X PBT*	5
100% PBT*	5

Table 4. Morpholino and sgRNA Sequences

Morpholino or sgRNA	Sequence
emp3b splice blocking	5'- GGGTTTGCCATACTCACCTTCTCCA-3'
emp3b ATG	5'-ATCAGAAGAGACGCCATGCTGAAGA-3'
erbb3b splice blocking	5'-TGGGCTCGCAACTGGGTGGAAACAA-3'
Exon 3 Target sgRNA	5'-ACAAGGAGAGCTCAGACCTGTGG-3'
Exon 4 Target sgRNA	5'-GGCAAAAGCCTGGCAGACGCCGG-3'

Vita

Jessica MaeAda White was born on December 18, 1997, in Portsmouth, Virginia to Thomas and Triganza White. She is currently an American resident and citizen. Jessica graduated from I. C. Norcom High School, Portsmouth, Virginia in 2016 with an advanced diploma. She received her Bachelor of Science (B.S.) from the Department of Biology at Virginia Commonwealth University in December 2019. During her time as an undergraduate student, she was a University Student Scholar, VCU Merit Scholar and the recipient of a Beazley Foundation Scholarship. She also performed research in the laboratory of Dr. Wenheng Zhang. She began the Master of Science in Biology program in August 2020. During her Master's program she has worked as a graduate research assistant in the laboratory of Dr. Erich Damm and has been a teaching assistant for introductory biology and advanced molecular laboratory courses at Virginia Commonwealth University. She plans to continue her journey through higher education to pursue her M.D. or Ph.D.