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A Structure/Function Analysis of Nhsl1b in Facial Branchiomotor Neurons

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A Structure/Function Analysis of Nhsl1b in Facial Branchiomotor Neurons

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

by

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List of Abbreviations

Abi	Ableson interactor
CT	C-terminus
Dlg	Disks large homolog
FBMN	Facial branchiomotor neuron
FMN	Facial motor neuron
Fz	Frizzled
GFP	Green fluorescent protein
Gukh	Guanylate kinase holder
hsp70l	Heat shock cognate 70-kd protein, like
HSPC300	Haematopoietic stem/progenitor cell protein 300
Isl1	Islet1
MLF	Medial longitudinal fasciculus
MO	Morpholino oligonucleotide
mRFP	Membrane red fluorescent protein
Nap	Nck-associated protein
NHS	Nance-Horan Syndrome
Nhsl1b	Nance-Horan syndrome-like 1b
OLe	Octavolateralis efferent

PCP	Planar cell polarity
PDGF	Platelet-derived growth factor
PDZ	PSD95/Dlg1/ZO-1
PSD95	Postsynaptic density protein 95
r4	Rhombomere 4
Scrib	Scribble
Sra	Specifically Rac-associated protein
UTR	Untranslated region
Vang, Vangl	Van gogh, Van gogh like
WASP	Wiskott-Aldrich Syndrome protein
WAVE	WASP family verprolin homologous
WHD	WAVE homology domain
ZO-1	Zona occludens

Abstract

A STRUCTURE/FUNCTION ANALYSIS OF NHSL1B IN FACIAL BRANCHIOMOTOR NEURONS

By John Ojumu, B.S.

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The goal of this study was to identify critical regions of a novel gene, Nance-Horan syndrome-like 1b (*nhsl1b*). It was previously discovered that C-terminal truncation of the Nhsl1b protein in *nhsl1b* mutants resulted in a loss of migration in the facial motor neurons of the hindbrain (Walsh et al. 2011). As *nhsl1b* expresses many isoforms, multiple targets were investigated in order to determine which transcript bears the largest impact on the motor neurons. Using confocal microscopy to observe immunostained embryos, we examined a mutation in an *nhsl1b* transcript that encodes a WHD, a domain that is known to function within the actin nucleation and polymerization pathways. In situ hybridization and injection of antisense morpholino

oligonucleotides indicate that it is not the WHD but another transcript (^{ex1b}*nhsl1b*) that is necessary for migration. The control experiments for rescuing the mutant phenotype have successfully been performed, but inducing expression of full length *nhsl1b* in the *nhsl1b* mutants is proving difficult.

Chapter I: Introduction

In order for the brain to form a proper neural circuit, axons must be able to navigate to the correct target and location of innervation. The cell body of a neuron may migrate as well so that it will receive its extracellular cues and be in the area that axons are searching for them in. This is important because human congenital disorders such as epilepsy, schizophrenia, and lissencephaly are the result of aberrant or failed neuronal migration.

Growth and development of motor neurons in the zebrafish brain

The facial branchiomotor neurons or facial motor neurons (FBMN/FMNs) represent an excellent model for studying neuron migration. Born at 16 hours post fertilization (hpf) in the zebrafish hindbrain, the FBMNs start migration near the ventral and medial borders of the neural tube at 17 hpf and conclude their movement by 48 hpf. While beside the floorplate, they undergo a caudal migration from rhombomere 4 (r4) to r6 and some as far as r7 (Chandrasekhar, Moens, Warren Jr, Kimmel & Kuwada, 1997). The axons of the FBMNs trail behind the migrating cell body in close apposition to the medial longitudinal fasciculus (MLF) and pathfind dorsolaterally to exit the nervous system at r4 (Chandrasekhar, 2004).

Due to such a stereotypical migration pattern, defects in neuron migration can easily be determined by the impaired movement of the FBMNs. Additionally, the zebrafish embryo is transparent and the hindbrain lies close enough to the dorsal surface to allow for easy imaging

and optical classification via fluorescent microscope without the need to penetrate masses of tissue (Long, Meng, Wang, Jessen, Farrell & Lin, 1997). Together, these allow for the immediate and accurate identification of complications within the motor neuron migration system.

To facilitate this process, the analysis of the motor neurons was made more simple through use of the transgenic line, Tg(isl1:GFP)rw0 (Higashijima, Hotta & Okamoto, 2000), that expresses GFP within the trigeminal (nV) neurons in r2 and r3, the facial branchiomotor neurons (nVII) and octavolateralis efferent neurons (OLe) in r4-r7, the glossopharyngeal (nIX) in r7 and the vagus (nX) nerves in r8 and onwards.

The facial motor neurons in r4 share their space with the octavolateralis efferent (OLe) neurons of the vestibuloacoustic and lateral line systems (Higashijima, Hotta & Okamoto, 2000). These OLe neurons are also born in r4 and migrate along the same path as the FBMNs but send a contralateral dendrite across the midline to contact their mirrored population as well as a few posteriorly directed axons (Higashijima, Hotta & Okamoto, 2000). Due to their similarity in location as well as behavior in response to genetic mutation, the FBMNs and OLe neurons will be discussed as though representative of a single population of motor neurons.

The facial branchiomotor neurons innervate muscles derived from the second branchial arch, also called the hyoid arch (Chandrasekhar, 2004). In humans, these muscles control facial expression as well as muscles in the cheek and pharyngeal regions. In zebrafish, these muscles are responsible for some movements of the jaw, throat, and opercle (Diogo, Hinits & Hughes, 2008), but defects within the migration of the FBMNs cause the cell bodies and dendrites to be improperly located, thus disrupting the circuit. This leads to impairment on the zebrafish's

ability to eat food which, in the wild, may lead to its death, but in a laboratory setting, it is not a lethal defect (Chandrasekhar, unpublished).

Protein interactions within the hindbrain

There are a number of genes that are responsible for the migration of FMNs and they can be categorized as cell and non-cell autonomous, though they are not mutually exclusive. Cell autonomous proteins are required to be expressed within the migrating body, while non cell autonomous proteins are required in the environment through which the cell is migrating. Adhesion and recognition molecules are important in order for the neurons to be able to migrate (Keller, 2002; Porcionatto, 2006) as well as identify with other cells to prevent migration past rhombomere boundaries (Guthrie, 2007). As such, it is important to establish cellular polarity to orient the cells and to form the structure of the neural tube, of which a critical component is transcription factors.

As a regulatory mechanism, transcription factors control the activation of the genes necessary for migration to occur. Islet1(Isl1) is key among these as the predominant marker and initiator for motor neurons (Pfaff, Mendelsohn, Stewart, Edlund & Jessell, 1996), followed by factors such as *hox*, *valentino*, retinoic acid, and *krox*-20 which control rhombomere identity, patterning, and segmentation (Prince, Moens, Kimmel & Ho, 1998; Moens & Prince, 2002; Oxtoby & Jowett, 1993; Chandrasekhar, 2004). Additionally, Hoxb1a has been shown to regulate Prickle1b (Rohrschneider, Elsen & Prince, 2007), a member within a group of proteins that serve to align cells.

This class of proteins, necessary for migration of the motor neurons, is the planar cell polarity (PCP) proteins. The PCP signaling pathway is thought to align cells with their neighbors within the plane of an epithelium. In this way, PCP is thought to be a form of cell-to-cell communication that transmits polarity information for uniform orientation of cells with the epithelial plane. It was found that in Drosophila, when the cells are lined in a single plane, these proteins function to orient the cell along axes, such as the anterior-posterior axis (Strutt, 2003).

In Drosophila, Van gogh (Vang) aligns itself asymmetrically within the cell, opposite to Frizzled (Fz), with Fz in the distal membrane and Vang occupying the proximal membrane (Adler, 2002). Based on their localization within the cells, these proteins signal to its adjacent neighbors and establish the directionality within the plane (Klein & Mlodzik, 2005). This establishes a polarity within the cell and plane that allows for such actions as the uniform development of the wing hair fibers on the distal edge of each cell (Strutt and Strutt, 2002). The removal of PCP proteins can then lead to the improper localization of the wing hair within the cell (Wong & Adler, 1993; Taylor, Abramova, Charlton & Adler, 1998).

The PCP pathway is conserved within vertebrates, as shown by the organization of stereocilia bundles within the cochlea (Kelly & Chen, 2007). In zebrafish, the removal of Vang-like 2 (Vangl2), a vertebrate Vang homolog, leads to a block in motor neuron migration as well as convergence extension defects (Bingham, Higashijima, Okamoto & Chandrasekhar, 2002). A similar defect in FMN migration was made for the other core PCP components, including Frizzled3a, Prickle1b, and Celsr2. The elimination of Scribble (Scrib), a large scaffold protein with leucine rich repeats and four PDZ domains, has been shown to have a similar but lesser

phenotype to Vangl2 mutants (Montcouquiol, Rachel, Lanford, Copeland, Jenkins & Kelley, 2003; Wada et al., 2005). The similarity in expression domains and the presence of a PDZ-binding domain on the carboxyl terminus (C-terminus) of Vangl2 suggest that Vangl2 and Scrib undergo protein-protein interactions (Murdoch et al., 2003). In vertebrates, PCP proteins function to extend the body axis in gastrulation and convergent extension movements (Heisenberg & Tada, 2002). Additionally, though not arranged in a single plane, the neuroepithelium displays aspects of planar polarity and the neural tube requires PCP proteins in order to form (Ciruna, Jenny, Lee, Mlodzik & Schier, 2005) and close correctly (Murdoch, Doudney, Paternotte, Copp & Stanier, 2001; Curtin et al., 2003; Williams, Yen, Lu & Sutherland, 2014).

In a forward genetic screen for mutants with FBMN migration defects, a mutation in the Nance-Horan syndrome-like 1b (*nhsl1b*) gene was identified in zebrafish. Nhsl1b bears homology with Nance-Horan syndrome (NHS) protein in humans and the Guanylate-kinase holder (Gukh) protein in Drosophila. Similar to Gukh, zebrafish Nhsl1b was shown, by immunoprecipitation, to physically interact with Scribble and PSD95; moreover, Nhsl1b and Scrib genetically interact in FBMN migration (Walsh, Grant, Morgan & Moens, 2011). Given the similarity in phenotypes and the interaction with the PCP protein Scribble, available evidence suggests that Nhsl1b is an effector of PCP signaling in FMN neurons during migration. Indeed, Nhs1b is required cell autonomously in FMNs during migration (Walsh et al., 2011).

Exploring the Nance-Horan Syndrome family

Nance-Horan Syndrome (NHS) was first described in humans and is characterized by congenital cataracts, facial dysmorphisms, and mental retardation (Burdon et al., 2003). There is a paralog, *NHSL1*, with no known defects that have been reported on in humans. There are two homologs to *NHSL1*, *nhsl1a* and *nhsl1b*, due to the genomic duplication that occurred in zebrafish (Woods et al., 2000). *nhsl1b* is located on chromosome 20 and codes for 8 exons, with an alternatively spliced fifth exon (Walsh et al., 2011). Additionally, the first exon has at least 5 alternative exon 1s (exon1, 1a, 1b, 1c, and 1e) (sequences in Appendix A), each with their own 5' UTR and translational start sites (Figure 1). Unless otherwise indicated, base pair and amino acid numbers are based on Nhsl1b with exon1, to be referred to as ^{ex1}Nhsl1b. The body of the protein (exon 2 - exon 8) is 1498 amino acids long and contains no known domains or motifs except for a nuclear localization sequence (276-291) and a serine rich region (524-970) and proline rich region (973-1089) in exon 6, an exon which represents more than 65% of the protein.

While the exact locations of the interaction sites of PSD95 and Scribble are also unknown, truncation of the last 389 AAs (*nhsl1b*^{fh131}) leads to a complete block in the FBMN migration out of r4 (Walsh et al., 2011). Additionally, the size of exon 6 has exploited with a reverse genetics process, known as TILLING (Draper, McCallum, Stout, Slade, & Moens, 2004), to identify two additional and more severe nonsense mutations, *nhsl1b*^{fh280} and *nhsl1b*^{fh281}, both of which truncate more than 1150 amino acids from Nhsl1b and lead to impaired migration (Walsh et al., 2011). It is not yet known what the smallest fragment of the Nhsl1b protein is that can still carry out its functions within the cell, but this suggests that some portion

of the C-terminus is important for its function, presumably due to post-translational modification, localization, and/or protein-protein interaction. In support of this, Walsh et al. (2011) found that Nhsl1b protein is still made in *nhsl1b*^{fh131} mutants, albeit a truncated form.

The C-terminus is critical for the mechanism of migration, but it is also important to determine if there is any significance in which first exon is expressed. Brooks et al. showed that there is a high degree of genomic conservation within the NHS family, both human paralogs and homologs across different species (Brooks et al., 2010). Thus, like human NHS (*hNHS*), when exon1 is present in the *nhsl1b* transcript, the translated protein is capable of forming the full N-terminal WAVE Homology Domain (WHD). The WHD in the NHS family is encoded by the first exon in conjunction with exon 2; all *nhsl1b* transcripts will contain a latter portion of the domain, but it will not code for the fully functional WHD (Brooks et al., 2010).

The WHD is present on the N-terminus of the three human WAVE [(Wiskott-Aldrich syndrome protein) WASP family Verprolin-homologous] proteins (Brooks et al., 2010) and directly binds between HSPC300 and Abi1 (Ableson-interactor protein 1) which itself is bound to Nap1 (Nck-associated protein 1) which binds to Sra-1 (specifically Rac1-associated protein) (Gautreau, Ho, Li, Steen, Gygi & Kirschner, 2004) (Figure 2A). These proteins or their orthologs (Soderling, Binns, Wayman, Davee, Ong, Pawson & Scott, 2002; Le, Mallery, Zhang, Brankle & Szymanski, 2006) form a heteropentameric complex (HSPC300/WAVE/Abi/Nap/Sra) that mediates membrane-localized actin nucleation and polymerization through Rac and Nck (Eden, Rohatgi, Podtelejnikov, Mann & Kirschner, 2002; Stradal, Courtney, Rottner, Hahne, Small & Pendergast, 2001). This actin modification occurs through the rest of the WAVE protein, which contains a medial proline rich region and C-terminal WASP homology, central/cofilin and acidic

(WCA) regions (Machesky et al., 1999; Rohatgi et al., 1999). The WASP homology or verprolin homology region interacts with G-actin for addition to the actin filament via the acidic region, which binds to the Arp2/3 complex and an actin filament and activates actin nucleation (Miki & Takenawa, 1998; Machesky & Insall, 1998) (Figure 2B).

As the WHD is incapable of binding to Sra and Nap on its own (Innocenti et al., 2004), the loss of Abi leads to the elimination of PDGF-stimulated Rac-mediated membrane ruffling, an indicator of a migration-viable cell (Scita et al., 1999), while the loss of specific isoforms of WAVE phenocopies Abi loss as well as impairs directed migration and axon growth (Yan et al., 2003; Tahirovic et al., 2010). Complementary to this, knockdown of NHS in human cell lines leads to loss of cellular shape and excessive lamellipodia formation around the cell, but overexpression causes widespread localization of the Arp2/3 complex (Brooks et al., 2010).

Though there are multiple members of the NHS and WAVE families, they are not thought to function redundantly in all cases or be capable of mitigating or overlapping functions (Suetsugu, Yamazaki, Kurisu & Takenawa, 2003). Thus, the loss of a specific isoform of Nhsl1b may not cause as striking a phenotype if other isoforms are present. However, as only ^{ex1}Nhsl1b encodes the WHD, if the protein's migratory function is mediated through the WHD, then removal of the transcript and protein should be lead to a migration defect.

While the ability to knockdown/knockout a gene through antisense oligonucleotides, siRNA, mutants, etc. is required, a way in which to implement and observe the function and expression of an exogenous gene is also necessary. RNA injections tend to be at far greater concentrations than endogenously expressed initially, but as the cells divide, the RNA becomes so dilute or degraded that expression past a certain timepoint is considered to be negligible (Xu,

1999). While zebrafish undergo a great deal of embryonic development within the first five days post fertilization, such that protein from injected material could still be expressed, a more stable and controlled method of gene expression leads to more reliable results (Xu, 1999).

Integration into the zebrafish genome by only injecting DNA is inefficient, at less than 5% success (Stuart, McMurray & Westerfield, 1988), however the Tol2 system represents a simple and efficient (>50%) method of gene insertion (Kawakami et al., 2004). First discovered in the medaka fish (Kawakami, Koga, Hori & Shima, 1998), the Tol2 element, when coupled with a functional transposase, is capable of nonspecific genomic transposition (insertion, excision, and/or reintegration) in zebrafish (Kawakami et al., 2000; 2004) as well as other vertebrates (Kawakami & Noda, 2004b; Kawakami, Imanaka, Itoh & Taira, 2004c). This means that a gene placed between two Tol2 recognition sites can be expressed under the control of ubiquitous or specific promoters placed beside the gene or used in an enhancer or gene trap (Kawakami et al., 2004). Additionally, the transposase system can be used to disrupt endogenous transcripts via insertion into the middle of an exon, establish transgenic lines via germline integration (Kawakami et al., 2004), or to rescue mutant phenotypes if expressed within the correct tissue (Taylor et al., 2005).

The mutants of *nhsl1b*, *nhsl1b*^{fh131} and *nhsl1b*^{fh281}, have nonsense mutations that lead to 400 or 1150 amino acid truncations in the protein, respectively, which lead to a block in FBMN migration (Walsh et al., 2011). In order to introduce fragmented or the full length *nhsl1b* gene into zebrafish embryos, the Tol2 system is ideal for efficient integration. However, it is necessary to ensure that the concomitant genes accompanying *nhsl1b* between the Tol2 elements (Figure 3) do not interfere or have unexpected effects. *crest1*, an enhancer of the

islet1 promoter, and *hsp70l*, a minimal promoter, are found within the zebrafish genome and are not expected to have a deleterious effect and allow for tissue-specific expression in cranial motor neurons, such as FMNs.

The aim of this study is to identify which *nhsl1b* isoform is responsible for its function in neuronal migration and to determine which regions of the gene are capable of rescue and restoration of the mutant phenotype.

Chapter II: Materials and Methods

Fish Husbandry

Zebrafish were maintained and staged as previously described (Kimmel, Ballard, Kimmel, Ullmann, & Schilling, 1995). The Isl1:GFP transgenic line, registered as Tg(isl1:GFP)rw0 at The Zebrafish International Resource Center (ZIRC) (Higashijima, Hotta, & Okamoto, 2000) was maintained in the *AB background. The *nhsl1b*^{fh131} mutants have a nonsense mutation (E1219*) in exon 6 and were originally described in (Walsh et al., 2011), the *nhsl1b*^{fh281} mutants also have a nonsense mutation (Q408*) in exon 6, and *nhsl1b*^{fh353} has a truncating nonsense mutation, but in exon1 (E9*).

Genotyping

Heterozygous mutants for the three *nhsl1b* mutations (*fh131*, *fh281*, and *fh353*) were incrossed and the progeny was grown for at least 4 months. The adults' caudal fin were clipped using a scalpel and treated with a 1X Base solution (25 mM NaOH, 0.2 mM EDTA), then incubated at 95°C for 30 minutes followed by the addition of 1X Neutralization solution (40 mM Tris-HCl). PCR was conducted using TaKaRa ExTaq DNA polymerase and primers from Eurofins (fh131 Forward [F]: TCCAATTCTGATCCAACATCCTTCC;

fh131 Reverse [R]: CAGCACAGGTATGGGGTCCA;

fh281 F: GTGCAAAAGCACCTAAGCAGATTGGAA;

fh281 R: GCTGTGAAGAGCAAAAACCTCAGCA;

fh353 F: TTAAAATGCCGTTTCCCGAGAGAGCCCTC;

fh353 F2: TTAAAATGCCGTTTCCCGAGAGAGCCGGC

fh353 R: TGTCACTGGCGTGTCGGGATAGA). A restriction enzyme digest using HphI for fh131, SspI for fh281, XhoI for fh353 was incubated at 37°C overnight and run on a 2% agarose gel. The previous enzymes only cut wild type DNA. An alternative enzyme, NheI, utilizing the fh353 F2 primer cuts only mutant DNA.

Reverse Transcription-PCR

RT-PCR was performed on wild-type embryos at 10, 24, 48, and 72 hpf. RNA was extracted from approximately 30 embryos with the RNeasy Mini Kit (QIAGEN, #74104) and converted to cDNA using random primers with the Protoscript M-MuLV *Taq* RT-PCR kit (NEB, #E6400S). The *nhsl1b* exon 1s were amplified and run on a 3% agarose gel using first exon-specific forward and exon 3 or 4 reverse PCR primers:

^{ex1}nhsl1b F: GCTCGCTTCAGATTTGAACAGGATT

^{ex1}nhsl1b R: GAGAAAGTGGGCCCCTGTGAGTAGT

^{ex1a}nhsl1b F: AGAGGGAGGGAGTGTCAGTGTGTTT

^{ex1a}nhsl1b R: TGAAGATGCTGTGGACTTTTTGTCA

^{ex1b}nhsl1b F: ATACGCTCAGCACCTCTCCATAGTG

^{ex1b}nhsl1b R: TGAAGATGCTGTGGACTTTTTGTCA

^{ex1c}nhsl1b F: GTTGGCAAGAGGACACAGGTTTTC

^{ex1c}nhsl1b R: TTGGCAGGAGTCTTTCTCTTCCTCT

ex1enhsl1b F: AAGTGACCTGAAATTCATCTGCTGAC

ex1enhsl1b R: GCTCTCATTTGGCAGGAGTCTTTCT

RNA in situ hybridization

In situ probes were generated by PCR amplification of exon 1 specific sequences with a T7 RNA polymerase binding site incorporated into the reverse primer. PCR DNA template was used to obtain antisense RNA probes. The primers used to amplify *nhsl1b* exon 1s are as follows:

ex1nhsl1b F: GCTCGCTTCAGATTTGAACAGG

ex1nhsl1b R: GTATTTTAACCGATTTTGGATCGAG

ex1anhsl1b F: AGAGGGAGGGAGTGTCAGTGTG

ex1anhsl1b R: CTTCAAGGCATGACATGATTC

ex1bnhsl1b F: ATACGCTCAGCACCTCTCCATA

ex1bnhsl1b R: TGGGAAAGCAAGACAATGAGTT

ex1cnhsl1b F: GTTGGCAAGAGGACACAGGTT

ex1cnhsl1b R: CCTTGCGTTTGAAGTATTTAATG

ex1enhsl1b F: TGAAGAGGGTATGATGAGGGACA

ex1enhsl1b R: CTATGTAGTGCTGGGGCTCATAAA

Zebrafish embryos were fixed at 24 hpf in 4% PFA (in PBS) overnight (O/N) at 4°C and then washed 5x 5 minutes in PBSTw (1X PBS with 0.5% Tween-20) at room temperature (RT). Embryos were permeabilized with ProteinaseK for 5 minutes, refixed in 4% PFA for 1 hour at RT,

and washed 5x 5 minutes in PBSTw. The embryos were then prehybridized in hybridization buffer (50% formamide, 5X SSC, 0.1% Tween-20, citric acid to adjust pH to 6.0) for 1 hr at 65°C.

Digoxigenin-labelled probes against the exon 1s of *nhsl1b* and islet1 were created and diluted to 0.5 ng/uL in hybridization buffer. The prehybridization buffer on the embryos was replaced with prewarmed buffer plus probe and incubated O/N at 65°C. Buffer with probe was removed and stored at -20°C and the embryos were washed at 65°C with the following prewarmed solutions: 66% hybridization buffer, 33% 2X SSC for 5 minutes; 33% buffer, 66% 2X SSC for 5 minutes; 2X SSC for 5 minutes, 0.2X SSC + 0.1% Tween-20 for 20 minutes; 0.1X SSC + 0.1% Tween-20 for 20 minutes twice. These steps were followed by room temperature washes: 66% 0.2X SSC, 33% PBSTw for 5 minutes; 33% 0.2X SSC, 66% PBSTw for 5 minutes; PBSTw washes for 5 minutes.

The embryos were blocked with PBSTw with 2% sheep serum and 2 mg/mL bovine sheep albumin (BSA) for 1h at RT. Embryos were incubated in alkaline-phosphatase conjugated anti-digoxigenin diluted in the block solution (1:5000) O/N at 4°C, then washed 5x 15 minutes in PBSTw. Freshly made coloration buffer (100 mM Tris-HCl ph 9.5, 50 mM $MgCl_2$, 100 mM NaCL, 0.1% Tween-20) was used to wash the embryos 4x 5 minutes. A nitro-blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) combined stock solution was used at 20 uL per mL of colorization buffer as substrates for the detection of the digoxigenin-labelled probe. The NBT/BCIP in colorization buffer was refreshed hourly while the embryos were rocked in the dark at room temperature and then the buffer was refreshed before rocking the embryos O/N at 4°C. The reaction was stopped with a few quick washes in sterile water. Background staining

was decreased through the addition of 100% methanol to the embryos followed by graded washes of 75%, 50%, and 25% methanol (in PBSTw) and finally washed into PBSTw.

To prepare for image analysis, the embryos were deyolked and serially dehydrated with glycerol in 25%, 50%, and 75% glycerol (in PBS), or dehydrated and then deyolked, or only deyolked.

In situ cross sections

After embryos underwent in situ hybridization, the yolk was completely removed and the region of the hindbrain between and including the otic vesicles was cut out. These pieces were embedded in resin and 2 um thick coronal sections were cut every 10 um.

Morpholino injection

Antisense morpholino oligonucleotides (MOs) were injected at the 1-cell stage:

^{ex1b}nhsl1b ATG MO: TTGCAGGTGTAAAAGTTGGCATCCC, 3.3 ng;

^{ex1c}nhsl1b ATG MO: TGAGCGAAGTCCCGATGAACACCAT, 3.3 ng;

p53 MO: GCGCCATTGCTTTGCAAGAATTG, 3.3 ng. Morpholinos were co-injected with p53 MO to reduce MO-induced toxicity (Robu et al., 2007).

Plasmid and RNA injection

Using the Gateway system, a plasmid encoding full length ^{ex1}*nhsl1b* was subcloned into a pTol2 vector under control of crest1 enhancer and hsp70l minimal promoter with an N-terminally fused *mCherry* gene (Villefranc, Amigo, & Lawson, 2007). Capped transposase mRNA

was synthesized using mMessage mMACHINE (Ambion) from pTransposase (Kawakami et al., 2004). 60 and 100 ng/uL of transposase was mixed 1:1 with plasmid concentrations of 50 ng/uL and 200 ng/uL. Approximately 1 nL of the mixture was injected into one-cell zebrafish embryos.

Immunocytochemistry

To prevent pigmentation, at 22-24 hpf, embryos were placed in N-Phenylthiourea (PTU) diluted to 0.003% in fish water. The chorions were removed and embryos were fixed with 4% paraformaldehyde (in PBS) at 24 and 48 hpf O/N at 4°C. Embryos were washed in PBSTw (1X PBS with 0.5% Tween-20), permeabilized with acetone for 7 minutes at -20°C and then washed with PBSTw. Embryos were blocked with 4% goat serum (GS) and 4% bovine sheep albumin (BSA) in PBSTx (1x PBS with 0.25% Triton X-100) at room temperature for at least one hour or O/N at 4°C. Primary antibodies used were rabbit αGFP (TP401 Torrey Pines; 1:2000) and, where applicable, mouse αmCherry (1:1000) diluted in blocking solution and rocked O/N at 4°C. Embryos were washed with PBSTw 5x 30 minutes and secondary antibodies were added. Alexa Fluor 488 goat anti-rabbit IgG (H+L) and Alexa Fluor 568 goat anti-mouse IgG (H+L) were used at 3:1000 diluted in blocking solution and rocked O/N at 4°C. Embryos were washed with PBSTw 5x 30 minutes and then serially dehydrated in 25%, 50%, and 75% glycerol (in PBS).

Microscopy

Motor neuron migration of wild-type and mutant embryos was assessed via Zeiss Discovery.V8 stereoscope. Live images of embryo morphology were obtained on the stereoscope using a Zeiss Axiocam 105 color. For immunofluorescent and colorimetric analysis

of the motor neurons in the hindbrain, embryos were deyolked and mounted on microscope slides, ventral-side-down, in 75% glycerol. Images of motor neurons were obtained on a Zeiss Observer.Z1 spinning disk laser confocal microscope. A 63x objective was used in conjunction with a 488nm and 568nm laser for immunostained embryos. In situ hybridization images were taken using differential interference contrast (DIC) and brightfield microscopy on 20x and 63x (closeup) objectives. Sections of Z-stacks, no thicker than 35 um, were used to construct maximum intensity projections for the quantification of proportional motor neuron migration.

Chapter III: Results

The WHD is not required for proper FBMN migration

In order to determine whether the Wave Homology Domain (WHD) is essential in the migration of the facial motor neurons, a zebrafish mutant carrying a nonsense mutation exclusively in the exon1 of *nhsl1b* (*nhsl1b*^{fh353}), which is the transcript variant that encodes the WHD, was analyzed. A heterozygote, *nhsl1b*$^{fh353/+}$ incross gave rise to a majority of embryos that were capable of normal FBMN migration. Embryos displaying the most normal and well defined motor neuron migration were enzymatically digested and all 3 genotypes were present in that pool (Figure 5). To eliminate the presence of possibly confounding maternally-loaded ^{ex1}*nhsl1b* mRNA and proteins, *nhsl1b*$^{fh353/fh353}$ homozygous mutant fish were mated and their embryos imaged at 24 and 48 hpf (Figure 6). We found that FBMN migration proceeds normally in maternal-zygotic *nhsl1b*^{fh353} mutant embryos lacking the ^{ex1}*nhsl1b* transcript that encodes the WHD. Taken together, our results show that the WAVE homology domain in *nhsl1b* transcripts is dispensable for FBMN migration.

Temporal and spatial expression of the alternatively spliced exon 1s

Given the lack of a phenotype within *nhsl1b*^{fh353} mutants, it became important to determine the expression of all *nhsl1b* variants within the embryo during the period of migration (16 - 36 hpf). Using RT-PCR, it was shown that all *nhsl1b* isoforms are present at 10,

24, 48, and 72 hpf (Figure 7). However, the presence of an *nhsl1b* transcript does not automatically indicate that it is important for migration or even expressed within the motor neurons, an important distinction to make (Walsh et al., 2011).

To localize the expression pattern of the *nhsl1b* transcripts, RNA probes spanning the entirety of each of the exon 1s were utilized in an in situ hybridization. The *nhsl1b* mRNA is present throughout the brain, eyes and upper portions of the trunk for all splice variants (Figure 8A), but a darker signal in between the otic vesicles recapitulating motor neuron migration (arrows) is indicative of the probe's presence within the FBMN. From a dorsal view, only the ^{ex1b}*nhsl1b* probe mimicked the control *islet1* probe in its enrichment within the motor neurons (Figure 8B). Cross sections of in situ hybridized embryos show that only ^{ex1b}*nhsl1b* displays a non-uniform stain in the hindbrain; enrichment is visible within the cells at the location that should be occupied by facial motor neurons (Figure 8C).

Loss of function of ^{ex1b}*nhsl1b* phenocopies *nhsl1b*^{fh281}

Injection of translation blocking antisense morpholino oligonucleotides (MOs) into one-cell stage embryos is an efficient way to knockdown the translation of a particular gene transcript. However, they are known to cause MO-induced toxicity, leading to the death of cells within the embryo, including the neuroepithelium. To alleviate these concerns, the p53 MO, which blocks activation of apoptosis, can be co-injected (Robu et al. 2007). Importantly, loss of p53 does not affect neural development (Robu et al. 2007).

When p53 MO is not added, the embryos display a brain with cell death, thus all further MO injections were performed with co-injected p53 MO. As there are no significant domains

encoded by the other *nhsl1b* variants, ^{ex1c}*nhsl1b* was selected for a control MO injection (Figure 9A). Embryos injected with ^{ex1c}*nhsl1b* MO had FBMNs that underwent normal migration (Figure 9D). The ^{ex1b}*nhsl1b* MO was used to verify the enriched stain within the motor neurons seen in the in situ hybridization. The embryos injected with this MO displayed a phenotype similar to the C-terminal truncation mutations, *nhsl1b*^{fh131} and *nhsl1b*^{fh281} (Figure 6). The majority of the facial motor neurons completely fail to migrate out of r4 (Figure 9C).

Expression of *nhsl1b* in *nhsl1b*^{fh281} to rescue migration

Our data suggests that FBMN migration is dependent on ^{ex1b}*nhsl1b*, largely because this is the only variant of *nhsl1b* expressed in FBMNs. Although, our analysis of mutants indicates that the ^{ex1}*nhsl1b* variant that encodes for the WHD at its N-terminus is not necessary, we do not know whether the ^{ex1}*nhsl1b* is sufficient to drive migration of FBMNs in *nhsl1b* mutants. To determine whether some or all *nhsl1b* variants are sufficient for rescue we made use of a genetic tool to express foreign genes in FBMNs using the motor neuron specific promoter Islet1. As discussed in the Introduction, transgenic expression can be achieved by injection of transposase and a plasmid containing transposase recognition sequences called Tol2 sites.

The full length *nhsl1b* pTol plasmid has an N-terminally fused fluorophore and is under the control of the islet1 promoter. This causes *nhsl1b* to be expressed in excess of normal endogenous quantities when present within the motor neurons. Two stable zebrafish lines expressing GFP or membraneRFP (mRFP) under the control of the islet1 promoter have shown that the GFP and mRFP fluorophores do not inhibit motor neuron migration (Figure 10). The constructs to be injected into these lines (Figure 4) will serve as controls for the rescue. To

verify that the mCherry fluorophore does not affect the migration of the FBMNs, we injected the isl1:mCherry into wild type isl1:GFP embryos and found no motor neuron defect; 93.05% of mCherry/GFP-expressing neurons were found in r6, 6.94% were found in r5 (n=72) (Figure 11A).

It is also necessary to establish that the migration of FBMNs is not blocked when *nhsl1b* is overexpressed in motor neurons. First, we injected isl1: GFP-^{ex1c}*nhsl1b* into isl1:mRFP fish and no migration defect was present; 87.22% of neurons expressing GFP/mRFP were found in r6, 6.02% in r5, and 6.77 in r4 (n=133) (Figure 11D). This allows us to visualize FBMNs overexpressing ^{ex1c}*nhsl1b* in GREEN next to the neighboring wild type FBMNs that are RED. We found that GFP-^{ex1c}*nhsl1b*-expressing FBMNs underwent proper migration. This suggests suggest that overexpression of Nhsl1b does not affect migration of wild-type neurons. Similarly, full length ^{ex1}*nhsl1b* fused with mCherry (isl1:mCherry-^{ex1}*nhsl1b*) was injected into isl1:GFP fish and the neurons still migrated correctly; 73.68% of neurons expressing mCherry/GFP were found in r6, 5.26% in r5, and 21.05% in r4 (n=19) (Figure 11B), showing once again that an excess of *nhsl1b* transcripts does not inhibit motor neuron migration. Finally, we injected a construct that only encoded for the C-terminus (CT) of *nhsl1b*, a truncated version that codes for the last 1166 bps (*nhsl1b*-CT). These are the very amino acids that are lost from *nhsl1b*^{fh131} mutants. This truncated form of *nhsl1b* was fused to GFP and injected into isl1:mRFP fish, resulting in neurons that underwent proper migration; 78.21% of neurons expressing GFP/mRFP were found in r6, 14.1% in r5, and 7.69% in r4 (n=390) (Figure 11C).

To prove that the Nhsl1b protein portion of the translated plasmid transcript is responsible for any changes seen in the rescue experiment and not the fluorophore, the isl1:mCherry plasmid was injected into *nhsl1b*^{fh281} mutants. Neurons were not seen to be

capable of migrating out of r4, regardless of whether they expressed red or green; 3.23% of neurons expressing mCherry/GFP were found in r5, 96.77% were found in r4 (n=62) (Figure 11E). Next, we injected isl1:mCherry-*nhsl1b*-CT into *nhsl1b*^{fh281} mutants. We reasoned that the C-terminus might be sufficient to rescue migration since this was the portion that was lost in *nhsl1b*^{fh131} mutants. However, we failed to successfully express mCherry-*nhsl1b*-CT in FBMNs in *nhsl1b*^{fh281} mutants. Finally, we injected isl1:mCherry-^{ex1}*nhsl1b* into *nhsl1b*^{fh281} mutants. Again, however, we were unable to obtain expression of mCherry-^{ex1}*nhsl1b* in FBMNs of *nhsl1b*^{fh281} mutants.

Chapter IV: Discussion

The *nhs1lb* gene is required for proper facial branchiomotor neuron migration in zebrafish and inhibited migration leads to a disruption in this neural circuit. The migratory function of Nhsl1b is believed to be mediated in part by its protein-protein interactions (Walsh et al 2011). For instance, Nhsl1b can physically interact with Scribble, a protein known for its role in planar cell polarity. In addition, the phenotype of *nhsl1b* mutants mirrors that seen in Scribble mutants and mutants in other PCP genes, indicating that Nhsl1b is a downstream effector of the PCP pathway. Additionally, the presence of an N-terminal WAVE homology domain in the Nhsl1b protein suggests that Nhsl1b may function through the regulation of the actin cytoskeleton, since WAVE proteins are known to play a role in promoting actin polymerization necessary for cell motility.

The WAVE Homology Domain does not function within the FBMN migration pathway

The migration of FBMNs in zebrafish lacking the ^{ex1}Nhsl1b protein looks identical to wild type (Figure 6). To show that hetero- and homozygous *nhsl1b*^{fh353} embryos give rise to motor neurons capable of undergoing the stereotypical migration, a heterozygous incross was screened for the most normal-looking migrants and digested for DNA analysis (Figure 5). To confirm that the nonsense mutation (E9*) was still in the correct location and coded for, an

additional restriction enzyme was used to genotype zebrafish with *nhsl1b*^{fh353}. The NheI enzyme cuts only mutant DNA and has been used to verify and complement genotyping with XhoI.

While wild type embryos were expected to show well defined migration, the fact that hetero- and homozygous *nhsl1b*^{fh353} shows that the WHD of ^{ex1}Nhsl1b is not required for nor involved in the migration of the FBMNs in the zebrafish hindbrain. It has been theorized that the WHD of hNHS functions as a regulatory mechanism for WAVE-mediated actin polymerization as the lack of hNHS leads to increased cell spreading; this cell surface area increase was due to excessive lamellipodia formation thought to originate from unchecked and unfocused activation of the Arp2/3 complex via WAVE (Brooks et al., 2010). In terms of migration, an unorganized distribution of lamellipodia leads to no net movement.

The uniqueness of the WHD within ^{ex1}*nhsl1b* suggested that even if other *nhsl1b* variants could compensate for the functions of the C-terminal portions of protein, no alternative transcript could take over for activity of the N-terminus of ^{ex1}Nhsl1b. The lack of a phenotype within the FBMNs and the body as a whole demonstrates that there are no lethal or gross morphological or reproductive defects resulting from the loss of the ^{ex1}*nhsl1b* transcript.

The localization and expression of *nhsl1b* mRNA highlights the importance of exon1b

Previous experiments with *nhsl1b* showed that its RNA transcripts were expressed before and after the time period for FBMN migration (16-48 hpf) (Walsh et al., 2011), however, which specific variants are present in this same window has not been examined. The RT-PCR for the five current *nhsl1b* transcripts displays the expression of all five before, during, and after FBMN migration (Figure 7). This means that no transcript variant can be immediately

disqualified for responsibility of the migratory function of *nhsl1b*. While ^{ex1}*nhsl1b* seems to increase its expression from 10 to 24 hpf and again between 48 and 72 hpf, ^{ex1c}*nhsl1b* and ^{ex1e}*nhsl1b* appear to both decrease from 48 to 72 hpf. This may be due to the non-neuronal function of the WHD of ^{ex1}*nhsl1b* in the rest of the body; as the embryo grows, more cells are born and need to migrate, leading to a higher number of cells with the WAVE and WASP pathways active.

Using in situ hybridization, the presence of the alternative exon 1s *nhsl1b* transcripts within the embryo during the time of motor neuron migration confirms the results of the RT-PCR. As the majority of the embryos' brains are stained at some basal level, detecting the presence of mRNA expression within the FBMNs requires extended incubation within the colorization buffer. This allows for the visualization of any enrichment within the motor neurons, which would indicate that the mRNA is expressed at background levels in the surrounding tissue as well as in higher quantities inside the FBMNs. Walsh et al. showed that *nhsl1b* is required cell autonomously (Walsh et al., 2011), suggesting that enrichment within the motor neurons themselves is a critical observation to make when analyzing in situ images. However, the lack of enrichment is not itself indicative of the absence of a transcript, given the brevity of the probes, the concentration within the cells, and the nature of in situ hybridization. The enrichment seen in embryos probed against ^{ex1b}*nhsl1b* implies that only the ^{ex1b}*nhsl1b* transcript is present within the FBMNs. Importantly, ^{ex1}*nhsl1b* transcripts were not found in FBMNs which explains why mutations in ^{ex1}*nhsl1b* did not cause a defect in FBMN migration. Blast analysis of exon1b of Nhsl1b did not reveal any known protein domains or post-transcriptional modifications, thus, it remains unclear whether exon1b confers a specific

function to the Nhsl1b protein, or whether this simply reflects alternative promoter usage by motor neuron- specific transcription factors for this variant in FBMNs.

The specific expression of ^{ex1b}*nhsl1b* in the FBMN may mediate its migratory function

Translation blocking MOs inhibit the production of proteins by binding to mRNA and preventing their translation (Nasevicius & Ekker, 2000). Zebrafish embryos reach one thousand cells at 3 hpf, right when zygotic transcription is beginning to increase activity (Mathavan et al., 2005). The MO concentration at that time is already diluted due to cellular division and will become further so as the embryo continues to divide and develop. The concentration and density of the MO is insufficient to bind every single mRNA molecule transcribed, leading to a knockdown, a partial or reduced knockout effect. Despite these dilute levels, morpholinos can cause MO-induced toxicity, leading to the death of nervous cells within the embryo (Ekker & Larson, 2001). This phenotype can be characterized by an opaque and "cloudy" brain due to the clustered, non-transparent dead cells. There also may be the failure to form the basement membrane which leads to neurons falling out of the neural tube and failing to progress through the surrounding tissue. In some cases, ventricular hypertrophy, fluid in the neural ventricles, may also occur, visible as a large and clear sac on top of the brain; to alleviate these effects, the p53 MO was co-injected (Robu et al., 2007), as cell death is not typically associated with mutations in *nhsl1b.*

The embryos injected with ^{ex1b}*nhsl1b* ATG MO had a majority of blocked r4 neurons, resembling the phenotype of the truncation mutants, while the embryos injected with ^{ex1c}*nhsl1b* ATG MO displayed no block in FMBN migration. This confirms the staining pattern

seen in the ^{ex1b}*nhsl1b* in situ image and supports the claim that ^{ex1b}*nhsl1b* is responsible for *nhsl1b*'s function in the migration pathway. It also provides support to the exclusive presence of ^{ex1b}*nhsl1b* within the FBMNs. If any other *nhsl1b* variants are present within the FBMNs, they are (i) not present in sufficient quantities to carry out the migratory function, (ii) not involved in the migration pathway, or (iii) not in possession of the necessary protein structure necessary to fulfill the role of *nhsl1b* in the motor neurons.

Recovery of migration in the FBMNs of *nhsl1b* mutants may prove difficult

It was necessary to inject the partial pieces of the mCherry-*nhsl1b* pTol plasmid to show that they do not block motor neuron migration. Knowing that the fluorophore itself did not cause any defects in FBMN migration of wild type embryos and did not cause migration in mutant embryos, testing the constructs that would be used to attempt a rescue of the mutant phenotype was the next required step. The CT construct is the exact portion of the gene that is truncated in the *nhsl1b*^{fh131} mutant. If the C-terminus with an N-terminally fused mCherry forms the correct tertiary structure, then it is possible that it could possess the proper binding domains necessary to reestablish *nhsl1b* functionality.

As the fluorophore and *nhsl1b* are under control of the islet1 promoter, presumably stronger expression than endogenous *nhsl1b* expression, it is also important to determine if overexpression of *nhsl1b* could lead to an inhibitory effect on FBMN migration. In human cell lines, overexpression of *hNHS* leads to impaired activation of WAVE, a key component in the actin-mediated cellular migration pathway (Brooks et al. 2010); similarly, overexpression of *Fz* leads to tissue polarity defects in the Drosophila wing (Strutt, 2001). When ^{ex1}*nhsl1b* and

^{ex1c}*nhsl1b* were expressed in isl1:GFP and isl1:mRFP embryos, respectively, the FBMN were capable of normal migration and any neurons that may have been lagging or stuck in r4/5 were rarely seen to be expressing the plasmid's fluorophore.

Due to the ^{ex1c}*nhsl1b* ATG MO injections and the offspring of *nhsl1b*^{fh353} incrosses, it can be concluded that these two variants are not required for FBMN migration. Rescue with either of these constructs would support two conclusions: (i) that the FBMN block resulting from the removal of ^{ex1b}*nhsl1b* is due to the solitary expression of that transcript in the motor neurons and (ii) that the N-terminus of *nhsl1b* is nonspecific and expression of any full length transcript is sufficient for FBMN migration. Rescue of the mutant phenotype with the CT construct would also show (ii) and go further to claim that the first 75% of the protein may not even be necessary for migration. This claim cannot be fully substantiated however, until the CT construct rescues migration after the Nhsl1b protein has been completely removed from the embryo; both *nhsl1b*^{fh131} and *nhsl1b*^{fh281} zebrafish still generate the protein in its truncated form (Walsh et al., 2011).

Future directions: Unfolding Nhsl1b

It has been shown that the WHD is not necessary for proper motor neuron migration. To complete the panel of required *nhsl1b* variants as well as substantiate the in situ hybridization results, an analysis of the knockout or knockdown of ^{ex1a}*nhsl1b* and ^{ex1e}*nhsl1b* should be conducted. MOs are acceptable, but to reduce the chance of off target effects and death in the neural tube, a more precise technique, such as CRISPR, may be attempted. Upon assessment, a closer examination of the *nhsl1b* isoforms whose loss leads to a phenotype may be able to

provide insight into a functional sequence, previously unrecognized by localization or modification standards.

Preliminary results suggest that the C-terminus construct is incapable of rescuing the *nhsl1b*^{fh281} phenotype. Though attempting a rescue with all variants has its merit, the critical rescue experiment to perform would be the injection of the full length ^{ex1b}*nhsl1b* construct. The lack of a rescued phenotype with what is deemed the principal *nhsl1b* transcript in FBMNs would strongly support the inability of a rescue. If integration into the genome poses an insurmountable obstacle, direct injection of mRNA can be attempted.

Assuming that the mutant phenotype is capable of being rescued, to establish which portions of Nhsl1b are important for its migratory function, a shifting but overlapping window of the gene can be used to rescue mutants. If expression of *nhsl1b*-CT is successful, using smaller and smaller fragments within that domain can restrict the possibilities of what may be a critical component in *nhsl1b*. However, as the structure of the C-terminus is not currently known, excessive truncation may lead to improper folding in the translated protein. Additionally, given that Nhsl1b physically interacts with Scribble and PSD95, it is possible that both sites are required for migration and as such there may be a gap in the sequence required to restore proper migration. This split would most likely present itself as a partially rescued motor neuron block when either of the sequences is expressed, but as a more substantial rescue when the two individual sequences are expressed together.

Complementary to discovering the sequence required to obtain a rescued phenotype, resolving the structure of the Nhsl1b protein may provide an indication of how it functions in the motor neurons. An antibody against Nhsl1b was developed (Walsh et al., 2011) and showed

that Nhsl1b localizes to membrane protrusions, as does Scribble (Wada et al., 2005), and that they co-immunoprecipitate; it has not, however, been shown whether or not they colocalize *in vivo*. Determining the distribution of Nhsl1b and PCP proteins and the manner in which they are affected by mutations in the Nhs and PCP families will provide knowledge about the signal transduction pathway that governs motility and migration.

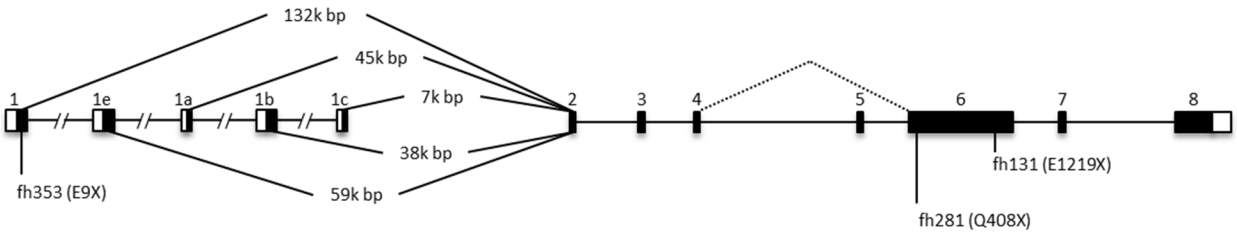

Figure 1. **The genomic structure of *nhsl1b***. White boxes represent alternative transcription start sites and/or UTR, black boxes are exons, all else are introns. The three mutations examined in this study are indicated.

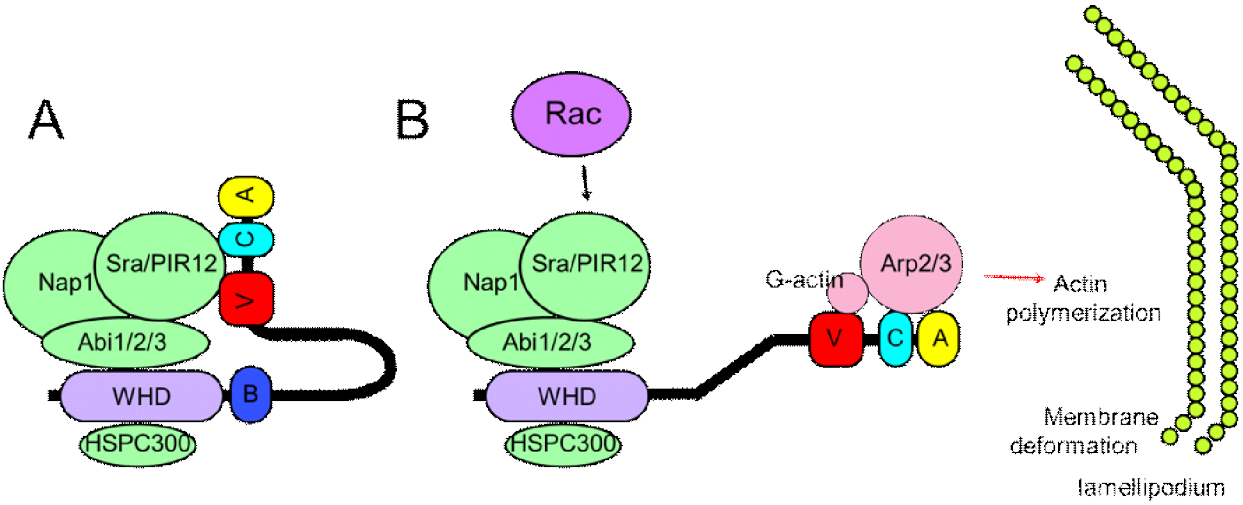

Figure 2. **Lamellipodium formation is mediated via the WAVE complex.** (A) The VCA of WAVE is auto-inhibited in the natural conformation, but interaction with Rac (B) frees the region to function in actin formation.

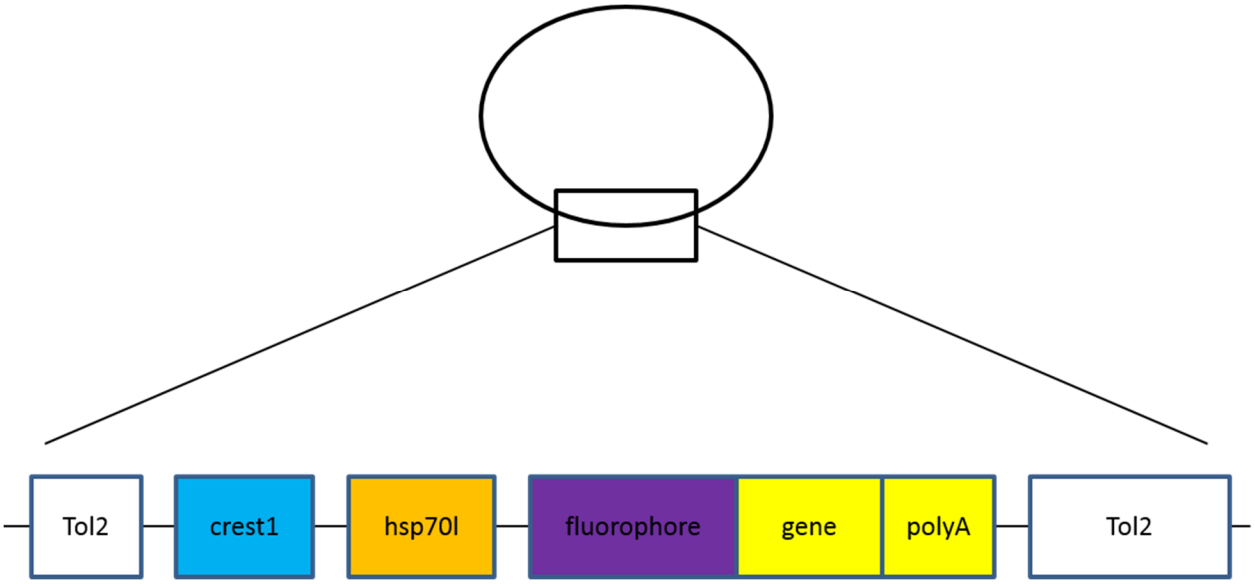

Figure 3. **A basic motor neuron pTol2 plasmid template.** Expression of the gene with an N-terminally fused fluorophore is under the control of isl1 and a polyA tail is present to ensure mRNA is made. Everything between the Tol2 sites will be inserted into the embryo's genome.

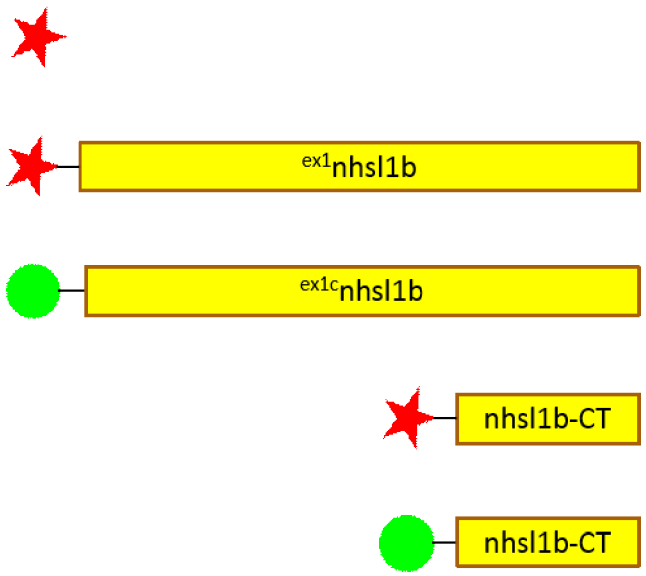

Figure 4. **The expressed regions of the injected plasmid constructs.** mCherry (star) and GFP (circle) are N-terminally linked to *nhsl1b* gene sequences.

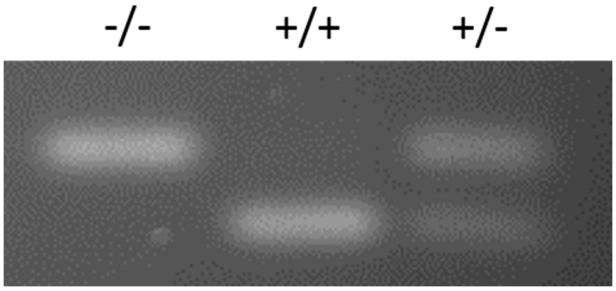

Figure 5. ***nhsl1b*^{fh353} genotype conducted on embryos with normal FBMN migration.** When using the XhoI restriction enzyme, the mutant embryo lacks the requisite nucleotide sequence for digestion; heterozygous embryos possess a subset of wild type DNA.

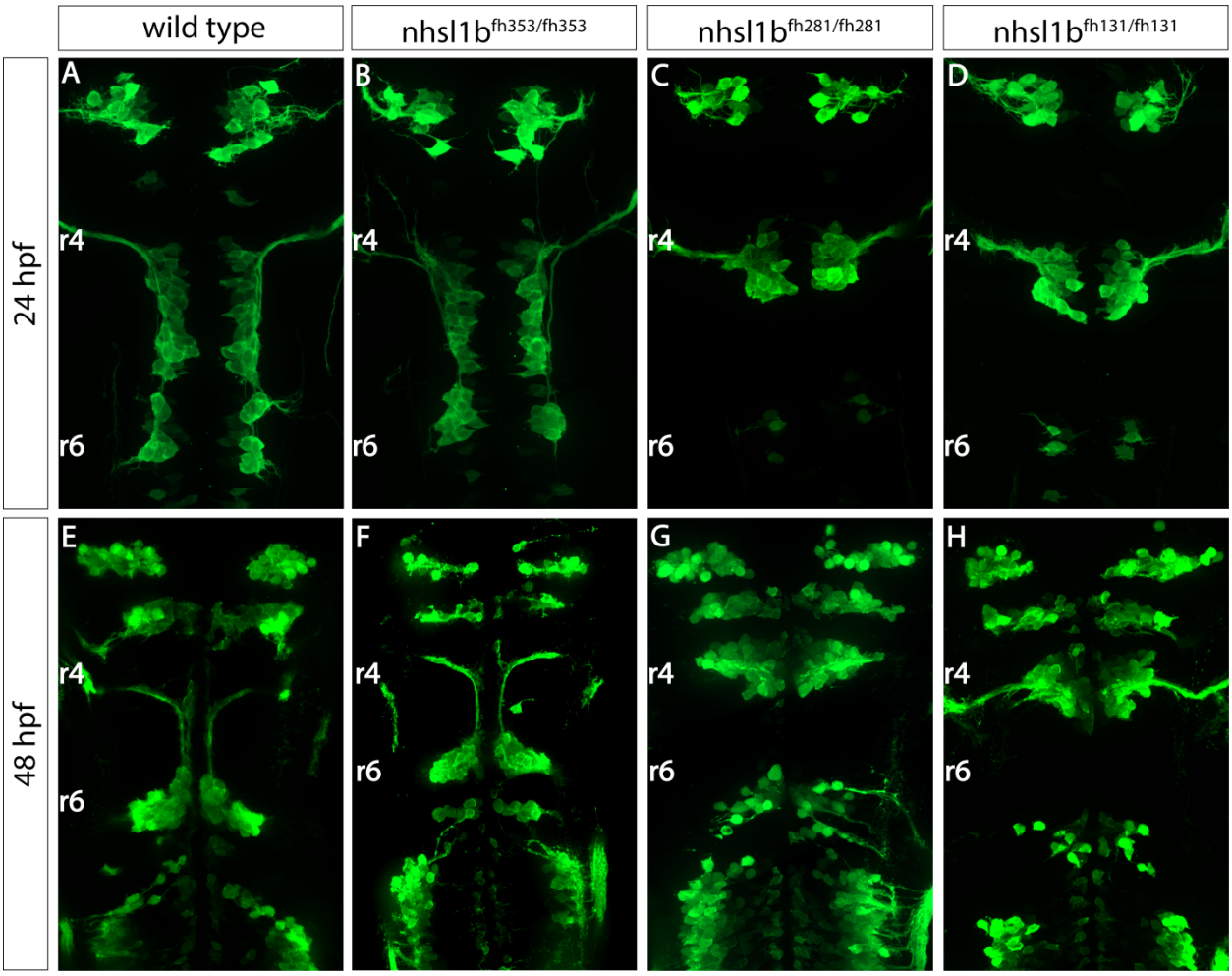

Figure 6. **Comparison of the FBMN phenotypes in *nhsl1b* mutants to wild type migration.** Display of wild type and mutant FBMN migration during (A-D) and after (E-H) migration. Motor neurons in embryos with *nhsl1b*^{fh281} or *nhsl1b*^{fh131} are unable to migrate out of r4. All mutant embryos are maternal-zygotic.

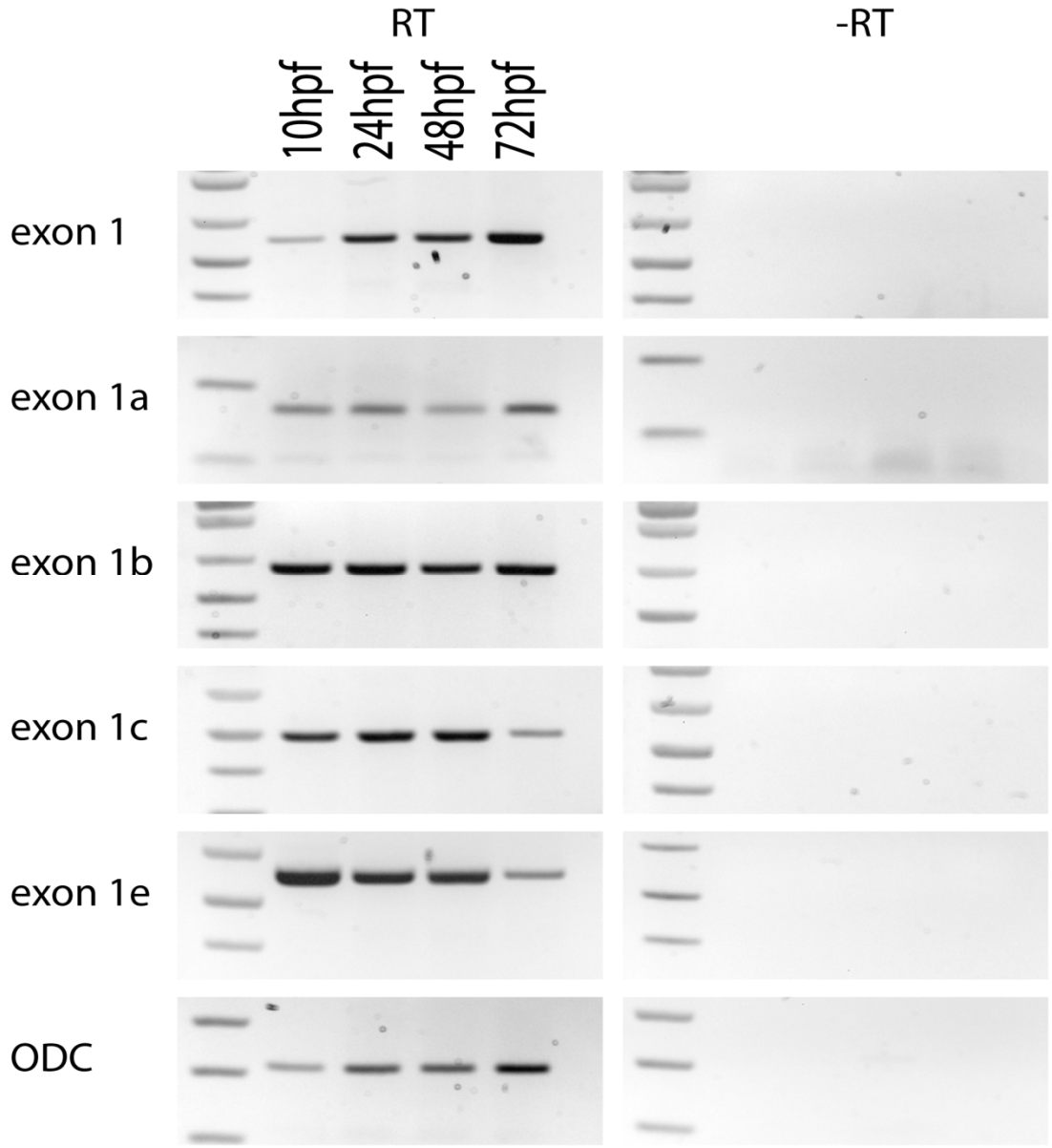

Figure 7. **All *nhsl1b* isoforms are expressed before, during, and after migration.** Transcription levels of ^{ex1b}*nhsl1b* remain steady while the others vary. ODC, ornithine decarboxylase control.

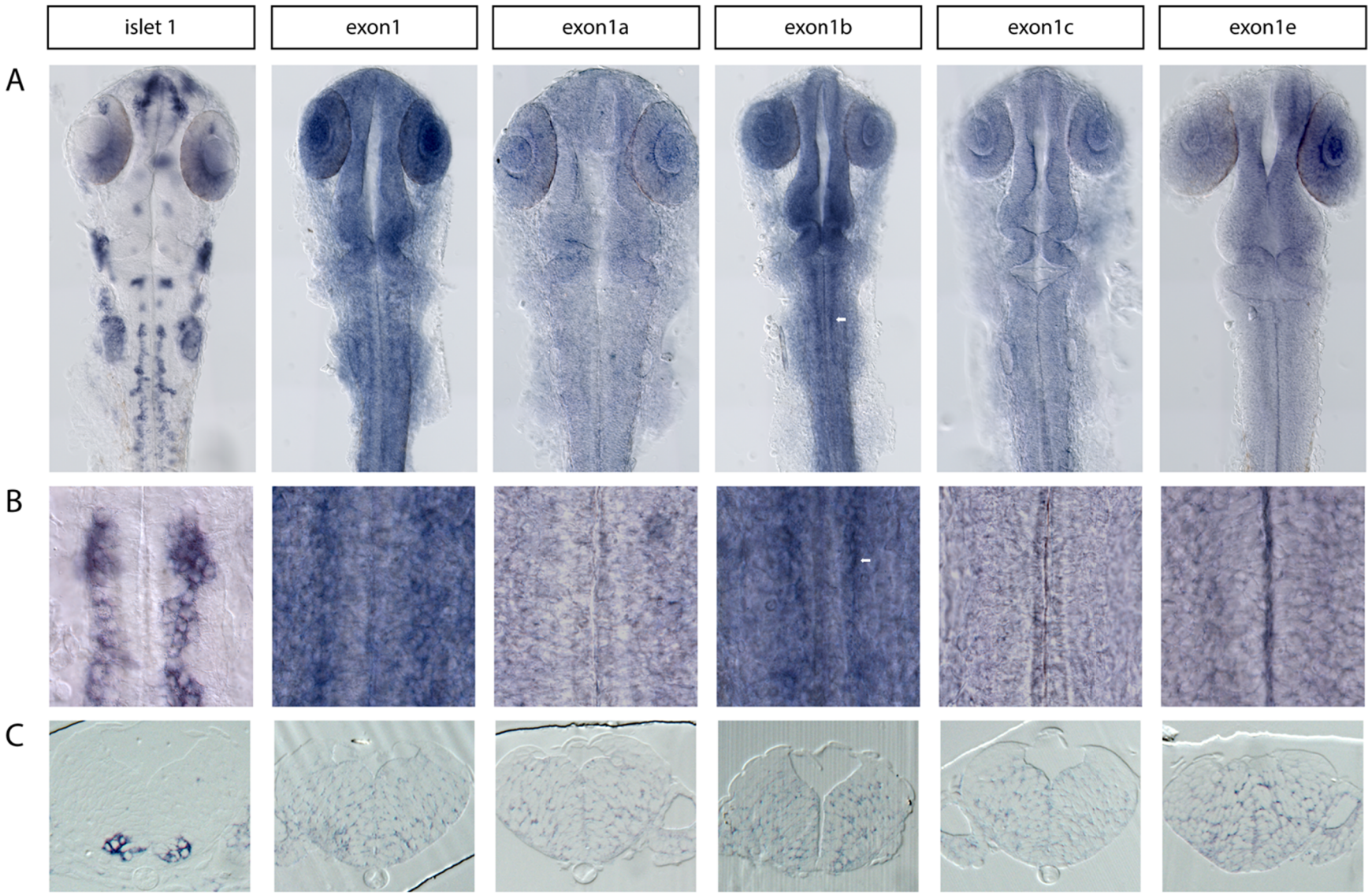
islet 1
exon1
exon1a
exon1b
exon1c
exon1e
A
B
C

Figure 8. ***nhsl1b* mRNA is expressed throughout the brains of 24 hpf embryos.** Dorsal view (A) with a 20x objective is able to distinguish a darker stain in exon1b (arrows) similar to islet1. A close-up of the area between the otic vesicles (B) with a 63x objective reveals deeper coloring around cells migrating in a column. Cross sections of the hindbrain (C); only exon1b displays enrichment of FBMN that is properly located at the ventral and medial borders of the neural tube, situated just above the notochord.

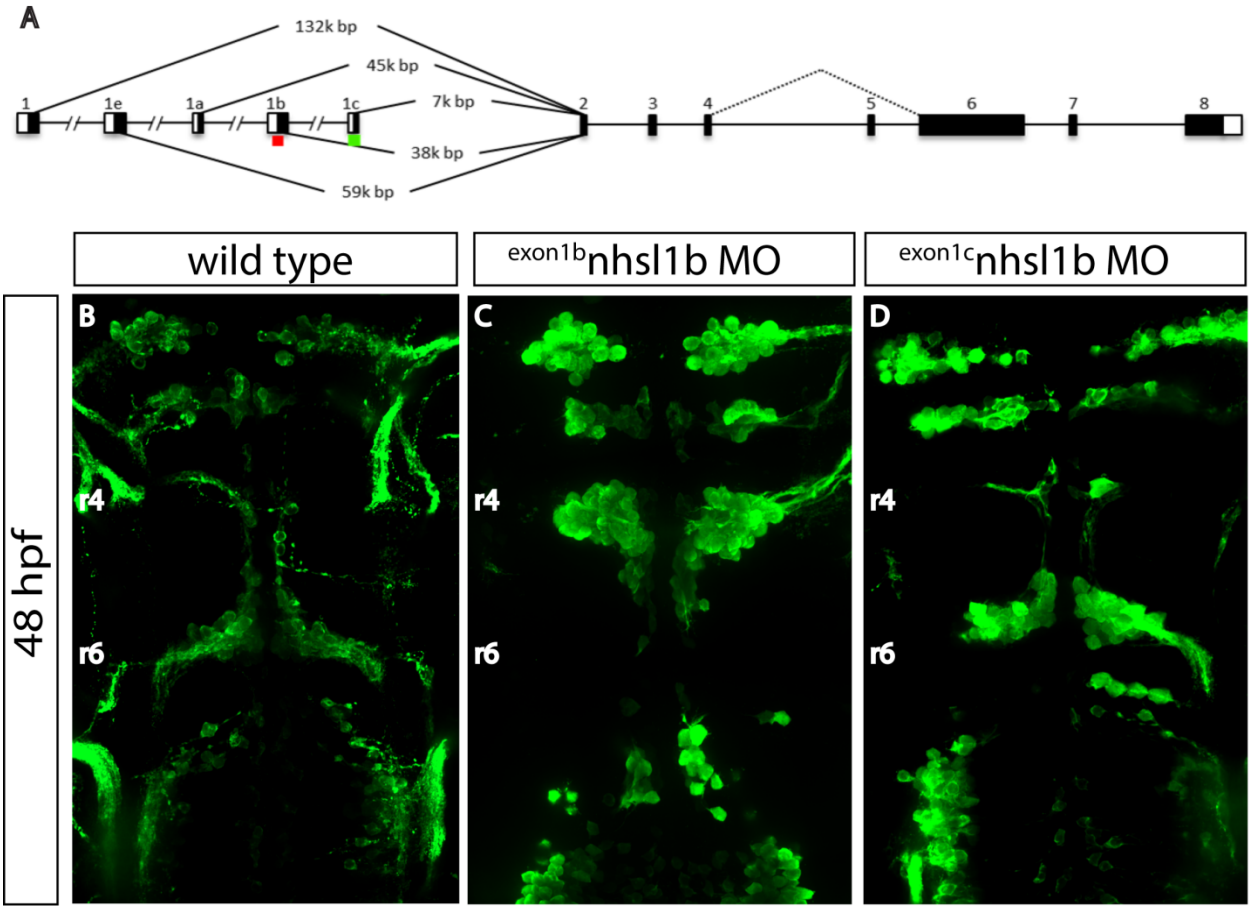

Figure 9. **Translation blocking MOs inhibit FBMN migration when targeted against ^{ex1b}*nhsl1b*.** (B) Wild type migration as reference; a majority of facial motor neurons are blocked in r4 for the ^{ex1b}*nhsl1b* MO (C), but neuron migration remains predominantly normal after the knockdown of the ^{ex1c}*nhsl1b* transcript (D).

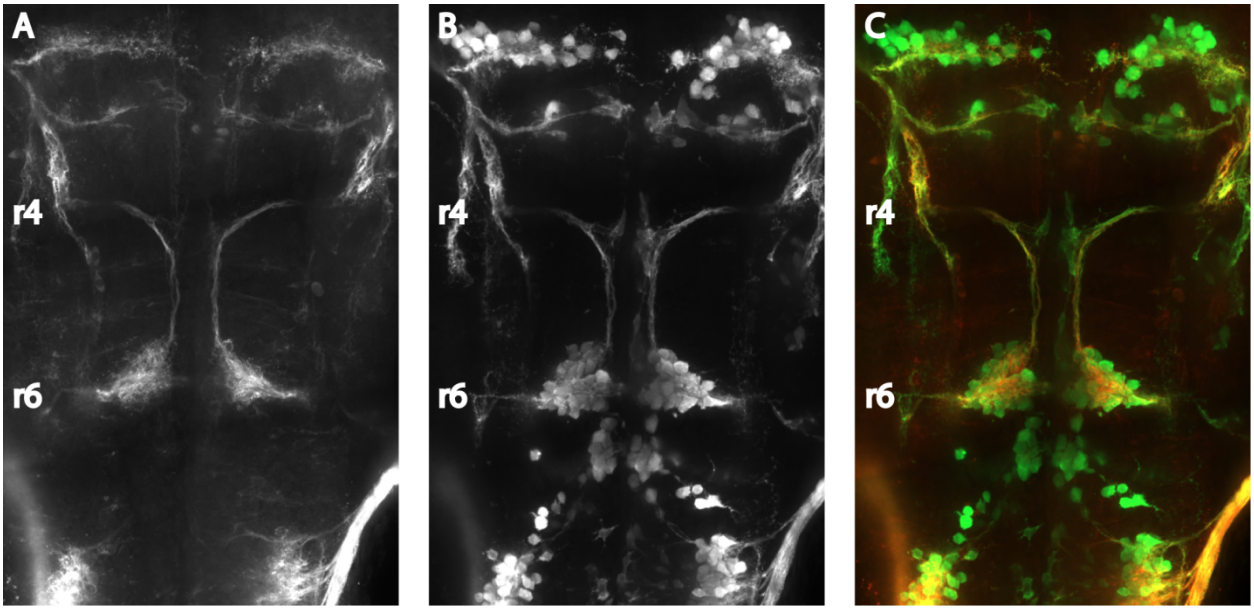

Figure 10. **Migration of FBMNs in a wild type embryo concurrently expressing GFP and mRFP under control of isl1.** Expression levels and localization of mRFP (A) and GFP (B) are unaffected by the presence of the other.

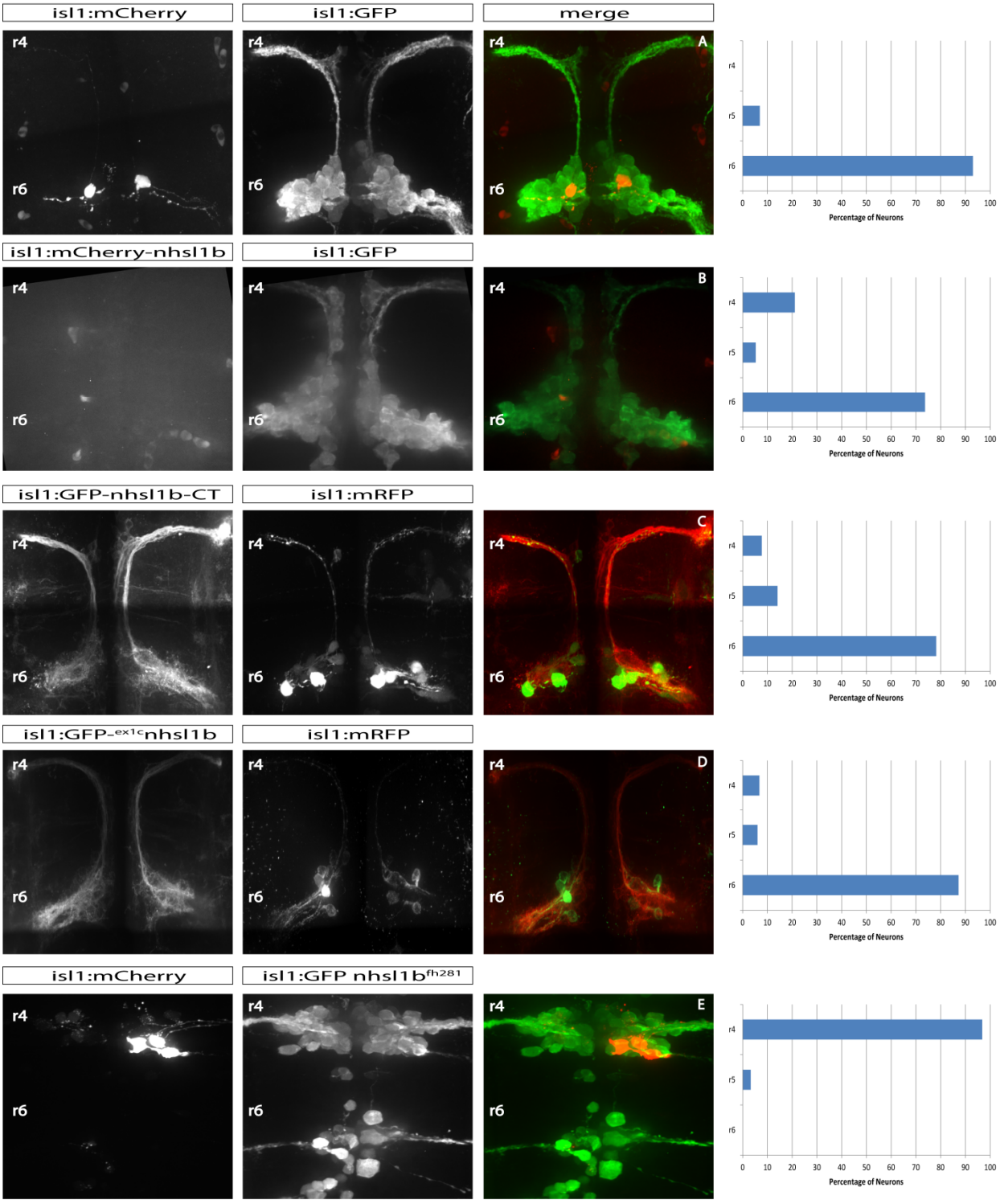

Figure 11. **Control injections do not cause a change in the FBMN migration pattern of the embryo.** (A,E) Fluorophore only, (C) *nhsl1b*-CT, (B,D) full length *nhsl1b*. Isl1:mCherry causes the least impact, 93% (A) and 96% (E), when injected; a GFP construct follows closely at 87% (D).

Works Cited

Works Cited

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Appendix A

ex1nhsl1b

ATGCCGTTTCCCGAGAGAGCCGTTGAGCCTCAGCTGCTGTGCCGGTTGAGGGGGAGCGATGGACCCGA
GAAGAGCTTTATAACACCGGACGGTCGCAAAGTTCGCAAACCGGTTTTGTTTTCCTCTCTGGAGGAAGTT
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ex1anhsl1b

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ex1bnhsl1b

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ex1cnhsl1b

ATGGTGTTCATCGGGACTTCGCTCAAGTCAGTCATTAAATACTTCAAACGCAAGG

ex1enhsl1b

ATGATGAGGGACAAGCGTTCTGGGTCTTTTAGGAGGGACAAGACAGAGAAGCCTGCGCCGATCTCTCG
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CCTTCACAACCACTCACACACTCCAGAGGGAGGAGATGATGAAGATGACAACTGGGTTTATGAGCCCCA
GCACTACATAG

Vita

John Ojumu was born October 21, 1988 in Alexandria, Virginia and is currently a US resident and citizen. After graduating from CD Hylton HS in 2007, he received his BS in Biology from VCU in 2010, where he was awarded the Provost Scholarship. He then received "real world" experience in a basic nursing school before returning to VCU for his MS in Biology.