A QUESTION OF IDENTITY: GENES THAT DISTINGUISH

MOTONEURONS FROM INTERNEURONS

by

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A DISSERTATION

Presented to the Department of Biology
and the Graduate School of the University of Oregon
in partial fulfillment of the requirements
for the degree of
Doctor of Philosophy

September 2012

DISSERTATION APPROVAL PAGE

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Title: A Question of Identity: Genes that Distinguish Motoneurons from Interneurons

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Degree awarded September 2012

DISSERTATION ABSTRACT

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Doctor of Philosophy

Department of Biology

September 2012

Title: A Question of Identity: Genes that Distinguish Motoneurons from Interneurons

The question of how a single cell can grow, divide, and ultimately acquire a distinct function within an adult animal is central to the field of developmental biology. An elegant way to address this question is by studying the specification of a specific cell type, for example, vertebrate motoneurons. For an animal to be able to move and behave appropriately, individual motoneurons (MNs) must correctly innervate specific muscles. For this to happen, MNs must first be specified and then must differentiate into distinct subtypes, each of which is classified in part by the muscle it innervates. MN subtype specification is dependent on both the acquisition of MN-specific characteristics as well as the failure to acquire characteristics specific to interneurons, cells that only innervate other neurons. The entire process of specification is initiated in progenitor cells and relies on the correct spatial and temporal expression of specific genes.

Previous work in various vertebrate models has identified some of the key genes involved in MN specification, most notably transcription factors such as *olig2*, *nkx6*s, *lhx*s, *mnx*s, and *islet1*. In this dissertation, I use the zebrafish model to demonstrate novel roles in MN specification for two of these families of transcription factors - the *lhx*s and the *mnx*s. I provide evidence that both *lhx3* and *lhx4* are

iv

necessary for normal MN and ventral interneuron (IN) development and work by

preventing MNs from expressing IN-specific characteristics. I also show that *mnx1*,

mnx2a, and *mnx2b* are necessary in MNs both to promote the acquisition of some MN

subtype-specific characteristics and to prevent the acquisition of some IN-specific

characteristics and appear to be working in part through interactions with *islet1*.

Finally, I identify an intermediate filament gene, *inab*, as being expressed in a subset

of zebrafish MNs and a ventral IN and as having a potential role in the axon outgrowth

of a specific MN subtype. Together, this work provides evidence for a mechanism of

MN specification dependent on the expression of genes that both promote aspects of

MN fate and inhibit aspects of IN fate.

This dissertation includes previously unpublished co-authored material.

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        proteins differentially affect motoneuron subtype identity and
        suppress acquisition of specific interneuron characteristcs', *submitted*.
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    Wilkes, D.E., Bennardo, N., Chan, C.W., Chang, Y.L., Corpuz, E.O., DuMond, J.,
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        N., Townsend, C.R., **Van Ryswyk, L.**, Watson, H.E., Wiedeman, A.E.,
        Rajagopalan, V., Asai, D.J. (2009). 'Identification and characterization
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ACKNOWLEDGMENTS

For Chris

TABLE OF CONTENTS

Chapter   Page

LIST OF FIGURES

LIST OF TABLES

CHAPTER I

INTRODUCTION

How a single cell can grow, divide, and develop into a complex adult animal is a question that has long fascinated developmental biologists. As cells grow and divide over the course of development, groups of cells emerge as progenitors of specific cell lineages. These progenitors go on to differentiate into a number of different cell types, each with distinct attributes. As subpopulations of cells are specified, they acquire specific characteristics that distinguish them from other cells. A well-studied example of the development of a specific cell type is vertebrate motoneurons - neural cells whose axons project out of the spinal cord to contact muscles.

For an animal to be able to move and to interact with its environment, individual motoneurons (MNs) must correctly innervate specific muscles. To do so, these MNs must differentiate into specific subtypes, each of which is classified by the muscle it innervates - this is a result of each cell's specific axon projection. And although axon projection is important in establishing the correct circuitry for locomotion, it is just one of the MN-specific characteristics that a MN must acquire as it differentiates. For a MN to acquire MN-specific characteristics and develop into the appropriate MN subtype, it must be specified correctly - a process that begins within progenitor cell populations.

The process of MN development can be broken down into three steps: First, specification is initiated in progenitor cells by the graded expression of morphogens that establish differential expression of sets of transcription factors, which in turn distinguish different progenitor populations (Ericson et al., 1996; Briscoe et al., 2000; Briscoe and Ericson, 2001; Lewis and Eisen, 2003). Next, as progenitor cells divide and become postmitotic, specification continues as the expression of particular combinations of transcription factors initiates programs of differentiation in individual cells (Briscoe and Ericson, 2001; Shirasaki and Pfaff, 2002; Lewis and Eisen, 2003). Finally, subtype specification occurs within populations of newly-born MNs, again regulated by the expression of specific transcription factors and other

1

downstream elements (Briscoe and Ericson, 2001; Shirasaki and Pfaff, 2002; Lewis and Eisen, 2003). Although many of the genetic components of MN development are known, how these genes interact with one another, and what roles they play in the different steps of MN development, is still being elucidated.

Proper MN development is also as much about *not* acquiring interneuron-specific characteristics as it is about acquiring MN-specific characteristics. Interneurons (INs) differ from MNs in a number of ways: because they synapse onto other neurons, their axons remain entirely within the spinal cord; and because they can be either excitatory or inhibitory, they release different neurotransmitters than MNs (Bernhardt et al., 1990; Higashijima et al., 2004). It has been shown that both MNs and INs can be produced from the same or closely-related progenitor domains (Kimmel et al., 1994; Shirasaki and Pfaff, 2002; Park et al., 2004), and in zebrafish, MNs and specific INs can even be siblings of one another (Park et al., 2004). Therefore, expression of a specific transcription factor in the MN progenitor population is not sufficient to distinguish a MN from an IN. Various knockdown and overexpression studies of genes involved in MN development reveal that different genes control the acquisition or repression of different MN-specific or IN-specific traits, respectively (Jurata et al., 2000; Lewis and Eisen, 2003). Active suppression of IN-specific characteristics appears to be necessary throughout the entire process of MN specification to assure proper MN development.

The process of MN development has been studied in many animals, both invertebrates and vertebrates (Arendt and Nubler-Jung, 1999; Shirasaki and Pfaff, 2002). Although much of the initial work on vertebrate MN specification has been done in the spinal cord of mouse and chick, the zebrafish has emerged as an ideal model in which to study questions of cellular identity. As vertebrates, zebrafish are remarkably similar to other models, but offer some unique advantages - the smaller overall number of cells in the spinal cord means that individual cells can be identified, and the changes in response to perturbations of the system can be analyzed on a single-cell level - and because of the rapid development of the embryo, often directly within the living animal (Eisen et al., 1986; Myers et al., 1986; Westerfield et al., 1986).

The zebrafish model confers an additional advantage to studying the acquisition of MN fate. Unlike other vertebrate models, the zebrafish motoneuron progenitor domain (pMN) generates INs as well as MNs - these cells can even be siblings of one another (Park et al., 2004). Therefore, studying the specification of zebrafish MNs allows for an even more precise analysis of what distinguishes MNs from INs than studying this in other models, such as mouse, in which the pMN domain generates only MNs, and the most closely-related INs arise from the adjacent p2 progenitor domain (Shirasaki and Pfaff, 2002). Already, work done in the zebrafish has revealed some genes that are involved in both the acquisition of MN-specific characteristics and the repression of IN-specific characteristics (Park et al., 2002; Cheesman et al., 2004; Hutchinson and Eisen, 2006; Hutchinson et al., 2007).

There are a few different ways in which a single gene can influence MN development. A MN-specifying gene can be expressed solely in MN progenitors, where it tends to affect a more general "MN versus IN" fate choice. A MN-specifying gene can also be expressed only in postmitotic MNs, where it tends to be involved in MN subtype specification. The genes expressed postmitotically especially tend to be involved in the acquisition of MN-specific characteristics and the repression of closely-related IN-specific characteristics. Finally, a MN-specifying gene can be expressed in both progenitors and postmitotic neurons, in which case it usually affects both general and subtype-specific MN development. Many of the genes identified to be involved in MN development are conserved across vertebrate species, and although they play similar roles in MN specification, they often have a slightly different functions in different animal species.

olig2 is an example of a transcription-factor encoding gene that is expressed in MN progenitors and has been shown to be involved in vertebrate MN specification (Mizuguchi et al., 2001; Novitch et al., 2001; Takebayashi et al., 2002). In both mouse and zebrafish, *olig2* is necessary for the formation of MNs (Park et al., 2002; Takebayashi et al., 2002). *olig2* is also sufficient to specify MNs, as its overexpression in both chick and zebrafish spinal cord generates ectopic MNs (Mizuguchi et al., 2001; Novitch et al., 2001; Park et al., 2002). This gene is clearly

working in MN progenitors to initiate programs of MN specification, and its general function is conserved across vertebrates.

The LIM homeodomain transcription factor *islet1* is an example of a gene that is expressed in postmitotic MNs (Korzh et al., 1993; Appel et al., 1995), and has been shown to be necessary for correct vertebrate MN specification (Pfaff et al., 1996; Thaler et al., 2004). In mouse, chick, and zebrafish spinal cords, *islet1* is necessary for the generation of MNs (Tsuchida et al., 1994; Pfaff et al., 1996; Hutchinson and Eisen, 2006). In the absence of *islet1* in zebrafish, the cells that would have become MNs instead acquire characteristics of neighboring INs. However, they maintain some attributes of MNs, and develop as MN/IN hybrids that simultaneously express some attributes of each cell type (Hutchinson and Eisen, 2006). *islet1* seems to be acting in post-mitotic MNs to maintain MN identity, as well as being able to initiate programs of MN specification.

As a final example, *nkx6* is a transcription factor-encoding gene that is expressed in both progenitor cells and postmitotic MNs (Sander et al., 2000; Vallstedt et al., 2001; Cheesman et al., 2004). It has been shown to be involved in both general MN specification (Sander et al., 2000; Vallstedt et al., 2001) as well as MN subtype specification (Cheesman et al., 2004; Hutchinson et al., 2007). In mouse and chick, the absence of *nkx6* leads to misspecification of progenitor cells and a drastic reduction in MN number (Sander et al., 2000; Vallstedt et al., 2001). In zebrafish, overexpression of *nkx6.1* is sufficient to generate ectopic MNs (Cheesman et al., 2004). In the absence of both zebrafish *nkx6*s, a specific MN subtype acquires characteristics of neighboring INs and these cells develop as MN/IN hybrids (Hutchinson et al., 2007). It is worth noting that these hybrid cells acquire IN characteristics that are distinct from the hybrids generated by *islet1* knockdown. *nkx6* is crucial for both initiating a program of MN specification as well as refining MN subtype specification, and its function is generally conserved across vertebrates.

Although transcription factor-encoding genes such as *olig2*, *islet1*, and *nkx6* have been shown to be involved in both MN formation and MN subtype specification, other genes must be involved in the process as well. No single gene appears to be responsible for a complete fate switch between MN identity and IN

identity. Each of these loss-of-function and gain-of-function studies affects MN development slightly differently, indicating that each gene is potentially involved in a slightly different aspect of the specification process. Identification of either new transcription factors or downstream genes is crucial to fill in the gaps in the current knowledge of genetic pathways specifying MN fate.

Previous work in both mouse and chick has suggested two additional families of transcription factors that may be involved in MN specification - the LIM homeodomain transcription factor-encoding genes *lhx3* and *lhx4*, and the homeobox transcription factor-encoding gene *mnx1* (Sharma et al., 1998; Tanabe et al., 1998; Arber et al., 1999; Thaler et al., 1999; Thaler et al., 2002; William et al., 2003).

lhx3 and *lhx4* are expressed in mouse MNs and are required for proper subtype specification for a certain class of MNs (Sharma et al., 1998). In the absence of both of these *lhx* genes, MNs with axons that exit the neural tube ventrally are converted into a different class of MNs with axons that exit the neural tube dorsally (Sharma et al., 1998), and conversely the overexpression of *lhx3* and *lhx4* is sufficient to turn MNs with dorsally-exiting axons into MNs with ventrally-exiting axons (Sharma et al., 1998; Thaler et al., 2002). *lhx3* is expressed in zebrafish MNs as well as some INs (Appel et al., 1995; Kimura et al., 2006; Batista et al., 2008), and I have contributed to work showing that the same is true for *lhx4* (Hutchinson et al., *in preparation*). Although these genes are likely to be involved in zebrafish MN specification, their additional expression in INs hints at a distinct role for these genes in zebrafish MN development.

mnx1 is expressed in MN progenitors and postmitotic MNs in the mouse, and knocking it down causes MNs to transiently acquire characteristics of neighboring INs (Arber et al., 1999; Thaler et al., 1999). A homolog in chick, *MNR2*, is expressed in MN progenitors in the chick spinal cord, and its overexpression is sufficient to induce ectopic MNs (Tanabe et al., 1998; William et al., 2003). Zebrafish have a homolog of *mnx1*, as well as two *MNR2* paralogs, *mnx2a* and *mnx2b*, and I have contributed to work showing that all of these genes are expressed in zebrafish MNs (Wendik et al., 2004; Van Ryswyk et al., *in preparation*). Again, though the *mnx* genes

are likely involved in zebrafish MN specification, their expression in an IN suggests a distinct role for these genes in zebrafish MN development.

Here I present work that demonstrates roles for *lhx* and *mnx* genes in zebrafish MN specification, as well as the results of a microarray screen to uncover more genes that are potentially involved in MN specification and/or differentiation. In Chapter II, I show that zebrafish *lhx3* and *lhx4* are expressed in both MNs and INs, and are together necessary for the subtype specification of a specific MN subtype - this chapter includes material coauthored with S. A. Hutchinson, S. D. Seredick, J. C. Talbot, and J. S. Eisen. In Chapter III, I show that *mnx1*, *mnx2a*, and *mnx2b* are dynamically expressed in zebrafish MNs and an identified IN, and are necessary for the subtype specification of a specific MN subtype - this chapter includes material coauthored with S. D. Seredick, S. A. Hutchinson, and J. S. Eisen. Finally, in Chapter IV I provide the first expression and functional analysis of a neuronal intermediate filament gene, *inab*, that is potentially involved in the acquisition of axon morphology for a particular subtype of zebrafish MNs.

CHAPTER II

LHX3 AND LHX4 PREVENT MOTONEURONS FROM CO-EXPRESSING INTERNEURON

CHARACTERISTICS

The work described in this chapter was co-authored by S. A. Hutchinson, S. D.
Seredick, myself, and J. C. Talbot. We worked with J. S. Eisen on experiment design,
and were responsible for the majority of data collection, data analysis, and writing. J.
S. Eisen also contributed to writing, and the work was carried out in her laboratory.

INTRODUCTION

Vertebrate motoneurons and interneurons have distinct morphological and
biochemical characteristics, yet they are generated from closely-related ventral
neural tube progenitors and, in some cases, they are siblings derived from the same
progenitor. Despite intense study, it is still unclear how discrete motoneuron and
interneuron identities are determined during vertebrate development. Here we
take advantage of the ability to recognize individual motoneurons (MNs) and
interneurons (INs) in embryonic zebrafish to explore this issue.

MNs are specifically generated from the progenitor of motoneuron (pMN)
domain in the ventral spinal cord. In contrast, INs are generated from the adjacent
p3 and p2 domains (Briscoe et al., 2001), and from the pMN domain (Park et al.,
2004), depending on their subtypes. Combinations of LIM homeodomain (LIM-HD)
proteins have been shown be pivotal in the fates of MNs and INs in both vertebrates
and invertebrates (Appel, 1999; Shirasaki and Pfaff, 2002; Thaler et al., 2002). Two
LIM-HD proteins, Lhx3 and its paralog Lhx4, are implicated in proper specification
of pMN domain-derived MNs and p2 domain-derived V2 INs. Lhx3 and Lhx4 have
both overlapping and distinct functions during development of the pituitary (Mullen
et al., 2007), however, these proteins appear to have redundant functions during
neural development. For example, misexpression of either Lhx3 or Lhx4 is sufficient
to cause development of ectopic MNs in mouse spinal cord (Sharma et al., 1998), as
long as the cells also express *MNR2* (Tanabe et al., 1998) or Islet1 (Thaler et al.,
2002). In the absence of both Lhx3 and Lhx4, MNs retain MN characteristics, but

their subtype identity is altered and they extend axons toward muscle targets appropriate for their new identity (Sharma et al., 1998). Similarly, misexpression of Lhx3 is sufficient to specify ectopic V2 INs in chick ventral spinal cord (Tanabe et al., 1998; Thaler et al., 2002). However, whether Lhx3 or Lhx4 is required for IN specification remains untested.

We followed development of individually identified MNs and INs in the zebrafish spinal cord to investigate the roles of Lhx3 and Lhx4 in their development. Development of the zebrafish spinal cord is very similar to that of other models such as mouse and chick except that, early in development, zebrafish have many fewer cells of each type (Lewis and Eisen, 2003; Lewis, 2006). For example, in zebrafish the pMN domain initially generates only 3-4 MNs in each spinal cord hemisegment. These cells are referred to as primary motoneurons (PMNs) and each of them can be individually identified based on the position of its cell body, the order in which it grows out its axon, the region of muscle it innervates, and its gene expression pattern (Lewis and Eisen, 2003). Later, the pMN domain generates additional MNs, referred to as secondary motoneurons (SMNs); whether SMNs are individually identified remains unresolved. In contrast to what has been described in amniote vertebrates, the zebrafish spinal cord pMN domain generates INs as well as MNs (Park et al., 2004; Shin et al., 2007). Here we focus on two of these interneurons - KA', which contacts the ventricle lumen and helps drive the spinal central pattern generator (Martin et al., 1998; Wyart et al., 2009), and VeLD, an IN of unknown function with a long descending axon. Like the early-developing PMNs, these INs can be individually identified based on the positions of their cell bodies, their axon morphologies, and their gene expression patterns (Bernhardt, 1990; Eisen, 1991a; Lewis and Eisen, 2003; Park, 2004; Batista et al., 2008). The zebrafish ventral neural tube also contains a p2 progenitor domain, similar to amniote vertebrates (Briscoe and Ericson, 2001). The earliest-developing V2 INs - V2a and V2b - can be individually identified, however, many more V2 neurons are generated over time and it is as yet unknown whether these later-developing V2 INs are also individually identified (Kimura et al., 2006; Batista et al., 2008; Kimura et al., 2008).

We show that similar to Lhx3 (Appel et al., 1995; Kimura et al., 2006; Batista et al., 2008), Lhx4 is expressed in MNs and INs derived from both the pMN and p2 domains. Our results also provide evidence that Lhx3 and Lhx4 are differentially required for development of distinct MNs and INs. They are unnecessary for specification of pMN domain-derived VeLD INs and the first set of p2 domain-derived V2 INs. However, they are required for specification of later-developing p2 domain-derived V2a INs. Lhx3 and Lhx4 are also necessary for normal development of pMN domain-derived SMN, PMN, and KA' neurons. We show that loss of Lhx3 and Lhx4 results in PMNs that develop as hybrids that possess morphological and neurotransmitter characteristics of both PMNs and INs. These studies support a model in which Lhx3 and Lhx4 are involved in distinguishing MN and IN fates and provide new evidence about their roles in the fates of specific INs.

METHODS

Embryos

Wild-type, *Tg(olig2:egfp) vu1* (Park et al., 2004), and *Tg(vsx1:gfp) nns5* (Kimura et al., 2008) zebrafish (*Danio rerio*) embryos were collected from natural crosses of adults, raised at 28.5°C, and staged by hours postfertilization (hpf) at 28.5°C and gross morphology (Kimmel et al., 1995).

Zebrafish *lhx4* cDNA islolation

Potential zebrafish *lhx4* sequence was obtained from zebrafish genomic sequence in the Sanger database (http://www.sanger.ac.uk/Projects/D_rerio/). Specific primers (forward 5'-CACACGGCGAAAGAACTCACG-3') and reverse (5'-TTTG CCCACACCGAACACTG-3') were designed to 5' and 3' UTR sequences and used to amplify *lhx4* cDNA from single stranded cDNA from 24 hpf zebrafish.

Phylogenetic analysis

MacVector software, using the Clustal W algorithm, created a maximum likelihood tree. Sequences included on the tree were gathered from GenBank.

RNA *in situ* hybridization

RNA *in situ* hybridization followed by NBT/BCIP staining was performed as described in Appel and Eisen (Appel and Eisen, 1998). RNA in situ hybridization followed by fluorescent staining was performed as described in Talbot et al. (Talbot et al., 2010). RNA probes include *lhx3* and *islet2a* (Appel et al., 1995), *chat* (Tallafuss and Eisen, 2008), *gad1b* and *gad2* (collectively referred to as *gad*) (Higashijima et al., 2004a), *vsx2* (Kimura et al., 2006), *tal1* (Batista et al., 2008), *tal2* (Yang et al., 2010), and *lhx4.*

Immunohistochemistry

Production of Lhx3 and Lhx4 antibodies

To prepare Lhx3 and Lhx4 antisera, cDNAs corresponding to amino acids 237-397 of Lhx3 or amino acids 241-389 of Lhx4 were cloned into the *pET28a* vector. Amino acids 237-397 of Lhx3 and amino acids 241-389 of Lhx4 correspond to the C-terminal ends of the proteins, C-terminal to the homeodomain; these represent the most diverged regions of these proteins. Polyclonal antisera raised against these regions were expected to recognize these proteins differentially. Fusion protein expression was induced in bacteria by 1mM IPTG and purified over a nickel column according to manufacturers instructions (QIAGEN) under native conditions. Purified proteins were used to immunize rabbits.

Antibody staining

The following primary antibodies were used: monoclonal anti-Islet (Korzh et al., 1993) recognizes Islet1 and Islet2 proteins (1:200; 39.4D5, Develomental Studies Hybridoma Bank), polyclonal anti-GABA (1:1000; Sigma), polyclonal anti-Gad (1:500; Abcam ab11070), zn1 monoclonal (1:200; Trevarrow et al., 1990), znp1 monoclonal (1:1000; Trevarrow et al., 1990), anti-Alcam monoclonal (1:4000; previously known as zn5, zn8, anti-DM-GRASP, anti-neurolin; Trevarrow et al., 1990), polyclonal anti-Lhx3 (1:500), and polyclonal anti-Lhx4 (1:500). The following secondary antibodies from Invitrogen-Molecular Probes (Eugene, Oregon) were used: goat anti-mouse Alexa-488 (1:1000), goat anti-mouse IgG_1 Alexa-488 (1:500), goat anti-mouse IgG_{2a} Alexa-488 (1:500), goat anti-mouse IgG_{2b} Alexa-546

(1:500), and goat anti-rabbit Alexa-546 (1:1000). Embryos were fixed for 3.5-4.0 hours in 4% paraformadehyde and 1x Fix Buffer (Westerfield, 2007) at 4°C. Embryos were blocked in 1xPBS, 5%NGS, 4mg/mL BSA, 0.5% Triton X-100 for 1 hour at room temperature then incubated in primary antibody diluted in block overnight at 4°C. Embryos were washed at room temperature for 1.5 hours in PBS + 0.1% Tween-20, incubated in secondary antibody diluted in block for 4 hours at room temperature, and then washed for 1.5 hours at room temperature in PBS + 0.1% Tween-20. Embryos were stored in 4% paraformaldehyde until analyzed.

Microscopy

Images of embryos were captured on a Zeiss Axioplan equipped with a digital camera, or a Zeiss Pascal confocal microscope. Adobe Photoshop was used to adjust brightness and contrast of images.

Morpholino injections

To create embryos with reduced Lhx3 or Lhx4, translation blocking morpholinos (MOs) were designed by Gene Tools (Philomath, Oregon) to the translation start sites of *lhx3* and *lhx4*: *lhx3* MO (5'-GTTCTAACAACATTCTGGCGATA AA-3') and *lhx4* MO (5'-GCAGCACAGCCGCACTTTGCATCAT-3') to positions +7 through +31 in *lhx3* or *lhx4* genomic DNA respectively. Several nanoliters of 2.5mg/mL *lhx3* MO or 5mg/mL *lhx4* MO were injected into 1-cell stage embryos as described in Lewis and Eisen (Lewis and Eisen, 2001). Morpholino specificity was established by using mis-match control MOs: *lhx3* MO 5-mis (5'-GTTGTAAGAACATT GTGGCCTTAA-3'), and *lhx4* MO 5-mis (5'-GCACCACCCCGGACTTTCCATGAT-3'). To knock down both Lhx3 and Lhx4, *lhx3* and *lhx4* MOs were co-injected into 1-cell embryos at the same concentrations listed above. Embryos looked generally healthy and had little or no Lhx protein remaining (Figure 1).
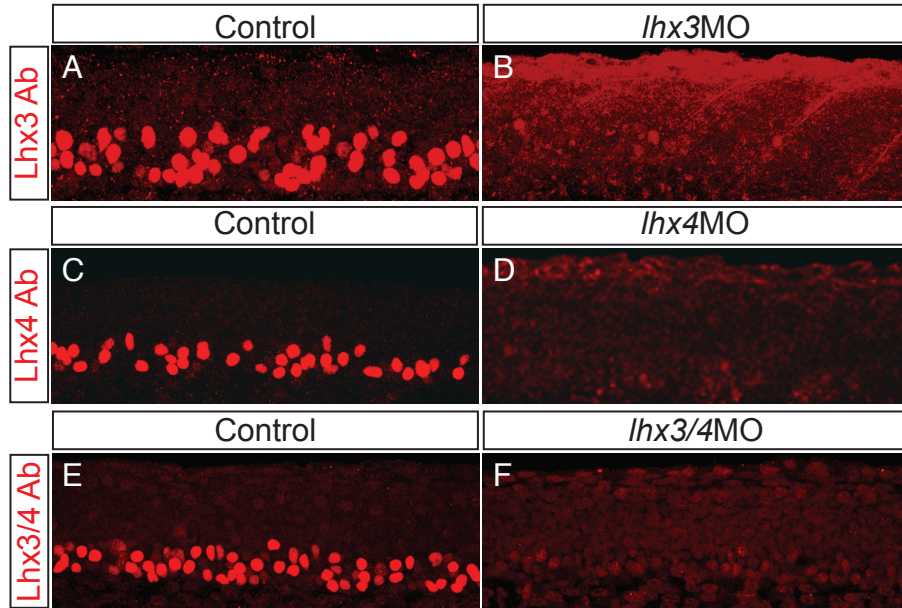
Figure 1. Morpholinos targeting *lhx* family genes are specific and effective at knocking down protein
(**A-F**) All images are lateral views of embryo trunks oriented with anterior to the left, unless otherwise noted. Control embryos are labeled for antibodies against Lhx3 (A), Lhx4 (C), or Lhx3 and Lhx4 (E). Embryos injected with *lhx3* MO lack Lhx3 labeling (B), embryos injected with *lhx4* MO lack Lhx4 labeling (D), and embryos injected with a combination of *lhx3* and *lhx4* MOs lack both Lhx3 and Lhx4 labeling (F).

RESULTS

Lhx4 is expressed in motoneurons and ventral interneurons

We isolated a full length zebrafish *lhx4* gene that has 1171 base pairs and 390 amino acids (Figure 2A). In phylogenetic analyses the protein encoded by this gene clusters with other Lhx4 proteins, and not with Lhx3 proteins, indicating it is an Lhx4 homologue (Figure 2B). Like other Lhx3 and Lhx4 proteins, the zebrafish Lhx4 protein has two LIM domains and a homeodomain that are highly conserved (Figure 2C).

To identify neural tube cell types that may require Lhx4 function, we performed RNA *in situ* hybridization on zebrafish embryos of various stages. Like *lhx3*, *lhx4* is expressed in two medial stripes of cells in the region that will form ventral spinal cord, beginning at the 2-3 somite stage [s; 11 hours postfertilization (hpf)] (Figure 3A, B). At 18 and 24 hpf, *lhx4* is expressed in a subset of *lhx3*-positive
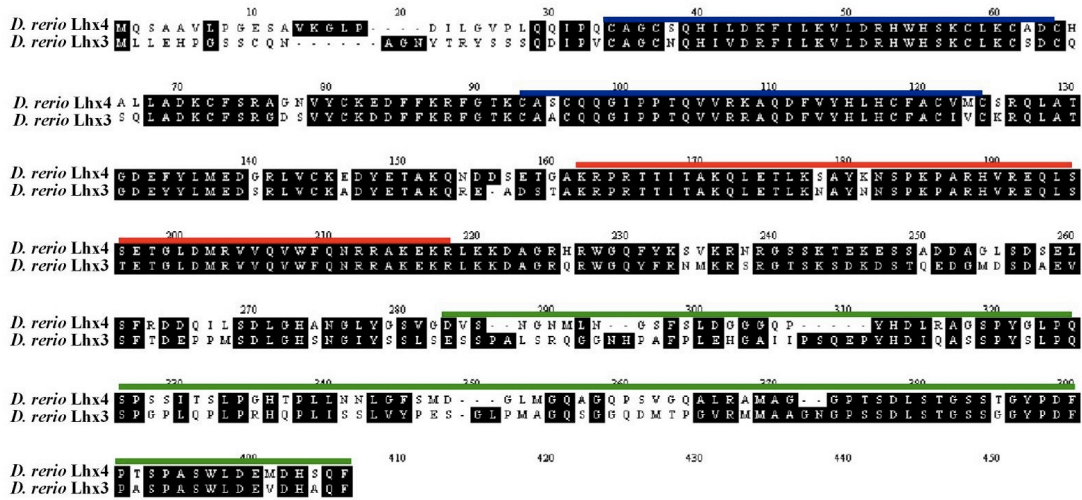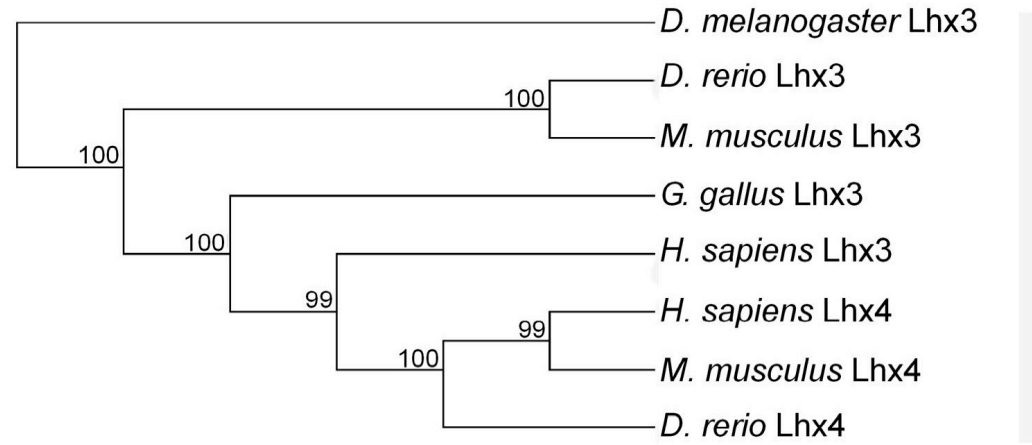
Figure 2. Zebrafish *lhx4* is closely related to other vertebrate *lhx4* homologues
(A) Alignment of zebrafish Lhx3 and Lhx4 proteins. Blue line indicates LIM domain 1, red line indicates LIM domain 2, and green line indicates homeodomain. (B) Phylogeny of Lhx3 and Lhx4 proteins showing that zebrafish Lhx4 groups with other vertebrate Lhx4 proteins. (C) Comparison of amino acid similarity in the conserved domains of Lhx4 proteins from mouse and pufferfish as well as Lhx3 from zebrafish. The percent of amino acids that are identical to the amino acids in zebrafish Lhx4 was calculated for the two LIM domains and the homeodomain (HD) of each protein.

spinal cord cells (Figure 3C, D; data not shown). The *lhx3* and *lhx4* co-expressing cells are ventral to *lhx3* only cells (Figure 3K, L) that are probably p2 domain-derived INs that have previously been shown to express *lhx3* (Kimura et al., 2006; Batista et al., 2008). In contrast, the positions of the *lhx3* and *lhx4* co-expressing cells suggest that they are primarily pMN-derived MNs and INs that have also previously been shown to express *lhx3* (Appel et al., 1995). By 28 hpf, *lhx3* and *lhx4* appear to be almost entirely co-expressed (Figure 3M). Thus at this stage *lhx4*, like *lhx3*, is expressed in MNs as well as pMN-derived and p2-derived INs. In addition to expression in the spinal cord, *lhx4* is also expressed in *lhx3*-positive cells in the pituitary and in a dorsal IN population between the eyes (Figure 3E, F) that is *lhx3*-negative.

We examined localization of Lhx3 and Lhx4 proteins using polyclonal antibodies we generated to the C-terminal ends of the two proteins. These antibodies label cells in patterns that closely mirror *lhx3* and *lhx4* mRNA expression

Figure 3 (next page). *lhx4* is expressed in many cells in the ventral neural tube (**A,B**) Flat-mounted 11 hpf embryos with yolk removed. Both *lhx3* (A) and *lhx4* (B) are expressed beginning at 11 hpf in two medial stripes of cells. (**C-F**) Images taken at 24 hpf at the level of somites 8-12. Both *lhx3* (C) and *lhx4* (D) are expressed in the MN domain of the ventral neural tube. However, *lhx4* is expressed in fewer cells than *lhx3* (black asterisks indicate labeled cells in C and D). The anterior edge of the head (black arrows) expresses both *lhx3* (E) and *lhx4* (F) RNAs at 24 hpf, but *lhx4* is expressed in a dorsal set of cells between the eyes (black arrowhead) that do not express *lhx3* (E,F). (**G-J**) Islet antibody (green) labels both Islet1 and Islet 2. Dorsal green cells are Rohon-Beard sensory neurons. Images are z-projections of confocal images of one half of the neural tube at the level of somites 8-12. Lhx3 antibody labels all Islet positive PMNs at 18 hpf, as well as several other cells in the ventral neural tube (G). At 24 hpf (H) all Islet+ cells are still co-labeled with Lhx3 antibody, and there are many more cells that are labeled with Lhx3 alone in the ventral neural tube. In contrast to Lhx3 antibody, Lhx4 antibody-positive cells are all co-labeled with Islet antibody at 18 hpf (I), suggesting that Lhx4 is expressed only in PMNs at this time point. By 24 hpf, all cells labeled with Islet antibody are also co-labeled with Lhx4 antibody, but there are also many cells in the ventral neural tube that are labeled with Lhx4 antibody alone (J). (**K-M**) *lhx4* RNA (green) and *lhx3* RNA (red) label cells in the ventral neural tube. At 20 hpf, *lhx4* RNA is expressed in fewer cells than *lhx3* RNA (K). At 24 hpf, *lhx4* RNA is expressed in more dorsal cells that also express *lhx3* RNA (L). At 28 hpf *lhx4* is expressed in almost all cells expressing *lhx3* (M).
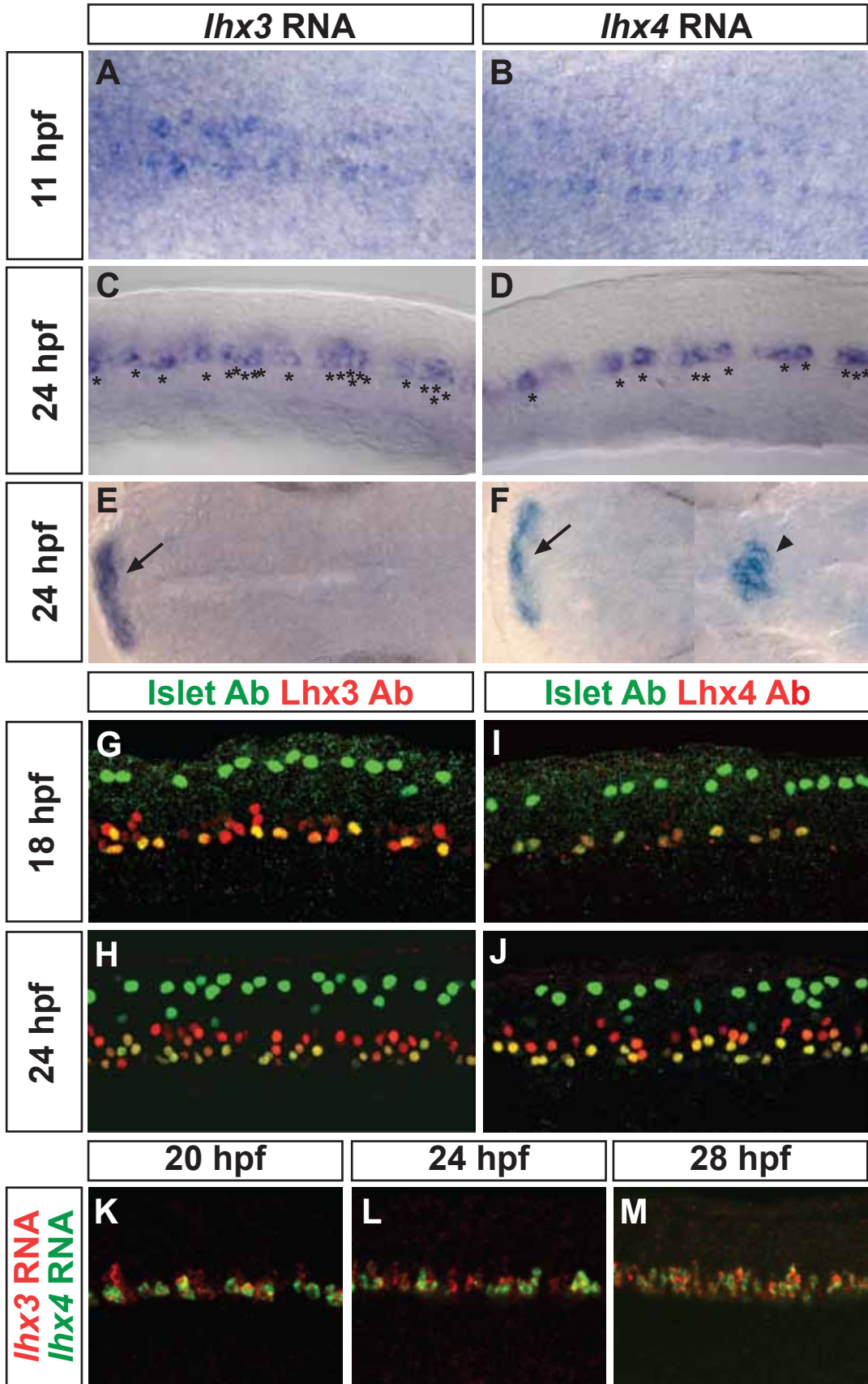
(Fig. 3G-J), Lhx3 antibody label is abolished by knockdown using *lhx3* MO, and Lhx4 antibody label is abolished by knockdown using *lhx4* MO (Figure 1A-D). In addition, we isolated one antibody that reacts with both Lhx3 and Lhx4 (referred to as Lhx3/4 antibody), indicated by labeling of the dorsal IN population in the head in addition to the entire set of *lhx*-positive ventral spinal neurons (data not shown). Lhx3/4 antibody label is only abolished when *lhx4* and *lhx3* MOs are co-injected (Figure 1E, F).

In zebrafish, *lhx3* mRNA is expressed in all PMNs, in VeLD INs (Appel et al., 1995) and initially in all V2 INs (Kimura et al., 2006; Batista et al., 2008). To learn whether Lhx4 is expressed in PMNs, we double-labeled embryos with Lhx3 or Lhx4 antibody and an antibody that recognizes Islet1 and Islet2 proteins (Korzh et al., 1993), and thus labels PMNs at 18 hpf, and PMNs plus some SMNs at 24 hpf (Figure 3G-J). Lhx3 and Lhx4 were both expressed in all Islet-positive cells in the ventral spinal cord at 18 and 24 hpf, indicating they are expressed in all PMNs and at least some SMNs. At 18 hpf, Lhx4 antibody labeled exclusively Islet-positive cells, suggesting that initially Lhx4 is only expressed by PMNs. In contrast, at 18 hpf Lhx3 antibody labeled more cells than Islet antibody, consistent with expression in pMN-derived and V2 INs (Appel et al., 1995; Batista et al., 2008). By 24 hpf, both Lhx3 and Lhx4 were expressed in many Islet-negative cells in the ventral spinal cord (Figure 3H, J). These data indicate that, similar to Lhx3, Lhx4 is expressed in PMNs, SMNs, and in pMN domain-derived and p2 domain-derived ventral INs at 24 hpf.

Lhx3 and Lhx4 are unnecessary for early-developing p2 domain-derived interneurons but required for later-developing V2a interneurons

Lhx3 is expressed in p2 domain-derived V2 INs (Sharma et al., 1998; Batista et al., 2008), but whether it is necessary for their differentiation remains unknown. In zebrafish, *lhx3* is initially expressed in both V2a and V2b INs; it is later maintained in V2a neurons but downregulated in V2b neurons (Batista et al., 2008). We found that *lhx4* is co-expressed with *lhx3* in p2 domain-derived INs (Figure 3). Thus, we hypothesized that in the absence of Lhx3 and Lhx4, prospective V2a neurons might develop as V2b neurons. To test this hypothesis, we knocked down

Lhx3 and Lhx4 in the *Tg(vsx1:GFP)nns5* transgenic line in which both V2a and V2b neurons express GFP (Kimura et al., 2008). We then examined expression of *vsx2* which marks V2a neurons, and *tal1* which marks V2b neurons (Batista et al., 2008) at 22 hpf, after individual p2 domain progenitors have generated the first V2a and V2b sibling pairs (Kimura et al., 2008). Contrary to our hypothesis, we found no change in the number of V2a or V2b INs (Table 1; Figure 4A-D), indicating Lhx3 and Lhx4 are unnecessary for formation of the first pair of V2a and V2b sibling neurons.

Because the number of V2 neurons increases over time (Kimura et al., 2006), we also examined expression of *vsx2* at a later time point. After 24 hpf there are 2 *vsx2*-positive V2a neurons that have slightly different positions (Figure 5A), consistent with what has been described previously (Kimura et al., 2006). MO knockdown of Lhx3 and Lhx4 resulted in the absence of one of the *vsx2*-positive neurons (Figure 5B). This result is consistent with our observation with Lhx3 and Lhx4 are unnecessary for formation of the first V2a neuron, and also provides evidence that Lhx3 and Lhx4 are required for formation of later-developing V2a neurons. Thus, Lhx3 and Lhx4 have distinct roles in V2a INs that develop at different times.

Lhx3 and Lhx4 are necessary for formation of pMN domain-derived MNs

In mouse embryos with a targeted deletion of both Lhx3 and Lhx4, MNs are present but their subtype identities are altered. In this case, MNs extend axons toward muscle targets appropriate for their new identities, but inappropriate for their identities when either Lhx3 or Lhx4 is present (Sharma et al., 1998). To

Table 1. Lhx3 and Lhx4 are required for formation of KA's but not other ventral spinal cord interneurons

IN type	stage	control	*lhx3* & *lhx4* MOs
V2a	24 hpf	1.0 $\pm$ 0.1; n=5 embryos	0.9 $\pm$ 0.2; n=15 embryos
V2b	24 hpf	1.0 $\pm$ 0.2; n=6 embryos	1.0 $\pm$ 0.2; n=10 embryos
VeLD	20 hpf	1.0 $\pm$ 0.3; n=7 embryos	0.9 $\pm$ 0.3; n=10 embryos
KA'	28 hpf	3.8 $\pm$ 1.4; n=8 embryos	*1.3 $\pm$ 0.7; n=17 embryos
KA"	28 hpf	2.8 $\pm$ 0.5; n=8 embryos	3.0 $\pm$ 0.8; n=17 embryos

Average number of interneurons per segment at mid-trunk.
*p-value < 0.0001
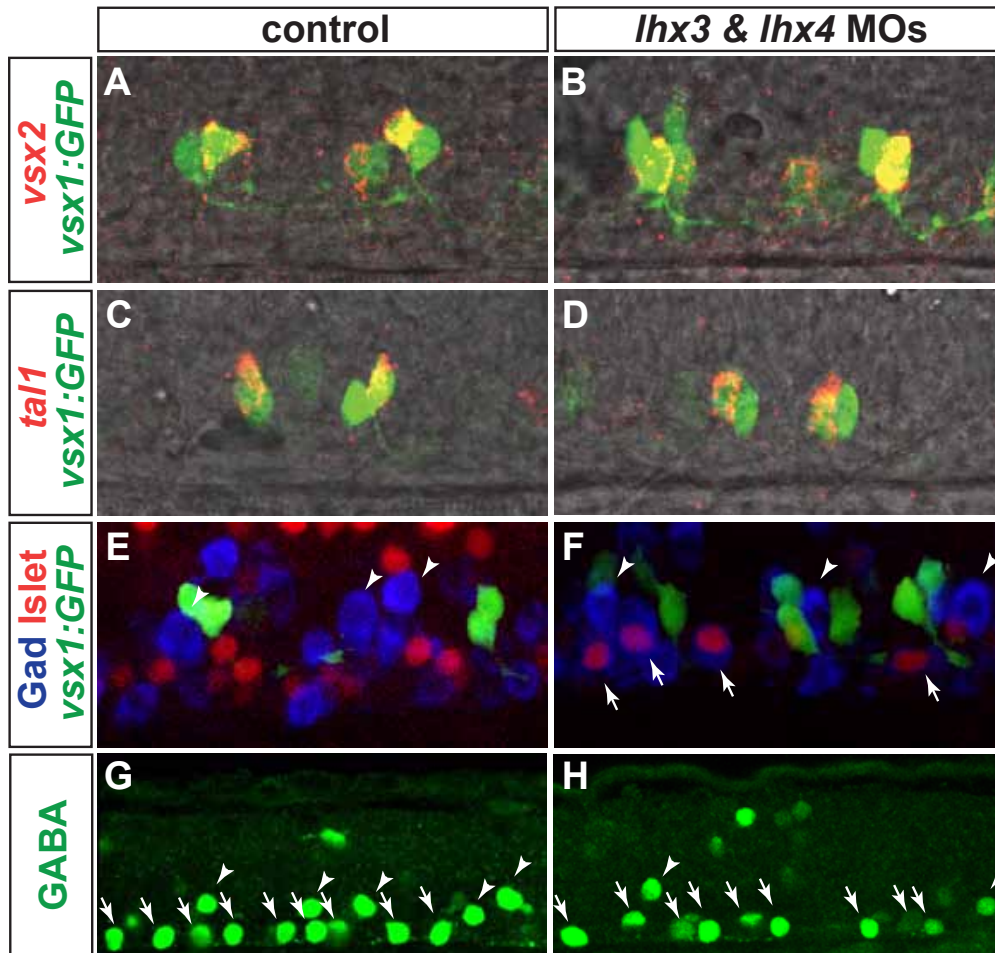
Figure 4. Lhx3 and Lhx4 are required for KA' interneuron formation
(**A-D**) Single optical slices from confocal images taken of 22 hpf embryos at somite
level 8-12. (**A,B**) *Tg(vsx1:GFP)^nns5* embryos co-labeled with GFP (green) and *vsx2*
RNA (red) identify V2a INs. The number of V2a INs in embryos injected with *lhx3*
and *lhx4* MOs (B) are indistinguishable from control embryos (A; Table 1). (**C,D**)
Tg(vsx1:GFP)^nns5 embryos co-labeled with GFP (green) and *tal1* RNA (red) identify
V2b INs. The number of V2b INs in embryos injected with *lhx3* and *lhx4* MOs (D) are
indistinguishable from control embryos (C; Table 1). (**E,F**) Z-projections of confocal
images taken of 20 hpf embryos at somite levels 8-12, representing one half of the
neural tube. *Tg(vsx1:GFP)^nns5* embryos co-labeled with Gad (blue) and Islet (red)
discriminate VeLD INs (GFP⁻/Gad⁺/Islet⁻; arrowheads), V2b INs (GFP⁺/Gad⁺/Islet⁻),
and PMNs aberrantly expressing Gad (GFP⁻/Gad⁺/Islet⁺; arrows). Very few V2b INs
have begun to express Gad by 20 hpf. The number of VeLD INs in embryos injected
with *lhx3* and *lhx4* MOs (F) are indistinguishable from control embryos (E; Table 1).
(**G,H**) Z-projections of confocal images from the medial spinal cord of 28 hpf
embryos at somite levels 8-12 labeled with GABA. KA' INs are labeled with
arrowheads and KA" INs are labeled with arrows. KA', but not KA" INs are reduced
in the embryos injected with *lhx3* and *lhx4* MOs (H) relative to controls (G; Table 1).
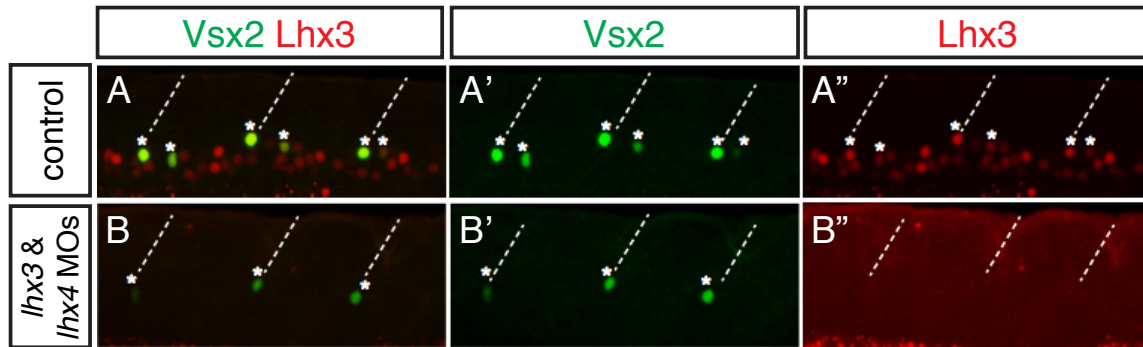
Figure 5. Later-developing V2 interneurons are lost with *lhx* knockdown
(**A,B**) Single optical slices from confocal images taken of 26 hpf embryos. Dotted lines mark segment boundaries. Two Vsx2+/Lhx3+ V2 neurons are visible in each hemisegment in control embryos (A). In the absence of both Lhx3 and Lhx4, the number of Vsx2+ V2 neurons is reduced to one per hemisegment (B).

determine whether MN identity was similarly altered in zebrafish lacking Lhx3 and Lhx4, we knocked down these proteins using MOs and assayed the axon trajectories of both SMNs and PMNs. Similar to mouse *lhx3* or *lhx4* single mutants, knock-down of either Lhx3 or Lhx4 function alone had no effect on SMN axon trajectories (Figure 6A-C). However, in contrast to mouse *lhx3* plus *lhx4* double mutants (Sharma et al., 1998), SMNs were absent from zebrafish *lhx3* plus *lhx4* MO embryos (Figure 6D), showing that these proteins are required for SMN formation.

As described earlier, both Lhx3 and Lhx4 are present in all PMNs, suggesting both proteins may also be required for PMN formation. To assess the role of Lhx3 and Lhx4 in PMN formation, embryos were labeled with zn1 and znp1 antibodies that reveal the axons of two PMNs, CaP and MiP (Trevarrow et al., 1990; Melancon et al., 1997). CaPs extend axons that innervate the ventral portion of each myotome, whereas MiPs extend axons that innervate the dorsal portion of each myotome (Lewis and Eisen, 2003). *lhx3* MO or *lhx4* MO injection alone had no effect on PMN axons (Figure 7A-C). Similar to SMNs, co-injection of *lhx3* MO and *lhx4* MO resulted in a reduction in the number of PMN axons (Figure 7D; Table 2). This reduction was sporadic along the length of the embryo, and in many cases differentially affected CaPs and MiPs (Figure 7). For example, Figure 7 shows one segment lacking both CaP and MiP axons, one segment with only a CaP axon, and one segment with only a MiP axon. These data suggest that PMN axons are able to form when Lhx3 and Lhx4
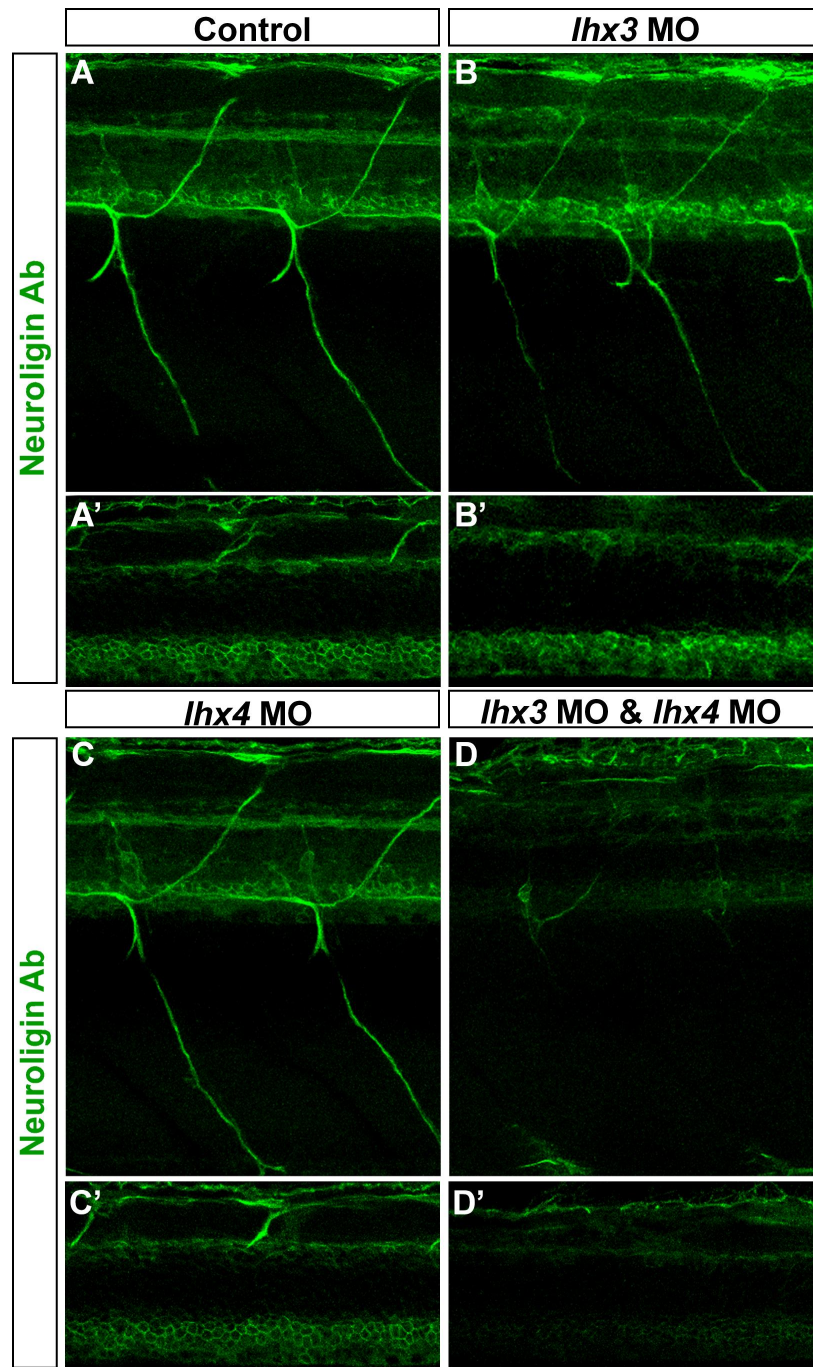
Figure 6. Lhx3 and Lhx4 are required for SMN formation
(**A-D**) Z-projections of confocal images of 72 hpf embryos taken at somite levels 8-12, representing one half of the neural tube and labeled with Neuroligin antibody. Control embryos (A) have both dorsally and ventrally projecting axons labeled by Neuroligin as well as many cell bodies (A') in the ventral neural tube. Both *lhx3* MO and *lhx4* MO-injected embryos have normal Neuroligin labeled axons (B,C) and cell bodies (B',C'). Embryos injected with both *lhx3* and *lhx4* MOs lack SMN axons (D) and cell bodies (D') labeled with Neuroligin antibody, leaving only floor plate cells labeled in the ventral neural tube.

are lost, albeit at a lower frequency than in control embryos.

The formation of some PMN axons in *lhx3* plus *lhx4* MO-injected embryos led us to hypothesize that cues that promote PMN formation are still present in the absence of Lhx3 and Lhx4. Islet1 is co-expressed in with Lhx3 and Lhx4 in PMNs and SMNs (Figure 3; data not shown). In addition, we showed previously that Islet1 is required for PMN and SMN formation (Hutchinson and Eisen, 2006). These data suggested that if Islet protein remains in *lhx3* plus *lhx4* MO-injected embryos, it could still promote the PMN fate. To test this possibility, we assayed Islet protein expression in *lhx3* plus *lhx4* MO-injected embryos and found that Islet protein was still present (Figure 8A-C). In mouse, Islet-1 is required for ventral spinal cord expression of Lhx3 and Lhx4 (Pfaff et al., 1996). To determine if this was the case in zebrafish we knocked down Islet1 using MOs and found that Lhx3 and Lhx4 expression remained and appeared normal (Figure 8D-F; see also Hutchinson et al., 2006). Together these observations suggest that in zebrafish, expression of Islet1, Lhx3, and Lhx4 are regulated independently. Thus, Islet1 can promote PMN development in the absence of Lhx3 and Lhx4.

Table 2. Lhx3 and Lhx4 are required for normal PMN axons

	Dorsal Projections	**Short Ventral Projections**	**Long Ventral Projections**
Control 28 hpf n=110 segments (11 embryos)	98.2%	0%	100%
***lhx3* MO 28 hpf** n=186 segments (22 embryos)	83.3%	2.7%	97.3%
***lhx4* MO 28 hpf** n=225 segments (28 embryos)	93.3%	4.9%	95.6%
***lhx3* & *lhx4* MOs 28hpf** n=481 segments * (37 embryos)	20.8%	10.5%	54.1%

***** Remainder of segments lacked all axons
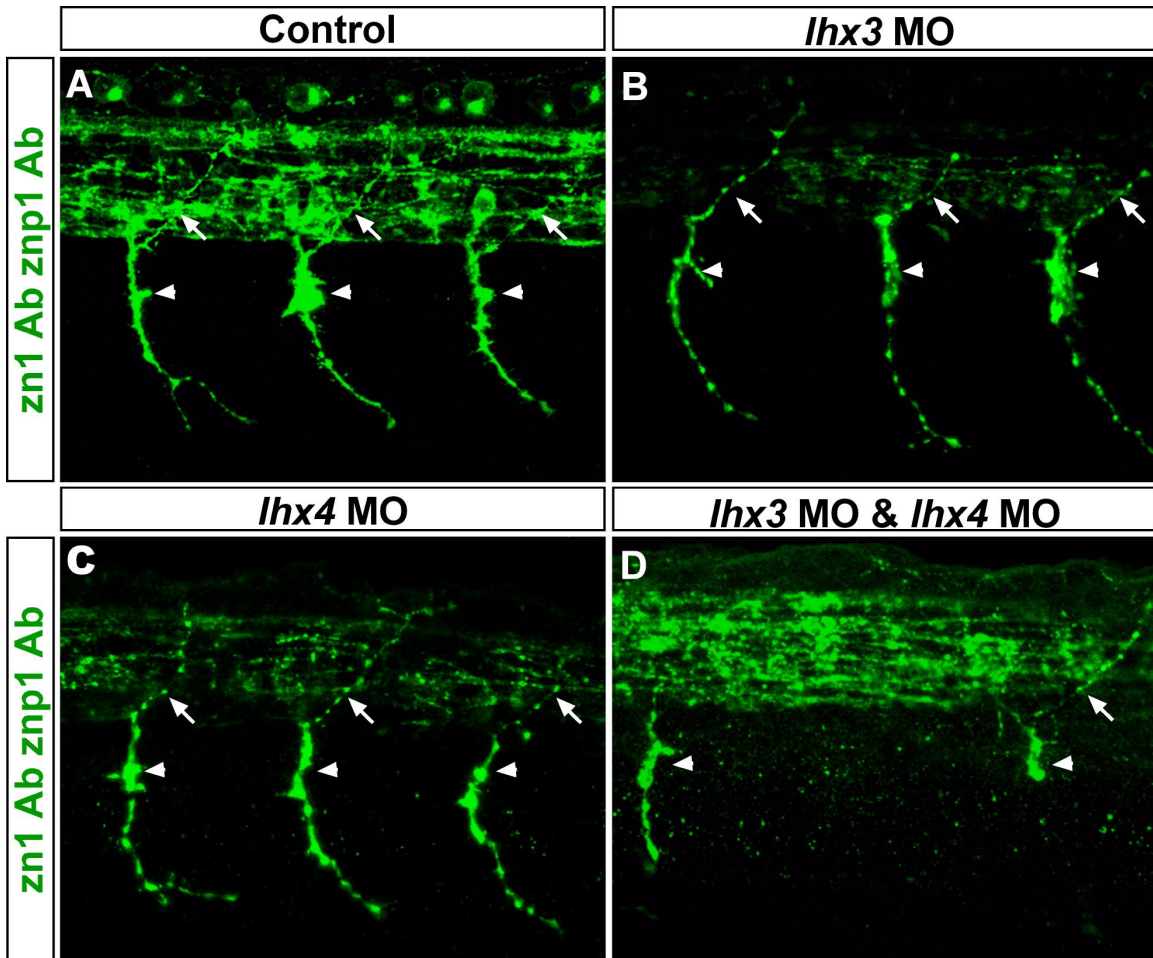
Figure 7. Lhx3 and Lhx4 are required for normal PMN pathfinding
(**A-D**) Z-projections of confocal images of 28 hpf embryos taken at somite levels 8-12 representing one half of the neural tube, co-labeled with zn1 and znp1 antibodies. Control embryos (A) have both dorsally-projecting axons (white arrowheads) and ventrally-projecting axons (white arrows). Both *lhx3* and *lhx4* single MO (B, C) -injected embryos have normal dorsally- and ventrally-projecting axons. Injection of both *lhx3* and *lhx4* MOs (D) results in random loss of either dorsally-projecting axons, ventrally-projecting axons, or both.

Lhx3 and Lhx4 prevent PMNs from expressing interneuron characteristics

Despite maintaining Islet protein expression, only some PMNs in *lhx3* plus *lhx4* MO-injected embryos form normal axons. Murine Islet1 is known to bind Lhx3 to promote MN fate (Thaler et al., 2002). In zebrafish, reduction of Islet1 results in PMNs developing as GABA-positive INs despite the presence of Lhx3 and Lhx4 (Figure 8; see also Hutchinson et al., 2006). These data suggest a combination of Islet, Lhx3 and Lhx4 is required to promote MN fate and inhibit IN fate, leading us to

hypothesize that PMNs in *lhx3* plus *lhx4* MO-injected embryos would express some characteristics of INs. To test this hypothesis, we co-labeled *lhx3* plus *lhx4* MO-injected embryos with antibodies to Islet and to GABA, a neurotransmitter expressed by KA', VeLD, and V2b neurons, but not by MNs. We found that in the absence of Lhx3 and Lhx4, most Islet-positive cells co-expressed GABA (Figure 9A, B), supporting our hypothesis. To our surprise, these cells also continued to express *chat*, which encodes the synthetic enzyme for acetylcholine, the normal MN neurotransmitter (Figure 9G, H). Thus, when Lhx3 and Lhx4 are lost, PMNs develop a hybrid phenotype in which they express biochemical characteristics of both MNs and INs.

To determine whether GABA-positive neurons in *lhx3* plus *lhx4* MO-injected embryos had a hybrid morphology to complement their hybrid biochemistry, we analyzed their axons. To our surprise, many of the GABA-expressing cells projected GABA-positive axons out of the spinal cord into the muscle. These axons co-labeled with zn1/znp1 antibodies, revealing that they were PMN axons (Figure 9F). To
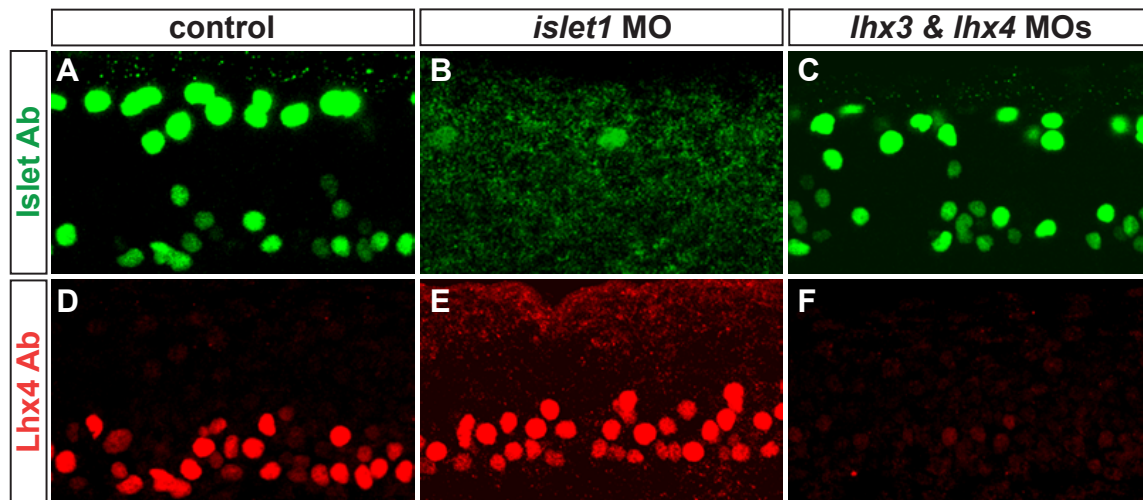
Figure 8. Islet and Lhx3/4 proteins are independently regulated in ventral spinal cord
(**A-C**) Staining with an antibody that recognizes both Islet1 and Islet2 (green). Islet expression is normal in control (A) and *lhx3* plus *lhx4* MO-injected embryos (C) but is absent from *islet1* MO-injected embryos (B). (**D-F**) Staining with an antibody that recognizes both Lhx3 and Lhx4 (red). Lhx4 expression is normal in control (D) and *islet1* MO-injected embryos (E) but is absent from *lhx3* plus *lhx4* MO-injected embryos (F).

examine PMN morphology in more detail, we labeled individual CaPs in *lhx3* plus

lhx4 MO-injected embryos by iontophoresis with rhodamine dextran. We found that

not only did CaPs have a hybrid biochemistry, they also had a hybrid morphology

with both a CaP-like axon that extended normally to ventral muscle and an IN-like

axon that extended caudally within the spinal cord along the same trajectory

normally followed by VeLD axons (Figure 9J). Thus, the phenotype of CaPs in the

absence of Lhx3 and Lhx4 suggests that they develop as hybrids that co-express

properties of PMNs and of VeLD INs. Together, these data show that Lhx3 and Lhx4

are required to prevent PMNs from co-expressing biochemical and morphological

characteristics appropriate for VeLD INs, but not for MNs.

**Lhx3 and Lhx4 are required for formation of specific pMN-derived
interneurons**

Despite having normal p2 domain-derived INs, the increase in GABA positive

cells in *lhx3* plus *lhx4* MO-injected embryos suggests that Lhx3 and Lhx4 may be

**Figure 9 (next page). Lhx3 and Lhx4 prevent PMNs from developing
interneuron characteristics**
(**A-H**) Z-projections of confocal images taken of 28 hpf embryos at somite levels 8-
12, representing one half of the neural tube. (**A-B**) Embryos co-labled with GABA
and Islet1/2 antibodies. Control embryos (A) had an average of 4.3+/-1.1 cells co-
labeled with GABA and Islet1/2 antibodies in the dorsal neural tube at somite levels
8-11 (n=7 embryos), but lacked co-labeled cells in the ventral neural tube. In
contrast, embryos injected with both *lhx3* and *lhx4* MO (B) had an average of 15.1+/-
5.0 cells co-labeled with GABA and Islet1/2 antibodies primarily in the ventral
neural tube (n=8 embryos, p-value=0.0003). GABA antibody labeled axons exiting
the neural tube in double MO-injected embryos (D) in contrast to the axons within
the neural tube in control embryos (C). The axons leaving the neural tube labeled
with GABA in double MO injected embryos are co-labeled with znp1 antibody (F) in
contrast to control embryos (E) where znp1 antibody alone labels axons leaving the
neural tube. (**G,H**) Confocal images of the ventral spinal cord of 24 hpf embryos at
somite levels 8-12 co-labeled with *islet2a* (green), which labels CaPs, a subset of
SMNs and dorsal sensory neurons, and *chat* (red). PMNs and SMNs express *chat* in
control embryos (G). PMNs and SMNs continue to express *chat* in embryos injected
with *lhx3* and *lhx4* MOs (H). (**I-J**) Dye injection of live PMNs at 28 hpf. (I) Control
CaP with a ventrally-projecting axon. (J) PMN in a double MO-injected embryo with
a ventrally-projecting axon leaving the neural tube and a caudally-projecting axon
within the neural tube.

24

Islet Ab GABA Ab

Control — A

lhx3 MO *lhx4* MO — B

Control	*lhx3* MO *lhx4* MO

GABA Ab — C, D

GABA Ab zn1 Ab znp1 Ab — E, F

chat islet2a — G, H

CaP — I, J

25
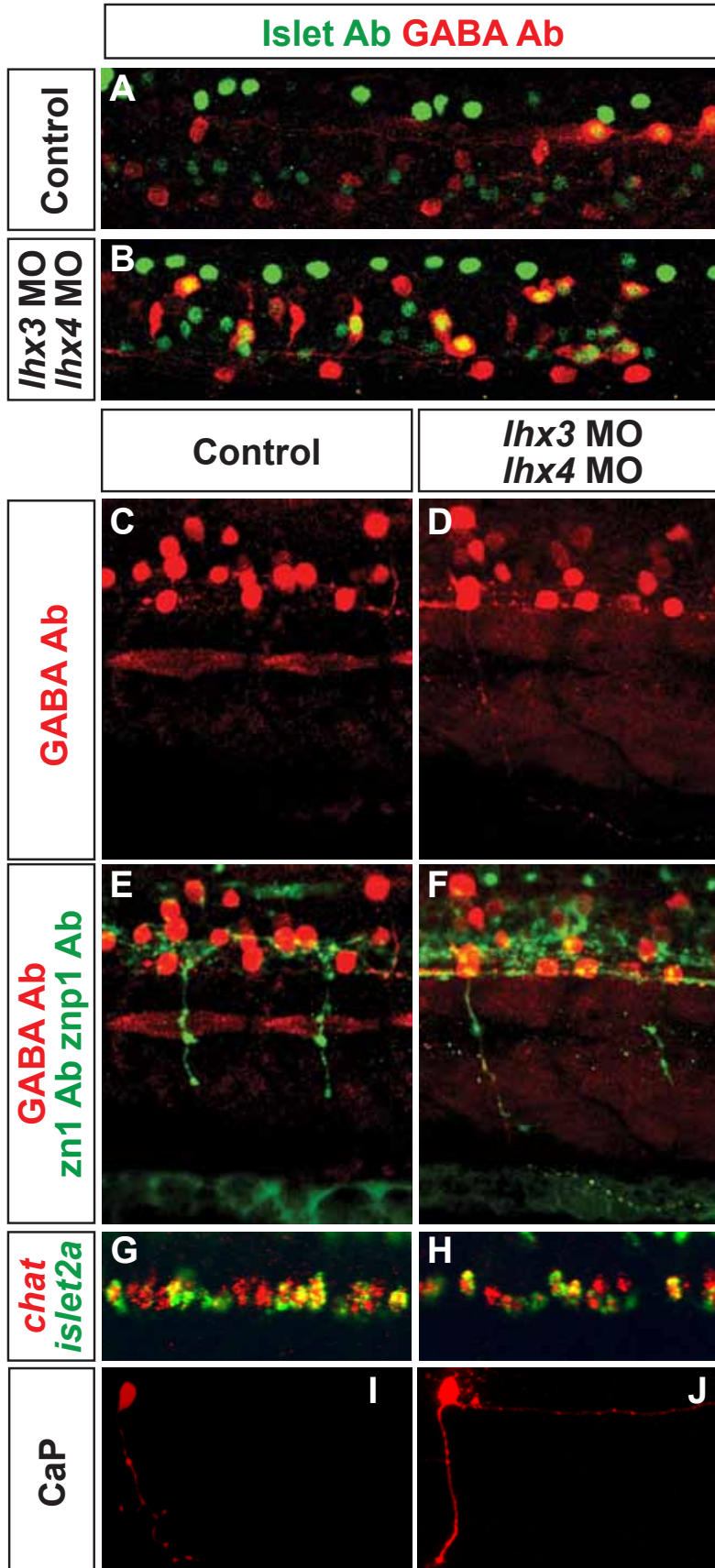
required for the formation of pMN domain derived INs. GABA and the biosynthetic enzymes that produce it - Gad1 and Gad2 - are normally expressed in the p2 domain-derived V2a INs, the p3 domain derived KA" INs, as well as the pMN domain-derived VeLD and KA' INs (Park et al., 2004; Batista et al., 2008). In the absence of Lhx3 and Lhx4 GABA, *gad1*, and *gad2* are all aberrantly expressed in PMNs (Figure 4; Figure 9). We know that p2 domain INs are unaffected (Figure 4A-D), suggesting a defect in either the p3 or pMN domain INs labeled by GABAergic markers. P3 domain-derived KA"s are located in the ventral-most spinal cord (Park et al., 2004), and were unaffected by the absence of Lhx3 and Lhx4 (Table 1), leaving only the pMN domain-derived INs as candidates for regulation by Lhx3 and Lhx4. VeLDs can be readily distinguished from KA' and KA" based on their lateral soma position and lack of both *Tg(vsx1:GFP)^{nns5}* and Islet labeling (Park et al., 2004). We found that the number of VeLDs was unaffected by the absence of Lhx3 and Lhx4 (Table 1; Figure 4E-F).

pMN domain-derived KA' somata are round and medially located, similar to KA", but are located more dorsally (Bernhardt et al., 1992). In contrast to the other IN types labeled by GABA, the number of KA's was significantly decreased in the absence of Lhx3 and Lhx4 (Table 1; Figure 4G-H). These results indicate that Lhx3 and Lhx 4 are required for promoting KA' IN fate in addition to inhibiting IN fate in PMNs.

Lhx3 acts in progenitors to regulate fate of pMN domain derived KA'

The loss of KA' INs in *lhx3* plus *lhx4* MO-injected embryos revealed that Lhx3 and Lhx4 are required for KA' IN fate. However, we do not know whether Lhx3 and Lhx4 are localized to KA' INs. We showed earlier that Lhx4 is exclusively expressed in PMNs at 18 hpf, but Lhx3 is expressed in Islet positive PMNs as well as some other cells (Figure 3). Previous work showed that *lhx3* is in VeLD INs (Appel et al*.,* 1995), however, it is possible that some of the Islet-negative cells expressing Lhx3 are KA' INs. We used two markers to investigate this possibility: *gad1/2* which is first expressed in KA's around 20 hpf (Shin et al., 2007), and *tal2* which is first expressed in the spinal cord just prior to 16 hpf (Pinheiro et al., 2004; Schafer et al.,

2007; Yang et al., 2010). We were unable to detect *lhx3* in any medially-located cells expressing *gad1/2* at 24 hpf, and detected *lhx3* in only a minority of cells expressing *tal2* at 16 hpf (8%, n=109 cells) (Figure 10B). These data indicate *lhx3* is not expressed in KA' INs.

Another way Lhx3 and Lhx4 could regulate KA' formation is by regulating KA' precursors. To explore this idea, we labeled *Tg(olig2:GFP)[vu12]* embryos with antibodies to Lhx3 and GFP at 12 hpf, a time during which KA' precursors are undergoing their final round of DNA synthesis (Shin et al., 2007). All Lhx3-positive cells at 12 hpf in *Tg(olig2:GFP)[vu12]* embryos were also GFP-positive, indicating Lhx3 is expressed in PMN/KA' progenitors (Figure 10A). These observations led us to consider that Lhx3 might exert an effect on KA' development via expression in KA' precursors or newly-born KA' INs. Together these results suggest that Lhx3 and Lhx4 expression in KA' and PMN precursors regulates formation of pMN domain-derived KA' INs and PMNs.

DISCUSSION

Our data are the first to describe the sequence, expression pattern and function of the zebrafish *lhx4* homologue. We find that in combination with its paralog *lhx3, lhx4* is required for formation of pMN derived MNs, similar to its function in mouse. Our analysis of individual, early born zebrafish neurons revealed a new role for Lhx3 and Lhx4 - specifically in the pMN domain progenitors promoting PMN fate while inhibiting KA' IN fate. These data are the first to analyze the role of Lhx3 and Lhx4 in vertebrate neurogenesis at the single cell level and provide a new understanding of how MN and IN fates are differentiated.

Analysis of Lhx3 and Lhx4 in other systems has shown Lhx3 and Lhx4 are co-expressed within the ventral neural tube (Sharma et al., 1998). Our studies analyzing Lhx4 expression patterns at the single cell level are the first to show that Lhx4 expression in the ventral neural tube is distinct from Lhx3 expression at both the mRNA and the protein level. As has been described previously for *lhx3* mRNA (Appel et al., 1995; Kimura et al., 2006; Batista et al., 2008), Lhx3 protein is expressed in pMN domain-derived MNs and INs as well as p2 domain-derived INs.

	12 hpf	16 hpf
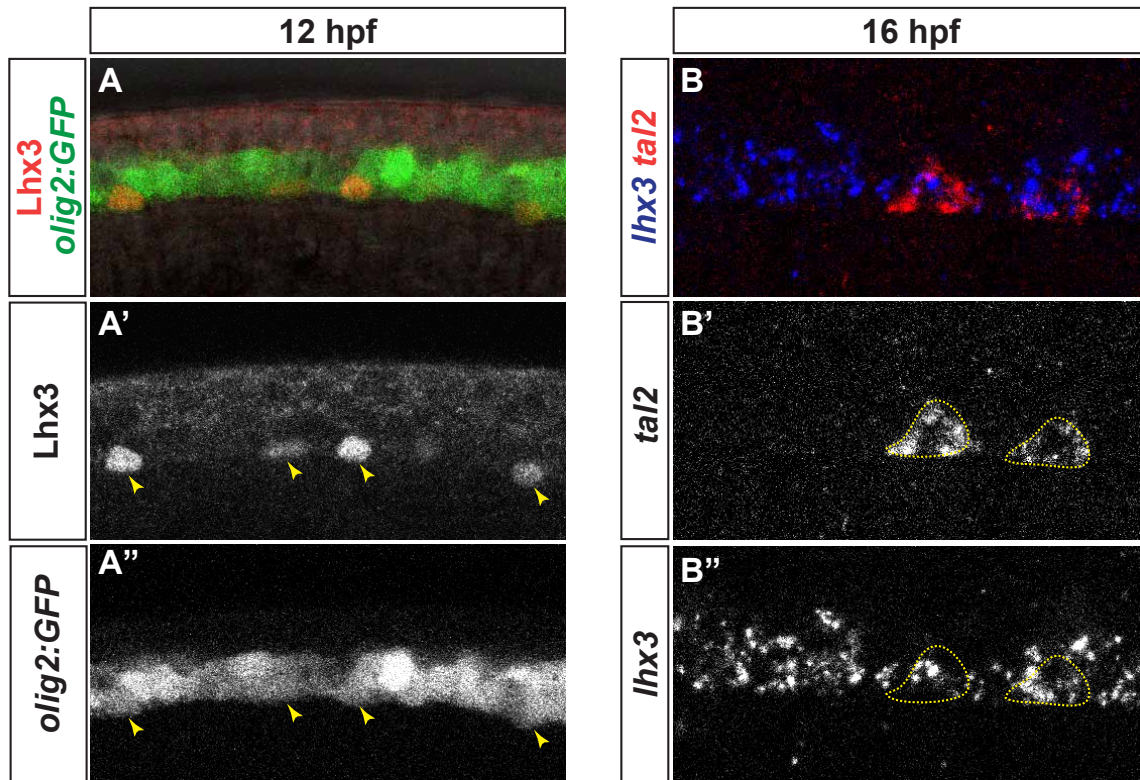
In contrast, at 18 hpf Lhx4 is expressed only in PMNs. Over the next few hours, both

Figure 10. Lhx3 may be expressed in immature KA' interneurons and KA' precursors
(**A**) At 12 hpf, all Lhx3⁺ cells (red, and A') express GFP (green and A") in *Tg(olig2:GFP)ᵛᵘ¹²* embryos. Lhx3⁺ cells are labeled with yellow arrowheads. (**B**) At 16 hpf, 8% of *tal2*⁺ cells (red and B'; n = 109 *tal2+* cells in 15 embryos) co-express *lhx3* (blue and B"). *tal2*⁺ cells are outlined in yellow.

pMN domain-derived and p2 domain-derived INs begin to express Lhx4, and by 28 hpf expression of Lhx4 has expanded so that both Lhx3 and Lhx4 proteins are expressed by the majority of MNs and INs. Our interpretation of the few cells that express only Lhx3 early in neuronal development is that they are newly generated and have not yet turned on Lhx4. Time-lapse imaging of doubly transgenic embryos in which the *lhx3* and *lhx4* promoters drive distinguishable fluorescent reporters will be required to test this hypothesis.

Previous studies showed that misexpression of Lhx3 in the chick spinal cord is sufficient to promote formation of V2 INs (Tanabe et al., 1998; Thaler et al., 2002). We show that in zebrafish, Lhx3 and Lhx4 are unnecessary for formation of the

earliest-generated V2 INs. These early V2 INs are generated as V2a and V2b sibling pairs that result from the final division of a *vsx1*-expressing progenitor (Kimura et al., 2008). V2as are excitatory, glutamatergic INs that normally maintain *lhx3* expression whereas V2bs are inhibitory, GABAergic INs that normally extinguish *lhx3* expression (Kimura et al., 2006; Batista et al., 2008). Despite expression of *lhx3* in V2as, Lhx3 and Lhx4 were unnecessary for V2a nor V2b formation. Other systems implicating Lhx3 in V2 neuron formation have focused on the sufficiency of Lhx3 and have not shown a necessity for Lhx3 in V2 IN formation (Thaler et al., 2002). Therefore it is possible that Lhx3 is sufficient, but not necessary for V2 IN formation in both systems. Another possibility we did not test is whether Lhx3 and Lhx4 are required to specify later-developing V2 neurons. Kimura and colleagues (Kimura et al., 2006) have shown that the number of V2 neurons increases over time, and that there are some clear morphological differences between V2 neurons born at different times. It is entirely possible that different temporal populations of V2 INs could be differentially affected by changes in Lhx3 and/or Lhx4 expression.

We have revealed a new, unpredicted role for Lhx3 and Lhx4 in pMN-derived KA' IN formation. KA INs extend cilia into the spinal cord central canal and have recently been shown to influence spontaneous forward swimming behavior by providing positive drive to the spinal cord central pattern generator (Wyart et al., 2009). It is still unclear why there are fewer KA's following Lhx3 and Lhx4 knockdown, because there does not appear to be an increase in another cell type. Intriguingly, previous data has shown that PMNs and KA's are siblings (Shin et al., 2007). These data, along with our data showing Lhx3 is expressed in pMN domain progenitors, leads to the hypothesis that in the absence of Lhx3 and Lhx4, these pMN domain progenitors undergo fewer divisions, leading to fewer KA' INs. Future studies further detailing the sibling relationship between PMNs and KA' INs, along with the timing of their divisions, will be important for further understanding of how Lhx3 and Lhx4 might regulate this relationship.

Lhx3 and Lhx4 are required for both pMN-derived PMN and SMN formation, however, the resulting cellular phenotypes differ. In mouse, Lhx3 and Lhx4 have differential effects of different classes of MNs, acting together to specify

development of MNs whose axons exit ventrally from the neural tube (Sharma et al., 1998). In the absence of Lhx3 and Lhx4, these cells are converted into MNs whose axons exit dorsally from the neural tube; thus Lhx3 and Lhx4 function as a switch to specify whether motor axons exit ventrally or dorsally (Sharma et al., 1998). We have not observed zebrafish MNs switching their axon trajectories in the absence of Lhx3 and Lhx4. Instead, in the absence of Lhx3 and Lhx4, SMNs fail to develop, whereas PMNs develop but express inappropriate characteristics. PMNs develop as hybrids that co-express biochemical and morphological features of both MNs and INs. In addition, they extend VeLD-like axons within the spinal cord and co-express GABA, the normal VeLD neurotransmitter. Clonal analysis has revealed that PMNs and VeLDs can be siblings (Park et al., 2004), suggesting that Lhx3 ahd Lhx4 may act in a progenitor to specify axon trajectory and neurotransmitter phenotype.

Other studies in zebrafish have also described hybrid PMN phenotypes, identifying other factors that may be regulated by Lhx3 and Lhx4. For example, knockdown of the Met receptor, which is expressed in CaPs starting at about 22 hpf, resulted in a similar hybrid phenotype (Tallafuss and Eisen, 2008), suggesting that Lhx3 and Lhx4 could regulate expression of *met*. However, we found that *met* expression in PMNs was normal in *lhx3* plus *lhx4* MO-injected embryos (data not shown), revealing that these Lhx transcription factors do not regulate expression of *met*. The identification of downstream targets of Lhx3 and Lhx4 signaling will be an interesting area of focus for future experiments.

Although studies in mouse have shown that in the absence of specific transcription factors MNs express IN characteristics or vice versa, these cells typically resolve and develop either as MNs or as INs. At least in some cases, the mechanism underlying this resolution is formation of distinct LIM domain transcription factor complexes in different types of neurons (Thaler et al., 2002; Lee et al., 2008; Gadd et al., 2011; Lee et al., 2012). For example, in MNs two LIM domain transcription factors, Islet1 and Lhx3, form a hexameric complex with a LIM interacting protein NL1 (aka Ldb1) that promotes MN fate. In contrast, in V2 INs Lhx3 forms a tetrameric complex with NL1 that promotes IN fate. In addition to NL1, a LIM only protein - LMO4 - is expressed in MNs and actively suppresses

formation of Lhx3-NL1 tetramers.   Zebrafish has two *lmo4* gene paralogs, *lmo4a* and *lmo4b*, however, neither of them is expressed in the pMN domain or in MNs (Thisse and Thisse, 2005). Zebrafish also has two *ldb1* paralogues, *ldb1a* and *ldb2b*, both of which are expressed broadly within the embryo at the relevant developmental stages (Toyama et al., 1998; Thisse and Thisse, 2004). The differences in expression of these genes may underlie the ability of zebrafish PMNs to co-express MN and IN properties, rather than resolving into either MNs or INs, as generally appears to be the case in mouse.

BRIDGE

Zebrafish Lhx3 and Lhx4 proteins play a complicated role promoting various aspects of MN development. They are clearly involved in preventing MNs from acquiring IN characteristics, but seem to be acting specifically in CaP MNs. Therefore, there must be other genes that are responsible for regulating MN development in the other identified MNs. The *mnx* family of homeobox transcription factor genes has been implicated in MN development, and here I show that the three zebrafish genes *mnx1*, *mnx2a*, and *mnx2b* work together to repress the acquisition of IN characteristics in a different identified MN, MiP.

CHAPTER III

ZEBRAFISH MNX PROTEINS DIFFERENTIALLY AFFECT MOTONEURON SUBTYPE
IDENTITY AND SUPPRESS ACQUISITION OF SPECIFIC INTERNEURON
CHARACTERISTICS

The work described in this chapter was co-authored by S. D. Seredick and myself, and we share first authorship. We worked with J. S. Eisen on experiment design, and were responsible for the majority of data collection, data analysis, and writing. S. A. Hutchinson generated antibodies. J. S. Eisen also contributed to writing, and the work was carried out in her laboratory.

INTRODUCTION

The ability of an animal to carry out behavior depends on precise innervation of each muscle by the appropriate motoneuron subtype. Motoneuron (MN) subtype identity is specified by the combination of transcription factors expressed by a cell during its differentiation, and recognized by characteristic features such as soma position, axon trajectory, and muscle innervation pattern. Specification of MN subtype identity has been well-studied (Shirasaki and Pfaff, 2002; Lewis and Eisen, 2003), however, we still have an incomplete picture of the molecular mechanisms regulating particular aspects of MN differentiation. Here we take advantage of the ability to recognize individual primary motoneurons (PMNs) in the spinal cord of embryonic zebrafish to explore the roles of Mnx family transcription factors in MN subtype specification.

Spinal cord neurons develop from distinct progenitor domains that are recognized by expression of specific transcription factors (Alaynick et al., 2011). Zebrafish PMNs are derived from the progenitor of motoneuron (pMN) domain (Park et al., 2004) and comprise three subtypes - CaP, MiP, and RoP - each of which can be distinguished based on soma position, axon trajectory, and muscle innervation (Eisen et al., 1986). A fourth PMN, VaP, is variably present, initially equivalent to CaP, and later dies (Eisen et al., 1990; Eisen and Melancon, 2001). Here we focus primarily on CaP, which innervates ventral mytome and MiP, which

innervates dorsal myotome. Initially both CaP and MiP express Islet1, a transcription factor required for PMN development. In the absence of Islet1, PMNs develop axon trajectories and express the neurotransmitter characteristic of VeLD interneurons (Hutchinson and Eisen, 2006), which are also derived from the pMN domain (Park et al., 2004). Later in development, CaP downregulates Islet1 and expresses a related protein, Islet2a. MiP also downregulates Islet1, but then reexpresses it about an hour later (Appel et al., 1995; Hutchinson et al., 2007). This second phase of Islet1 expression is regulated by Nkx6 transcription factors. In the absence of Nkx6 proteins, MiP can extend a ventral axon to the periphery that stops normally at an identified set of muscle fibers, the muscle pioneers, that separate dorsal and ventral muscle (Melancon et al., 1997). However, MiP fails to extend its normal axon collateral to dorsal muscle, and instead develops an interneuron (IN)-like axon within the spinal cord (Hutchinson et al., 2007). This IN-like axon often resembles axons of V2a INs (Kimura et al., 2006; Kimura et al., 2008). V2a INs are derived from the p2 domain situated just dorsal to the pMN domain (Alaynick et al., 2011). The p2 domain, which generates excitatory V2a and inhibitory V2b neurons, has been shown to be closely related to the pMN domain based on shared expression of a number of transcription factors (Shirasaki and Pfaff, 2002).

The vertebrate Mnx family comprises homeodomain transcription factors originally isolated in human and subsequently isolated in chick and mouse (Ferrier et al., 2001). Mnx2 [previously called MNR2 and Hlxb9l (Ferrier et al., 2001)] was isolated from a single chick cell induced to become a MN (Tanabe et al., 1998). Mnx2 is expressed in MN progenitors and in postmitotic MNs. Ectopic expression of Mnx2 is sufficient to induce MN differentiation in Islet1-positive spinal cord neurons; whether Mnx2 is necessary for MN differentiation has not been tested. Mnx1 [previously called Hb9 and Hlxb9 (Ferrier et al., 2001)] was isolated in mouse and shown to be necessary for normal differentiation of medial motor column MNs (Arber et al., 1999; Thaler et al., 1999). Mnx1 is expressed in postmitotic MNs. In its absence, medial motor column MNs still project axons to the periphery, but the axon projections are abnormal and the cells inappropriately express markers of V2 INs (Arber et al., 1999; Thaler et al., 1999).

We provide evidence for a novel role of Mnx proteins in zebrafish MN subtype specification. Zebrafish has three Mnx proteins - Mnx1 and two co-orthologs of Mnx2, Mnx2a and Mnx2b (Wendik et al., 2004) - all of which are expressed primarily in postmitotic neurons. We show that each Mnx family member is expressed in a distinct pattern in each PMN subtype, and that this pattern is dynamic during PMN differentiation. In contrast to early developmental stages in chick and mouse when Mnx expression within the spinal cord is exclusive to MNs (Tanabe et al., 1998; Arber et al., 1999; Thaler et al., 1999), two zebrafish Mnx family members are expressed in VeLD INs. We used morpholino antisense oligonucleotides [MOs (Eisen and Smith, 2008)] to knock down Mnx function and found, to our surprise, that CaPs and VeLDs developed normally. In contrast, Mnx proteins are required for normal MiP development. In their absence, the second phase of Islet1 expression is initiated at the appropriate time, but is lost a few hours later. MiPs in *mnx* MO-injected embryos express markers of V2a INs, similar to what has been reported in mouse mutants (Arber et al., 1999; Thaler et al., 1999). However, in contrast to mouse mutants, these MiPs also developed V2a-like axons in addition to peripheral axons projecting to muscle. Surprisingly, the peripheral axons of these MiPs did not extend to their normal dorsal muscle target, but instead projected ventrally along side normal CaP axons. The studies reported here extend our previous finding about the role of Islet1 in promoting formation of the normal MiP dorsal axon and preventing formation of a V2 IN-like axon (Hutchinson et al., 2007). They also reveal an unexpected role for Mnx proteins in preventing MiPs from becoming more like CaPs.

METHODS
Zebrafish

Wild-type (AB), *Tg(olig2:GFP)vu12* (Shin et al., 2003), *Tg(nrp1a:GFP)js12* (Sato-Maeda et al., 2008), and *Tg(vsx1:GFP)nns5* (Kimura et al., 2008) zebrafish were maintained in a laboratory breeding colony according to established protocols (Westerfield, 2007). Embryos collected from natural crosses were allowed to

develop at 28.5°C, and staged by hours postfertilization (hpf) according to morphological criteria (Kimmel et al., 1995).

Generation of transgenic fish lines

A 3-kb fragment of the *mnx1* promoter (Flanagan-Steet et al., 2005) was subcloned into *p5E-MCS* (Kwan et al., 2007). Multi-site Gateway® technology (Life Technologies; Eugene, OR, USA) was used to assemble an *mnx1:GAL4VP16:pA* construct flanked by *Tol2* terminal inverted repeats. *Tg(mnx1:GAL4VP16)* lines were generated by co-injecting plasmid DNA and *Tol2 transposase* RNA (Kawakami et al., 2004) into the yolk of one-cell stage embryos. Multiple founders were recovered and characterized and *Tg(mnx1:GAL4VP16)*[b1222] was chosen for this study because transgene expression faithfully mirrored endogenous *mnx1* mRNA expression (data not shown).

Morpholino injections

Approximately 2.5 nL of 100 μM translation-blocking morpholinos (Gene Tools, LLC; Philomath, OR, USA) against *mnx1* (5'-ACCTCACAAACAGATTAACGCCTCG -3'), *mnx2a* (5'-ACCTCACAAACAGATTAACGCCTCG-3') and *mnx2b* (5'-GACTTTTCCAT TGCAACACTTTTGT-3') were injected into 1- to 2-cell stage embryos; this was sufficient to suppress translation as assayed by whole-mount immunohistochemistry (Figure 11) without elevated cell death as assayed by acridine orange staining (data not shown). These MOs have been previously validated (Wendik et al., 2004).

Other previously validated MOs used in this study include: random control oligonucleotide (5'-N$_{25}$-3'), 2.5 nL of 100 μM *islet1* E2 (5'-TTAATCTGCGTTACCTGAT GTAGTC-3') plus 100 μM *islet1* E3 (5'-GAATGCAATGCCTACCTGCCATTTG-3') (Hutchinson and Eisen, 2006) to knock down *islet1*; 2.5 nL of 400 μM *nkx6.1* (5'-CGC AAGAAGAAGGACAGTGACCCG-3') (Cheesman et al., 2004) plus 400 μM *nkx6.2* (5'-CG CGCAAAACTCACCCGCACAGGGA-3') (Hutchinson et al., 2007) to knock down *nkx6.1* and *nkx6.2*; and 2.5 nL of 280 μM *lhx3* (5'-CATTCTGGCGATAAA-3') plus 280 μM *lhx4* (5'-GCAGCACAGCCGCACTTTGCATCAT-3') to knock down *lhx3* plus *lhx4*
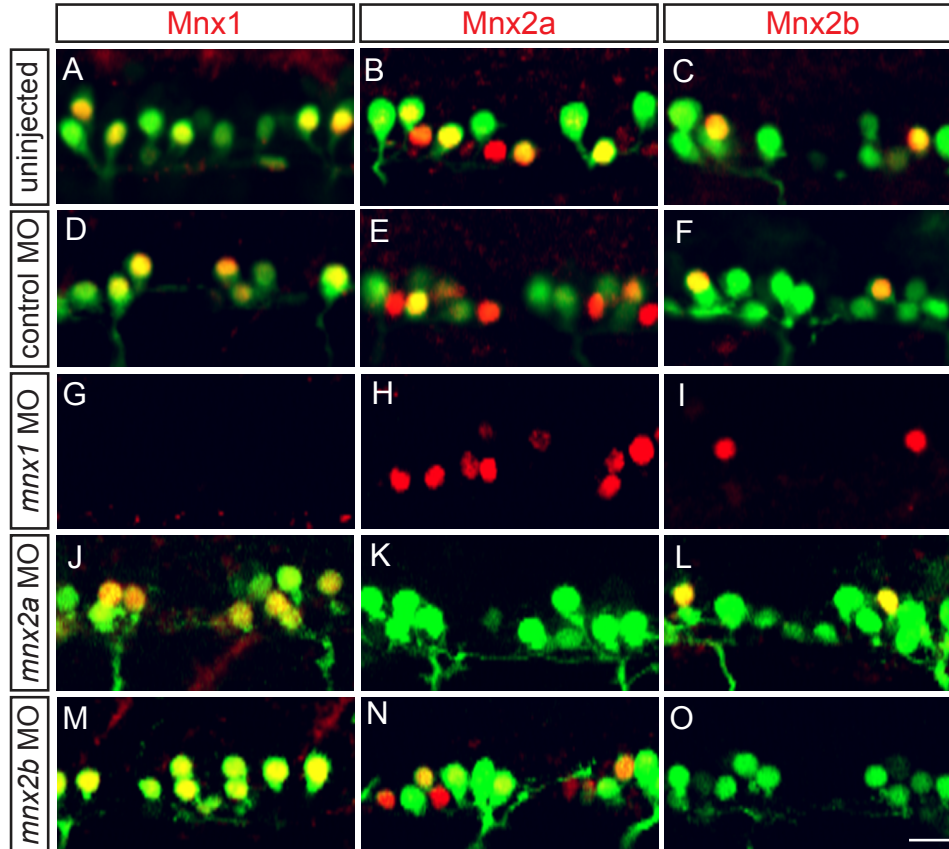
Figure 11. Morpholinos targeting *mnx* family genes are specific and effective in knocking down protein

(**A-O**) Single confocal slices of 24 hpf embryos. Uninjected, control MO-injected, and *mnx* MO-injected embryos labeled for antibodies against Mnx1 (A, D, G, J, M), Mnx2a (B, E, H, K, N), and Mnx2b (C, F, I, L, O). Embryos injected with *mnx1* MO lack Mnx1 antibody labeling (G), but maintain Mnx2a (H) and Mnx2b (I) antibody labeling. Embryos injected with *mnx2a* MO lack Mnx2a antibody labeling (K), but maintain Mnx1 (J) and Mnx2b (L) antibody labeling. Embryos injected with *mnx2b* MO lack Mnx2b antibody labeling (O), but maintain Mnx1 (M) and Mnx2a (N) antibody labeling. Scale bar: 20μm in A-O.

(Hutchinson et al., *in preparation*). Morpholino effectiveness was verified by whole mount immunohistochemistry.

Fluorescent RNA *in situ* hybridization

RNA *in situ* hybridization was performed according to standard protocols (Thisse and Thisse, 2008) with the following modifications: for 2-color fluorescent *in situ* hybridization, anti-sense probes were labeled with digoxigenin-UTP (Roche Applied Sciences, Indianapolis, USA) and dinitrophenol-UTP (Perkin-Elmer,

Waltham, MA, USA). Following overnight hybridization, unbound probe was removed with three 30 minute washes at 68°C in 50% formamide, 5x SSC and 0.1% SDS, followed by stringent washes in 50% formamide, 2x SSC and 0.1% Tween-20. Labeled probes were detected with HRP-conjugated anti-DIG (1:2000; Jackson ImmunoResearch, West Grove, PA, USA) or HRP-conjugated anti-DNP (1:2000; Perkin-Elmer) and stained with fluorescein, Cy3-, or Cy5-tyramide (1:100; Perkin-Elmer) for 1-10 minutes.

Probes used include *mnx1, mnx2a*, and *mnx2b* (Wendik et al., 2004); *chat* (Tallafuss and Eisen, 2008); *islet1*, *islet2a* and *lhx3* (Appel et al., 1995); *lhx4* (Hutchinson et al., *in preparation*); *gad1b* and *gad2* (collectively referred to as *gad*), *slc17a6a, slc17a6b*, and *slc17a7* (collectively referred to as *vglut*) and *slc6a9* and *slc6a5* (collectively referred to as *glyt*) (Higashijima et al., 2004a); *vsx2* (Kimura et al., 2006); and *gata3* (Batista et al., 2008).

Antibody generation

To prepare Mnx1 and Mnx2b antisera, cDNAs corresponding to amino acids 245-311 of Mnx1 or amino acids 224-301 of Mnx2b were His-tagged, over-expressed in *E. coli* and purified by nickel column chromatography under native conditions. These regions are C-terminal to the homeodomain, and are the most diverged regions of the gene family. Purified recombinant proteins were used to immunize rabbits, and the resulting antisera screened by whole mount immunohistochemistry. Attempts to generate antisera against Mnx2a were not successful.

Immunohistochemistry

Embryos were fixed for 2 hours in 4% paraformaldehyde and 1x Fix Buffer (Westerfield, 2007) at 4°C, and then treated with 0.5% Triton X-100 in 1x PBS for 15 minutes at room temperature. Embryos were blocked in 5% normal goat serum, 2.5% DMSO and 0.1% Tween-20 in 1x PBS before overnight incubation in diluted primary antibody at 4°C. Unbound primary antibodies were removed by washing for 2 hours in 1x PBS plus 0.1% Tween-20, followed by overnight incubation in

diluted secondary antibody at 4°C. Anti-Mnx1, anti-Mnx2a and anti-Mnx2b were detected with HRP-conjugated goat anti-rabbit and stained with fluorescein-, Cy3-, or Cy5-tyramide (1:100; Perkin-Elmer) for 1 minute; all other primary antibodies were detected with dye-labeled secondary antibodies.

Antibodies used include rabbit polyclonal anti-Mnx1 (1:1000) and anti-Mnx2b (1:1000), anti-Mnx2a (1:1000; AnaSpec, Fremont, CA, USA), anti-Lhx3 and anti-Lhx4 (Hutchinson, 2012), mouse monoclonal anti-Elavl3/4 (1:10,000; A21271, Life Technologies), anti-Gad (1:500; ab11070, Abcam, Cambridge, MA, USA) anti-GFP (JL-8; Clontech, Mountain View, CA, USA; or A-11120; Life Technologies), anti-Histone H3 (phospho S10) (1:1000; ab14955, Abcam), anti-Islet (39.4D5; DSHB, Iowa City, IA, USA), and anti-Nkx6.1 (F55A10; DSHB).

Subtype-specific cell labeling

To correlate cell morphology with gene expression, we injected *UAS:EGFP* plasmid with *Tol2 transposase* RNA and selected embryos with GFP-expressing cells for immunohistochemistry. Since our *mnx1* MO also suppressed expression from our *Tg(mnx1:GAL4VP16)* transgene, individual neurons in *mnx* MO-injected fish were dye-labeled with 5% tetramethylrhodamine-dextran (D-3308; Life Technologies) in 0.2 M KCl (Hale et al., 2011).

Image acquisition

All images were acquired on a Zeiss Pascal confocal microscope using a 40x water immersion objective. The brightness and contrast of images was adjusted using Photoshop CS5 (Version 12.0, Adobe Systems, Inc.; San Jose, CA, USA).

Quantification

All observations of PMNs were made in the mid-trunk region of the spinal cord adjacent to somites 8-12. We examined at least 30 segments from 10 embryos for each condition.

RESULTS

mnx family genes are dynamically expressed in primary motoneurons and VeLD interneurons

We characterized expression of *mnx1, mnx2a*, and *mnx2b* within the zebrafish spinal cord using RNA *in situ* hybridization. To determine in which PMNs each *mnx* gene is expressed, we simultaneously labeled for *islet1* mRNA, which is expressed in all PMNs before 14 hpf (Appel et al., 1995) and each of the *mnx* family members. At slightly later stages we included either *islet1*, which is expressed in RoP and MiP, or *islet2a*, which is expressed in CaP and VaP (Appel et al., 1995). After 20 hpf, *islet1* and *islet2a* are also expressed by smaller, more ventrally located secondary motoneurons (Korzh et al., 1993; Inoue et al., 1994; Appel et al., 1995) which we excluded from our analyses.

mnx1 is expressed in all four PMNs from 14-24 hpf (Figure 12A-D). *mnx2a* is initially expressed in only CaP and VaP from 14-18 hpf (Figure 12E, F). By 20 hpf, its expression has expanded to all four PMNs, a pattern that persists through 24 hpf (Figure 12G, H). *mnx2b* is initially expressed in all four PMNs from 14-18 hpf (Figure 12I, J). By 20 hpf, *mnx2b* expression is reduced to a single *islet1*+ PMN (Figure 12K, L). To learn the identity of the PMN that expresses *mnx2b* after 20 hpf, we injected *UAS:GFP* plasmid into *Tg(mnx1:GAL4)* embryos and processed them at 28 hpf for GFP immunohistochemistry. Based on its expression of *islet1* and dorsal axon, the Mnx2b+ PMN is MiP (Figure 12M).

In addition to expression in PMNs, *mnx1* and *mnx2b* are also expressed in a slightly more dorsal cell first visible at 14 hpf (Figure 12A, I). *mnx1* expression persists in this cell through 24 hpf, but *mnx2b* expression is extinguished around 20 hpf. The position and early appearance of these *mnx1*+ *mnx2b*+cells suggested that they were VeLD INs (Bernhardt et al., 1990; Eisen and Pike, 1991; Eisen, 1991). To test this, we labeled VeLDs by injecting the *UAS:GFP* plasmid into *Tg(mnx1:GAL4)* embryos, which reveals both the soma and the axon trajectory, and verified that they expressed Mnx1 by immunohistochemistry (Figure 12N). We also showed that *mnx1* is coexpressed with the VeLD marker *gad* (Bernhardt et al., 1992; Higashijima et al., 2004a; Hutchinson and Eisen, 2006; Batista et al., 2008), but not with *vglut*, a

39
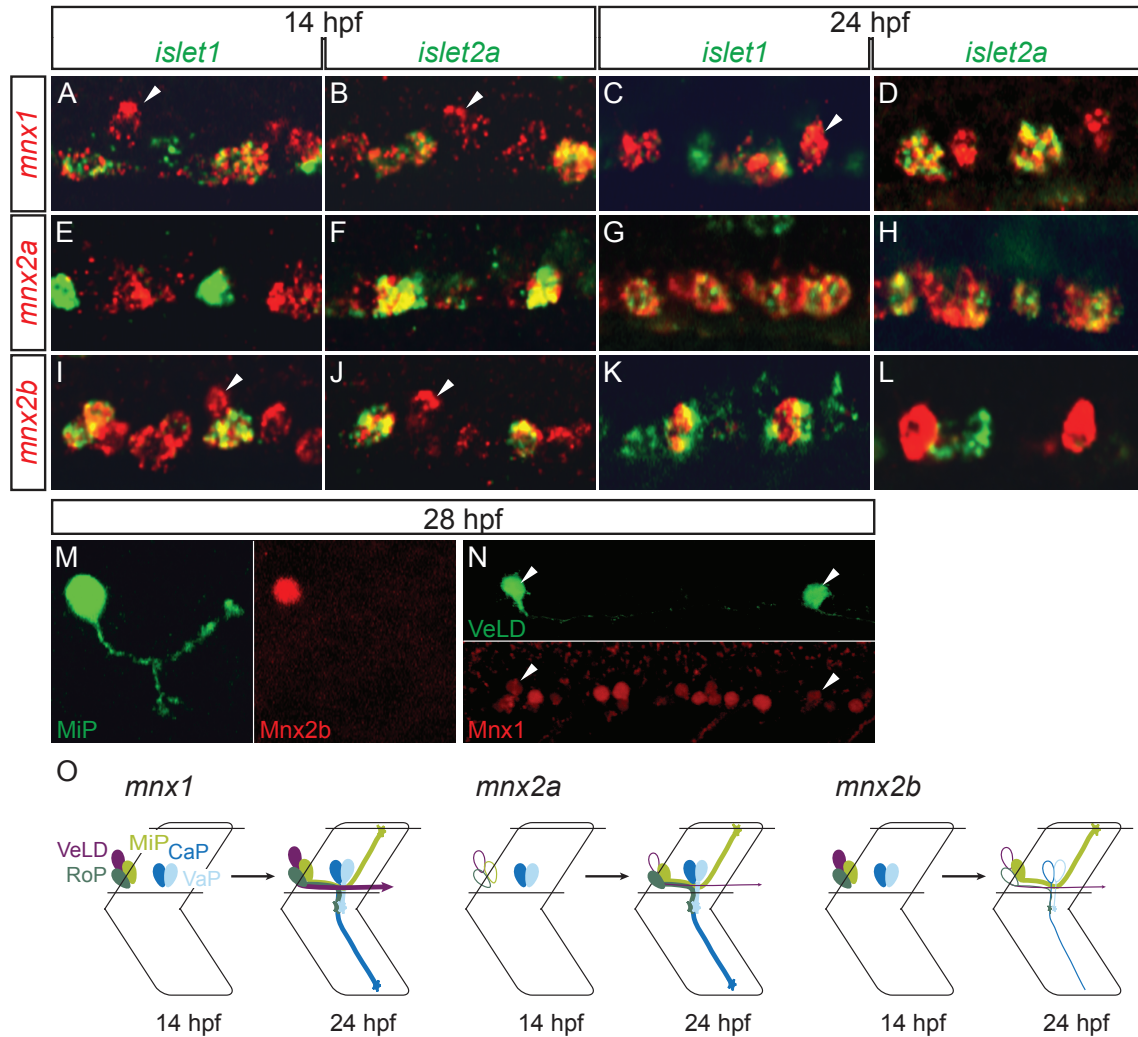
Figure 12. Zebrafish *mnx* family genes are dynamically expressed in PMNs and VeLD interneurons

(**A-L**) Single confocal slices of embryos labeled with probes for *mnx*s and *islet*s. At 14 hpf, *mnx1* is coexpressed with both *islet1* (A) and *islet2a* (B), *mnx2a* is only coexpressed with *islet2a* (F), and *mnx2b* is coexpressed with both *islet1* (I) and *islet2a* (J). The VeLD interneuron can be seen expressing both *mnx1* (arrowheads in A and B) and *mnx2b* (arrowheads in I and J). At 24 hpf, *mnx1* maintains coexpression with both *islet1* (C) and *islet2a* (D), *mnx2a* is coexpressed with both *islet1* (G) and *islet2a* (H), and *mnx2b* is coexpressed with only *islet1* (K). VeLD still expresses *mnx1* (arrowhead in C). (M-N) Z-projections of confocal images of embryos. Mnx2b protein colocalizes with the MiP soma (M), and Mnx1 protein colocalizes with the VeLD soma (N). (**O**) Schematic of dynamic *mnx* expression. *mnx1* is expressed in all PMNs and VeLD between 14 and 24 hpf; *mnx2a* is expressed in CaP/VaP at 14 hpf and all PMNs by 24 hpf; *mnx2b* is expressed in all PMNs and VeLD at 14 hpf and only in MiP by 24 hpf.

40

marker of excitatory V2a INs (Kimura et al., 2006) (Figure 13).

To explore the possibility that *mnx* genes are expressed by other INs with descending axons we looked for coexpression of *mnx1* and *vsx2* or *gata3*, markers of V2a and V2b fate, respectively (Batista et al., 2008; Kimura et al., 2008). Expression of *mnx1* and these markers was always mutually exclusive (Figure 13; data not shown), ruling out expression of *mnx* family genes in V2a and V2b INs. Based on its descending axon, early appearance and expression pattern, the IN positive for expression of both is *mnx1* and *mnx2b* is VeLD.
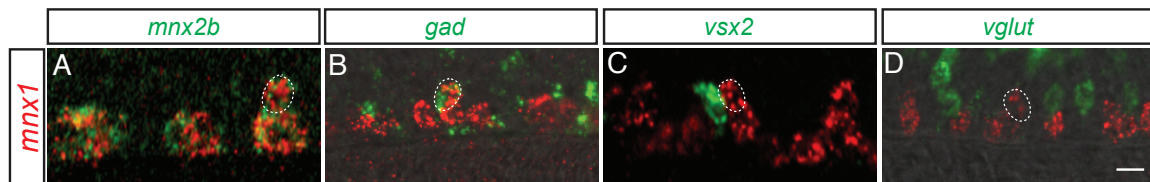
Figure 13. *mnx1* and *mnx2b* are both expressed in VeLD interneurons
(**A-D**) Single confocal slices of embryos. At 16 hpf, VeLD interneurons (outlined) coexpress *mnx1* and *mnx2b* (A) and at 24 hpf they express *gad* (B), but not *vsx2* (C) or *vglut* (D). Scale bar: 10μm in A-D.

In mouse and chick, members of the *mnx* gene family are expressed in MN progenitors prior to exit from the cell cycle. In zebrafish, PMNs and VeLDs adjacent to somites 5-15 emerge from *olig2:GFP*[+] progenitors in the pMN domain (Park et al., 2004), exit the cell cycle between 9-16 hpf (Myers et al., 1986), and then down-regulate *olig2* (Park et al., 2002), although GFP persists for a short time. To determine if zebrafish Mnx proteins are expressed in PMN progenitors, we examined expression in *Tg(olig2:GFP)*[vu12] embryos (Figure 14). We found Mnx1^{+} cells that were either GFP[-] or expressed low levels of GFP; these cells often coexpressed Elavl3, a marker of postmitotic neurons, but did not coexpress phosphohistone H3 (PH3), a marker of mitotic cells. Similarly, Mnx2a was often coexpressed with Elavl3 and never coexpressed with PH3, even though Mnx2a was expressed in some cells with high levels of GFP (Figure 14). Although *mnx2b* RNA was present as early as 14 hpf, we could not detect Mnx2b protein until at least 20 hpf, and then it was present only in MiPs (Figure 12M, O). Together these data are

most consistent with the idea that all three Mnx proteins are first expressed in postmitotic neurons and that expression of Mnx2a precedes expression of Mnx1.
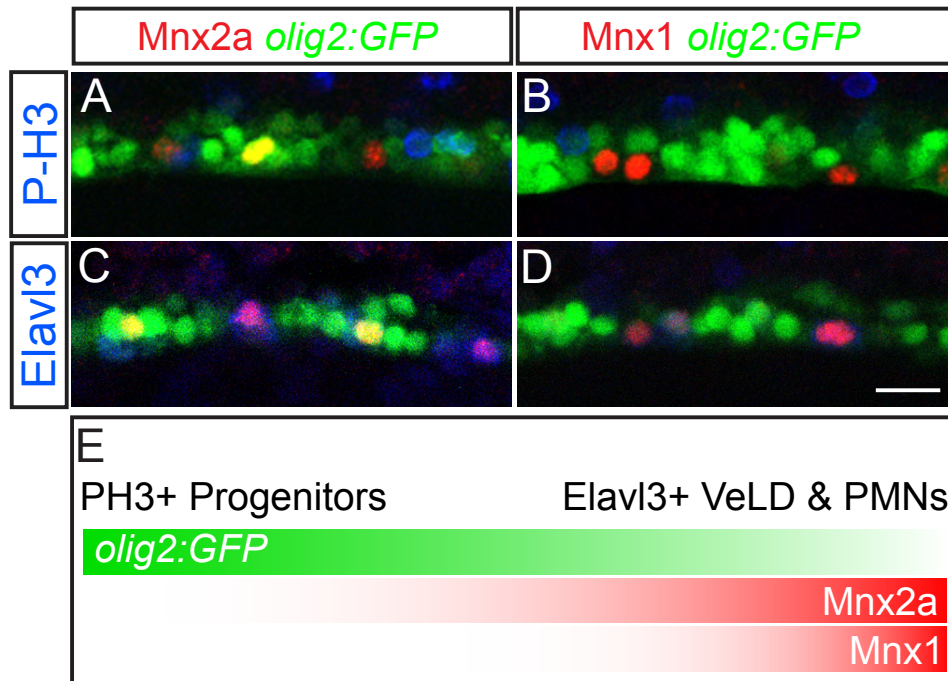
Figure 14. Mnx proteins are restricted to post-mitotic neurons
(**A-D**) Z-projections of confocal images of 14 hpf embryos. At 14 hpf, *olig2:GFP+* progenitors occasionally coexpress Mnx2a (A) but never Mnx1 (C). Elavl3+ neurons can be seen that coexpress either Mnx2a (B) or Mnx1 (D). Mnx1+ cells often co-expressed Elavl3, a marker of postmitotic neurons but did not co-express phosphohistone H3 (PH3), a marker of mitotic cells (0/70 Mnx1+ cells in 10 embryos). Similarly, Mnx2a was often co-expressed with Elavl3 and never co-expressed with PH3 (0/153 Mnx2a+ cells in 13 embryos). (**E**) Schematic of gene expression during transition from progenitors to post-mitotic neurons. Progenitors express PH3, whereas postmitotic neurons express Elavl3. *olig2* expression is initiated in progenitors, and downregulated as cells become postmitotic. Both *mnx1* and *mnx2a* expression is initiated after cells become postmitotic, with expression of *mnx2a* preceding expression of *mnx1*. Scale bar: 30µm in A-D.

mnx expression is independent of Islet1

Islet1 and Lhx3 cooperate to regulate Mnx1 expression in chick (Thaler et al., 2002; Lee et al., 2008). To learn whether this relationship is conserved in zebrafish, we injected MOs to knock down either Islet1 (Hutchinson and Eisen, 2006), or Lhx3 and Lhx4 (Hutchinson et al., *in preparation*), and examined *mnx* gene expression. Surprisingly, expression of all three *mnx* genes was unaffected by Islet1 knockdown

(Figure 15). Moreover, at 24 hpf only *mnx2b* expression was eliminated in the absence of Lhx3 and Lhx4 (Figure 15I), revealing that Islet1 and Lhx3 do not cooperate to regulate *mnx* expression in zebrafish.
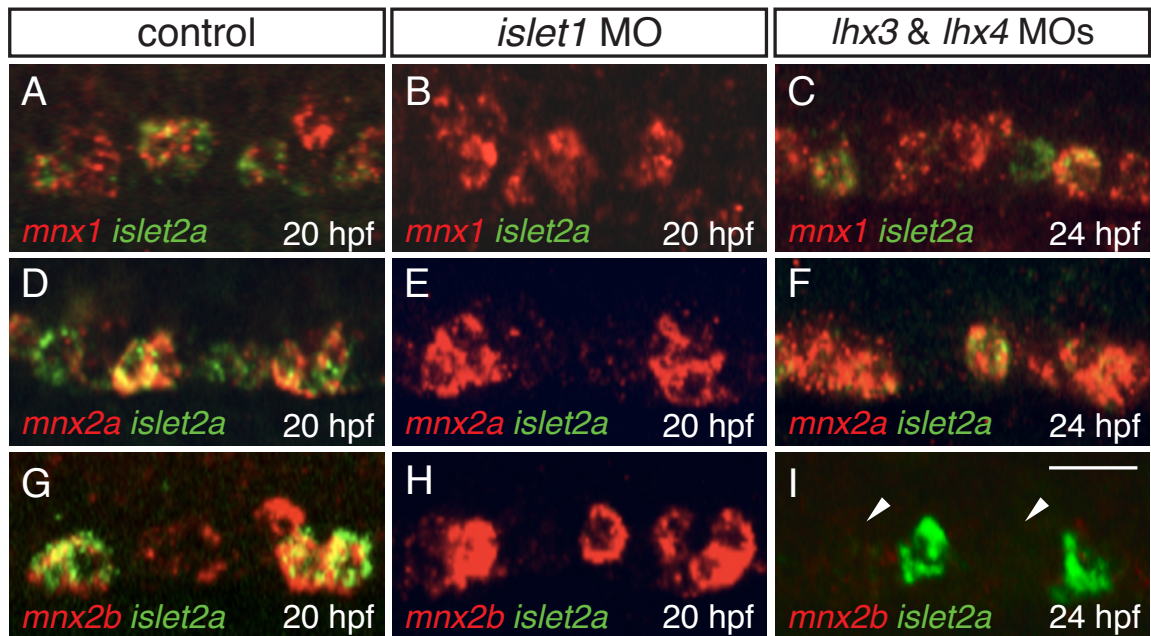
Figure 15. *mnx* genes are unaffected by Islet knockdown, and only *mnx2b* is affected by Lhx knockdown
(**A-I**) Single confocal slices of embryos. Control and MO-injected embryos. *mnx* genes are coexpressed with *islet2a* at 20 hpf (A, D, G). In the absence of Islet1, expression of *mnx1* (B), *mnx2a* (E), and *mnx2b* (H) persists, although *islet2a* expression is eliminated (Hutchinson and Eisen, 2006). In the absence of both Lhx3 and Lhx4 at 24 hpf, expression of *mnx1* (C) and *mnx2a* (F) persists, while expression of *mnx2b* is eliminated (arrowheads, I). Scale bar: 20μM in A-I.

Overexpression of Mnx proteins in chick induces formation of ectopic MNs expressing Islet, Lhx3, and other Mnx paralogs (Tanabe et al., 1998; William et al., 2003). To test the hypothesis that Mnx proteins regulate expression of *lhx* genes in zebrafish, we injected *mnx* MOs and examined expression of *lhx3* and *lhx4*. We found that neither *lhx3* nor *lhx4* expression was affected by the absence of Mnx proteins. We also eliminated expression of Mnx1, Mnx2a, and Mnx2b individually and examined expression of each *mnx* gene. We found no change in expression of any paralogs in the absence of any one *mnx* gene (data not shown), revealing that each member of the gene family is regulated independently of the others.

Mnx proteins are unnecessary for formation of primary motoneurons and VeLD interneurons

To test the function of Mnx proteins in PMN development, we used previously validated translation-blocking morpholinos to knock down *mnx1*, *mnx2a*, and *mnx2b* (Wendik et al., 2004) (Figure 11). To determine whether PMNs form in the absence of Mnx proteins, we assessed three markers of PMN identity: Islet (Hutchinson and Eisen, 2006), *chat,* which encodes an enzyme required to synthesize acetylcholine (Tallafuss and Eisen, 2008), and *nrp1a:GFP*, a transgene expressed in CaP and VaP at 18 hpf (Sato-Maeda et al., 2008) and in all PMNs at later stages. All three markers were expressed normally in the combined absence of Mnx1, Mnx2a, and Mnx2b (Figure 16A-D).

To assess whether VeLD development was compromised in the absence of Mnx proteins, we examined expression of Gad65/67, the biosynthetic enzyme for GABA, in *Tg(vsx1:GFP)^{nns5}* embryos. VeLDs express Gad65/67 but not *vsx1:GFP,* and can be uniquely identified based on their lateral position and soma shape. At 20 hpf, the number of VeLDs in the absence of all three Mnx proteins was indistinguishable from controls (Figure 16E, F). Moreover, at 28 hpf VeLDs were morphologically normal in the absence of Mnx proteins (data not shown). Together, these data provide evidence that Mnx proteins are not required for VeLD or PMN generation, and that both cell types acquire aspects of their mature identity in the absence of Mnx proteins.

Mnx proteins promote acquisition of some aspects of primary motoneuron subtype identity

Because *mnx* genes are expressed during and after the period of PMN subtype commitment (Eisen, 1991), we examined whether Mnx proteins play a role in subtype specification. Normally, all PMNs express *islet1* as they exit the cell cycle and then later express only one *islet* gene characteristic of their subtype: MiP and RoP express *islet1*, whereas CaP and VaP expresses *islet2a* (Appel et al., 1995) (Figure 17A). In the absence of Mnx proteins, CaPs inappropriately express both *islet1* and *islet2a* (Figure 17B). However, these cells form normal, ventrally-
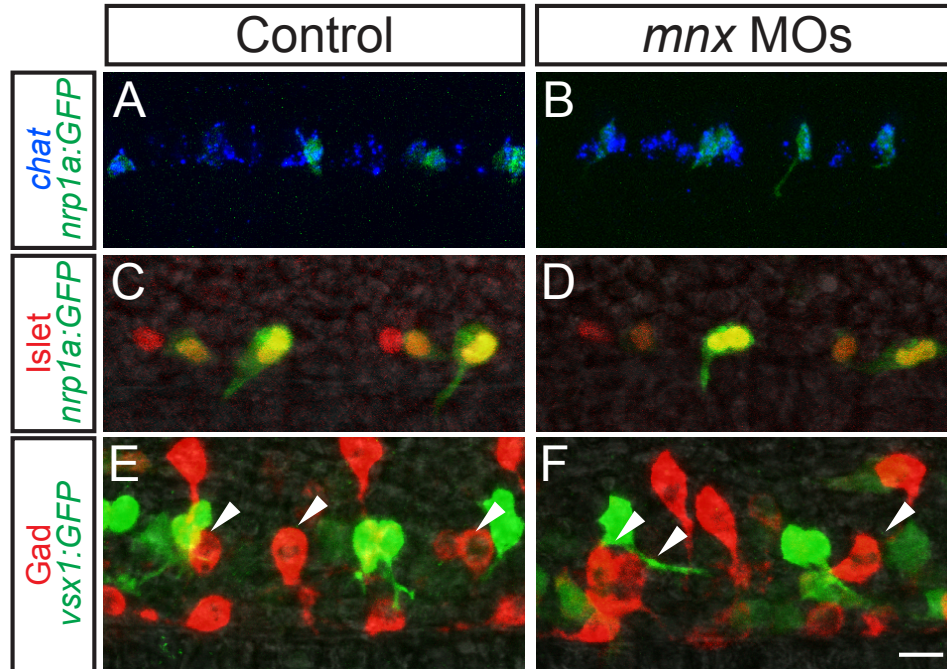
Figure 16. Mnx proteins are not required for formation of PMNs or VeLDs
(**A-F**) Control and *mnx* MO-injected transgenic embryos labeled with markers of PMN or VeLD identity. PMNs coexpress *chat* in control (A) and *mnx* MO-injected embryos (B). PMNs coexpress Islet in control (C) and *mnx* MO-injected embryos (D). VeLD IN numbers and distribution are the same in control (arrowheads, E) and *mnx* MO-injected embryos (arrowheads, F). Scale bar: 30μm in A-B, 25μM in C-D, 20μm in E-F.

extending CaP axons (Figure 17C,D), consistent with our previous finding that *islet1* and *islet2a* can play equivalent roles in CaP specification (Hutchinson and Eisen, 2006).

Strikingly, MiP dorsal axons were almost entirely absent from embryos lacking all three Mnx proteins (Figure 17C,D). To determine if a subset of the Mnx proteins is responsible for the MiP axon phenotype, we knocked down each Mnx protein singly or in pairs and counted the number of MiP axons in the mid-trunk. We saw no phenotype in the absence of any single Mnx protein, or in the absence of Mnx1 plus Mnx2a, or Mnx1 plus Mnx2b. However, in the absence of both Mnx2a and Mnx2b, MiP axons were absent from more than half the segments, and when present they were truncated (Table 3). Thus, all three Mnx proteins are required for proper MiP subtype specification and formation of normal, dorsally-projecting MiP axons, although the two Mnx2 paralogs appear to play a predominant role in this process.

Mnx proteins prevent MiPs from acquiring interneuron-like axons

In the absence of Mnx proteins, some MiPs failed to up-regulate expression of the *nrp1a:GFP* transgene. This, in conjunction with the absence of MiP axons from *mnx* MO-injected embryos, led us to consider whether in the absence of Mnx proteins MiPs developed as INs. Consistent with this idea, *nrp1a*:*GFP*+ descending IN axons were present in the ventral spinal cords of embryos lacking all three Mnx proteins, something never seen in control embryos (Figure 17C, D). To examine the morphology of MiPs in triple *mnx* MO-injected *Tg(nrp1a:GFP)js12* embryos in more detail, we labeled individual GFP-expressing cells in the MiP position with rhodamine-dextran and saw several phenotypes. Some MiPs initiated MN development by projecting a normal-appearing MiP ventral axon that stopped at the muscle pioneers, but instead of also projecting a collateral to dorsal muscle, these cells developed an IN-like axon that extended caudally many segment lengths within the spinal cord (Figure 17E-E"). Surprisingly, in some cases the ventral axons of these cells failed to stop at the muscle pioneers, instead extending as far ventrally as CaP axons (Figure 17F-F"). In other cases, we labeled GFP+ cells that had only a descending IN-like axon (Figure 17G-G"), or that had both a truncated MiP axon and a normal-appearing CaP axon (Figure 17H-H"). Based on these observations, we

Table 3. Mnx proteins are required for MiP formation

	CaP axons	MiP axons		
	normal	normal	truncated	absent
Control	**100%** n = 30 10 embryos	**97%** n = 85 18 embryos	**1%** n = 85 18 embryos	**1%** n = 85 18 embryos
***mnx2a & mnx2b* MOs**	**100%** n = 85 18 embryos	**26%** n = 85 18 embryos	**25%** n = 85 18 embryos	**49%** n = 85 18 embryos
***mnx1 & mnx2a & mnx2b* MOs**	**95%** n = 87 19 embryos	**11%** n = 87 19 embryos	**13%** n = 87 19 embryos	**76%** n = 87 19 embryos

Assayed at 28-32 hpf, segments 8-12 of *Tg(nrp1a:GFP)*; n = number of segments

conclude that Mnx proteins play two distinct roles in MiPs: they are required to prevent MiPs from developing as INs, and they are required to prevent MiPs from extending a ventral axon along the pathway normally followed by the CaP axon.

Mnx proteins prevent MiPs from acquiring V2a-like molecular characteristics

To ascertain whether MiPs in *mnx*-deficient embryos take on molecular as well as morphological characteristics of INs, we assayed for coexpression of IN and MN markers. We found that in triple *mnx* MO-injected embryos, MiPs coexpressed cholinergic and glutamatergic markers, a phenotype never seen in control embryos or in CaPs (Figure 18A, B). This hybrid neurotransmitter phenotype is specific, as expression of cholinergic and GABAergic or glycinergic markers was always mutually exclusive (Figure 19).

Figure 17 (next page). Mnx proteins promote acquisition of some aspects of PMN subtype identity and prevent MiPs from acquiring interneuron-like and CaP-like processes
(**A-D**) Control and *mnx* MO-injected embryos. In controls, *islet1* and *islet2a* expression is mutually exclusive at 18 hpf (A). In embryos injected with *mnx1*, *mnx2a*, and *mnx2b* MOs, *islet1* is coexpressed with *islet2a* in CaP and VaP. Control embryos have normal, ventrally-projecting CaP axons (arrows) and dorsally-projecting MiP axons (arrowheads in C). CaP axons (arrows) are normal in *mnx* MO-injected embryos whereas MiP axons (closed arrowheads) are absent and there are ectopic IN-like axons (open arrowheads in D). (**E-H"**) Rhodamine dextran-labeled MiPs in 28-32 hpf *Tg(nrp1a:GFP)* embryos injected with *mnx1*, *mnx2a*, and *mnx2b* MOs. Panels show rhodamine dextran labeling (no superscript), *nrp1a:GFP* (') and a merged image of the two channels ("). MiPs become hybrids with four morphologies. MiP-V2a hybrids have a descending V2a-like axon as well as a normal-appearing MiP ventral axon (E-E"). MiP-CaP-V2a hybrids have a descending V2a-like axon as well as a ventrally-projecting CaP-like axon (F-F"). "V2a" cells have a descending V2a-like axon (G-G"). MiP-CaP hybrids have a truncated MiP dorsal axon as well as a ventrally-projecting CaP-like axon (H-H"). All of these hybrids coexpress *nrp1a:GFP* (E", F", G", H"), as indicated by the yellow arrowheads. (**I**) Quantification of phenotypes shown in E-H. During normal pathfinding, MiP growth cones first extend ventrally to the horizontal myoseptum, where they pause before sprouting a collateral that extends along the dorsal myotome. The original ventral process is later retracted by 48 hpf, but is often present at the stages we examined (Eisen et al*.,* 1986; Melancon et al*.,* 1997). In the absence of Mnx proteins, none of the labeled MiPs had adopted either of these typical projections. Scale bar: 20µm in A-D, 40µm in E-H''.
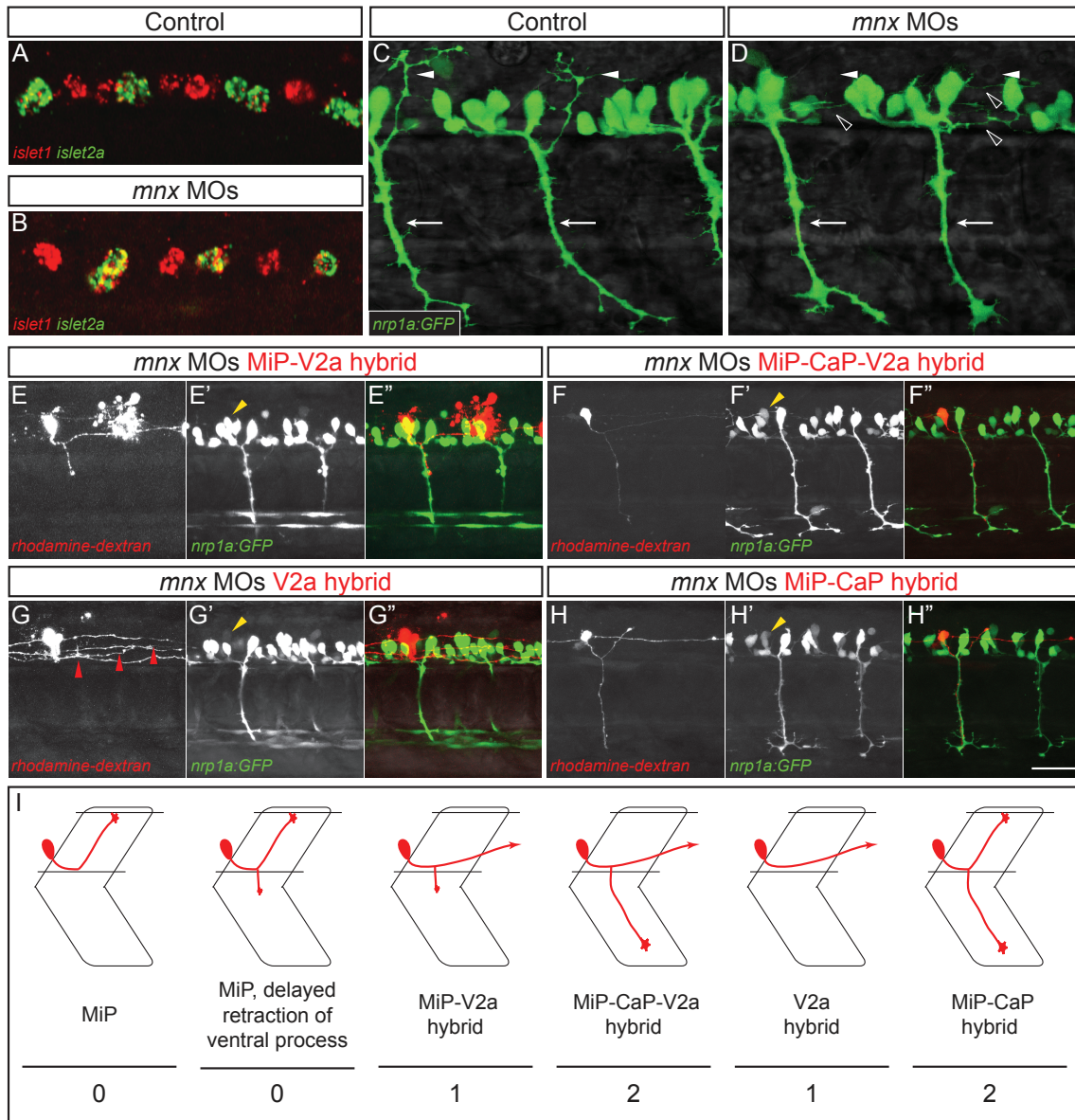
V2a INs are the only cells in the ventral spinal cord that express glutamatergic markers before 32 hpf (Higashijima et al., 2004a), thus we examined expression of *vsx2*, a definitive V2a marker (Kimura et al., 2008). In 22 hpf control *Tg(nrp1a:GFP)^{js12}* embryos, we found 1.2 *vsx2^+* cell per spinal hemisegment. In controls, expression of *vsx2* and the MN markers *islet1* and GFP was mutually exclusive (Figure 18C). In the absence of Mnx proteins, we found 2.3 *vsx2^+* cells per spinal hemisegment. Often, the extra *vsx2^+* cell weakly expressed GFP and was located near the somite boundary in the position occupied by the MiP soma (Figure 18D), suggesting the hybrid cells expressing both PMN and IN markers were MiPs. By comparison, *vsx2* was never expressed in CaPs, which continued to express *islet2a* in the absence of Mnx proteins (data not shown). These results suggest that Mnx proteins act to block expression of V2a IN markers specifically within MiPs.

***mnx* genes promote MiP subtype identity by maintaining expression of Islet1**

Previously, we found that in the absence of Nkx6.1 and Nkx6.2, MiPs often failed to form dorsal axons, and instead projected both their normal short ventral axon to the muscle pioneers and an IN-like axon within the spinal cord (Hutchinson
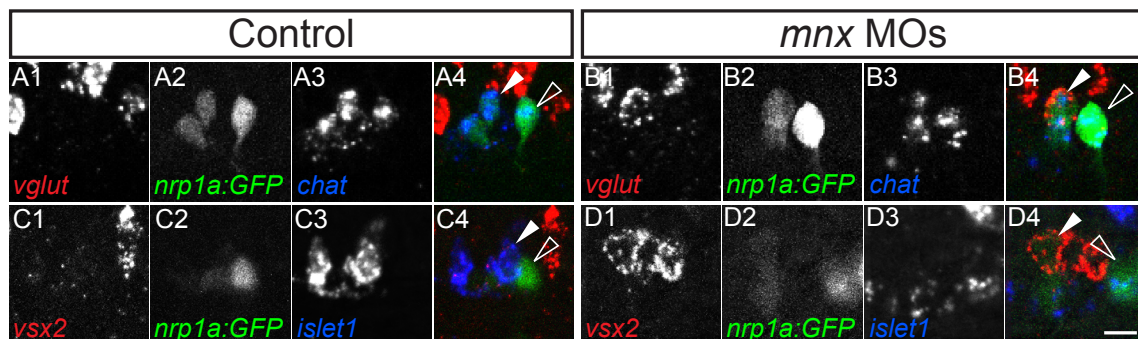
Figure 18. Mnx proteins prevent MiPs from acquiring V2a-like molecular characteristics

(**A-D**) Control and *mnx* MO-injected embryos at 22 hpf. MiPs (closed arrowheads) and CaPs (open arrowheads) are indicated in the merged panels. In control embryos, MiPs and CaPs express *chat* but never *vglut* (A). In *mnx* MO-injected embryos, MiPs coexpress both *chat* and *vglut*, while CaPs express *chat* but not *vglut* (B). In control embryos, MiPs express *islet1* but not *vsx2*, while CaPs express neither *islet1* nor *vsx2* (C). In *mnx* MO-injected embryos, MiP express *vsx2* but not *islet1* while CaPs express neither *vsx2* nor *islet1* (D). Scale bar: 10μm in A1-D4.
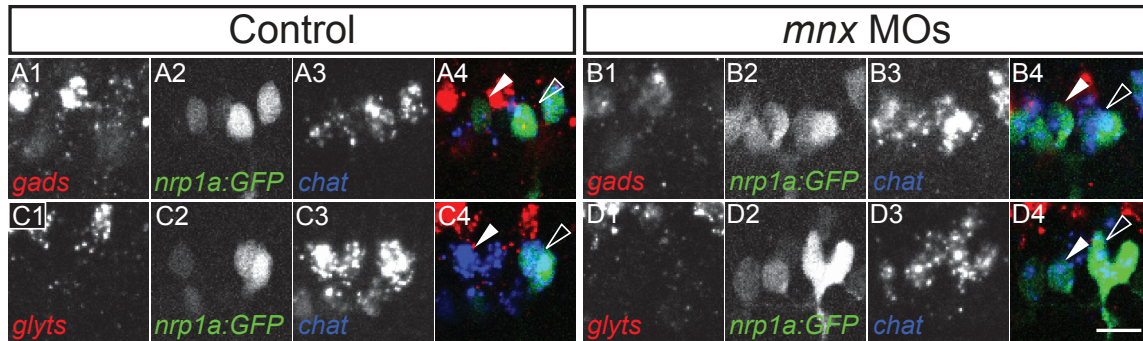
Figure 19. In the absence of Mnx proteins, neither MiPs nor CaPs aberrantly express GABAergic or glycinergic markers

(A-D) Control and *mnx* MO-injected embryos. MiPs (closed arrowheads) and CaPs (open arrowheads) are indicated in the merged panels. In control and MO-injected embryos, MiP and CaP express *chat* but not *gads* (A, B). In control and MO-injected embryos, MiP and CaP express *chat* but not *glyts* (C, D). Scale bar: 20μm in A1-D4.

et al., 2007). The similarity of the Nkx6 and Mnx knockdown phenotypes suggested that the genes might be part of the same pathway. To test this hypothesis, we injected *nkx6* MOs and examined Mnx expression, and we also injected *mnx* MOs and examined Nkx6 expression. We found that *mnx* expression was unaffected by the absence of Nkx6 proteins (data not shown). Similarly, *nkx6* expression was unaffected by the absence of Mnx proteins (data not shown). This indicates that Mnx proteins influence MiP development independently of Nkx6.

Nkx6 proteins exert their effect on formation of MiP dorsal axons by initiating a late, MiP-specific phase of Islet1 expression (Hutchinson et al., 2007), thus we examined whether this second phase of Islet1 was appropriately expressed in the absence of Mnx proteins. While expression of Islet1 at 18 hpf was normal in the absence of Mnx proteins, by 21 hpf Islet1 expression in MiPs was either absent or barely detectable (Figure 18D; Figure 20B, D). This suggests that the second phase of Islet1 expression in MiP is initiated correctly in the absence of Mnx proteins, but that Mnx proteins are necessary to maintain expression of Islet1 in MiP, and that continued Islet1 expression is necessary for MiP to form a normal dorsal axon.
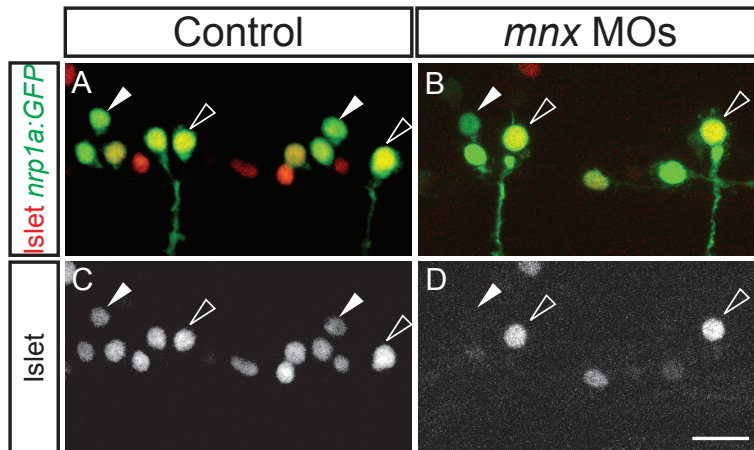
Figure 20. Mnx proteins maintain the second phase of Islet in MiPs
(**A-D**) Control and *mnx* MO-injected embryos at 21 hpf. MiPs (closed arrowheads) and CaPs (open arrowheads) are indicated. In control and MO-injected embryos, CaP expresses Islet (open arrowheads, A-D). MiP expresses Islet in control embryos (closed arrowheads, A, C) but not in MO-injected embryos (closed arrowheads, B, D). Scale bar: 20µm in A-D.

DISCUSSION

We show that the three Mnx transcription factors have dynamic expression patterns in each of the zebrafish PMN subtypes and in VeLD INs. Surprisingly, however, Mnx proteins appear dispensable for development of CaP MNs and VeLD INs. In contrast, Mnx proteins regulate both axon pathfinding and neurotransmitter specificity in MiP MNs.

Mnx expression in interneurons

Spinal cord expression of *mnx* genes was originally thought to be restricted to MNs (Tanabe et al., 1998; Arber et al., 1999; Thaler et al., 1999). More recently, a small population of Mnx1-expressing INs was identified in mouse (Brownstone and Wilson, 2008). Here we identify a class of ventral INs in zebrafish, VeLDs, that express two *mnx* genes, *mnx1* and *mnx2b*. Lineage tracing studies have demonstrated that VeLDs emerge from the same progenitor domain as PMNs, and can be PMN siblings (Park et al., 2004). The Mnx1-expressing INs in mouse are active components in the locomotor central pattern generator (Brownstone and Wilson, 2008; Kwan et al., 2009; Ziskind-Conhaim et al., 2010). Although these cells

51

have been extensively characterized during fictive locomotion in isolated spinal cord preparations (Hinckley et al., 2005; Wilson et al., 2005; Hinckley and Ziskind-Conhaim, 2006; Wilson et al., 2007; Ziskind-Conhaim et al., 2008; Hinckley et al., 2010), their exact role in the locomotor network in intact animals is unknown. Indeed, mouse Mnx1^{+} INs have proven resistant to genetic analysis, in part because their developmental provenance is entirely unclear.

It seems unlikely that VeLDs are the zebrafish equivalent of mouse Mnx1^{+} INs. VeLDs are born early, are GABA[+], and have ipsilateral, descending axons that extend many segments posterior to their somata (Bernhardt et al., 1992; Park et al., 2004; Hutchinson and Eisen, 2006). In contrast, although the Mnx1^{+} mouse INs may arise from the same domain as MNs (Alaynick et al., 2011), they are likely to be born later than MNs as they have not been described in lineage studies. Mouse Mnx1^{+} INs are glutamatergic and likely make strictly local projections to MN pools within the same segment (Hinckley et al., 2005; Wilson et al., 2005). We have also noticed some ventromedially-located Mnx1^{+} INs that appear at about 3 days postfertilization and do not seem to make projections to adjacent segments. Given the striking parallels between well-characterized components of the locomotor network in zebrafish and mouse (Higashijima et al., 2004b; Kimura et al., 2006), it will be important to follow development of these Mnx1^{+} INs *in vivo* to learn their origins. Assessing their role in zebrafish motor behavior should have implications for understanding the contribution of Mnx1^{+} INs to locomotion in other vertebrate species.

Despite expression in VeLDs, Mnx proteins appear unnecessary for VeLD development. However, we only assayed axon projection and neurotransmitter phenotype, thus our results do not rule out a role for Mnx proteins in regulating some other aspect of VeLD differentiation.

Mnx proteins promote MiP subtype identity

The acquisition of MiP and CaP subtype identity are differentially affected by the absence of Mnx1, Mnx2a, and Mnx2b. This results from a failure to regulate a late, MiP-specific phase of *islet1* expression appropriately in the absence of Mnx proteins. In the absence of high levels of Islet1, MiPs fail to form their characteristic

dorsal axons. This is reminiscent of the phenotype observed in the absence of Nkx6 (Hutchinson et al., 2007), but whereas Nkx6 proteins are required to initiate the late phase of *islet1* expression in MiP, Mnx proteins seem to be required to maintain high levels of Islet1 in MiP, similar to what has been reported in a mouse *Mnx1* knock out (Arber et al., 1999; Thaler et al., 1999).

Variability in the amount of Islet1 protein or the precise time at which it is cleared might also account for variability in MiP morphologies in the absence of Mnx proteins. One possibility is that MiPs that maintain Islet1 expression relatively late retain sufficient MN character to project an axon out of the spinal cord. In contrast, those that downregulate Islet1 relatively early might fail to express factors necessary to guide growth cones into the periphery. This is consistent with the finding that early expression of Islet1 in CaP is sufficient to permit axon growth into the periphery (Hutchinson and Eisen, 2006), and could be tested with photo-activatable morpholinos (Tallafuss et al., 2012) to block *islet1* translation at different times and assess the frequency with which MiP axons exit the spinal cord.

Surprisingly, when MiPs in *mnx* MO-injected embryos project axons into the periphery, most of them aberrantly extend to ventral muscle along a pathway normally reserved for CaP axons. Notably, MiP ventral axons normally stop at the muscle pioneers in the absence of Nkx6 proteins and late phase Islet1 expression (Hutchinson et al., 2007). Thus, while Mnx proteins promote formation of dorsal MiP axons by maintaining Islet1 expression, they exclude MiP axons from ventral muscle independently of Islet1. Motor axons navigate toward their appropriate muscle targets by following subtype-specific guidance cues. The cues that are differentially recognized by CaP axons and MiP axons are unknown. Our results suggest that Mnx proteins regulate expression of receptors that recognize cues that prevent the MiP growth cone from progressing ventral of the muscle pioneers, and thus prevent MiPs from becoming CaP-like.

Mnx proteins prevent MiP from acquiring V2a interneuron characteristics

In the absence of Mnx proteins MiPs, but not CaPs, form hybrids that have features of both MNs and INs. Based on expression of *vsx2* and *vglut* and axon

53

morphology, MiPs appear to have acquired features of zebrafish V2a INs (Kimura et al., 2006). As this phenotype is only observed in the combined absence of Mnx1, Mnx2a, and Mnx2b, the three zebrafish paralogs act redundantly to suppress the formation of MiP-V2a hybrids. The acquisition of V2a features is reminiscent of the phenotype of *mnx1* knockout mice in which Vsx2 is inappropriately expressed in a subset of Islet1^{+} MNs (Arber et al., 1999; Thaler et al., 1999). However, MN-V2a hybrids in *mnx1* knockout mice fail to project IN-like axons within the spinal cord and whether they express glutamatergic markers was not assessed. Our results suggest that zebrafish MiP-IN hybrids acquire a more complete set of V2a features. Regardless, our results reveal a conserved role for the *mnx* gene family in segregating MN from V2a IN cell fate in specific MN subtypes.

We previously reported that knocking down Islet1 resulted in PMNs developing as INs and that knocking down the Met receptor tyrosine kinase resulted in PMNs coexpressing MN and IN characteristics (Hutchinson and Eisen, 2006; Tallafuss and Eisen, 2008). In both of these cases, PMNs expressed the neurotransmitter GABA. Clonal analysis in zebrafish has revealed that PMNs can be siblings with either KA' or VeLD INs (Park et al., 2004), both of which express GABA (Bernhardt et al., 1992; Park et al., 2004). These observations supported a model whereby many factors expressed by PMNs cooperate to suppress acquisition of characteristics of closely-related INs derived from the pMN domain. Here we show that in the absence of Mnx proteins, PMN-IN hybrids inappropriately express V2a characteristics. These data support a model whereby postmitotic Mnx expression in PMNs suppresses acquisition of characteristics of more distantly-related INs from the adjacent p2 domain. A striking aspect of the PMN-V2a phenotype is that it is limited to MiP. This suggests that MiPs are more similar to V2a INs than are CaPs. This is consistent with the observation that in the absence of Nkx6 proteins, many MiP-IN hybrids have axons with a V2a morphology (Hutchinson et al., 2007). V2as, like PMNs, originate from a domain that expresses Nkx6.1 (Kimura et al., 2006), and continue to express Lhx3 after they exit the cell cycle (Batista et al., 2008). Moreover, recent lineage-tracing work in mouse has revealed that many V2a neurons have expressed *olig2* during their developmental history (Dessaud et al.,

2010; Chen et al., 2011), revealing that they may be even more similar to MNs than had been previously appreciated. A more detailed lineage analysis in zebrafish of the relationship between PMNs, VeLD INs, and V2a neurons could help resolve the relationships among these neurons.

BRIDGE

A number of transcription factors, among them the *lhx* and *mnx* families, have been shown to promote the acquisition of MN characteristics and repress the acquisition of IN characteristics. Although a few downstream elements have also been identified, such as sodium channels (Pineda et al., 2006), receptor tyrosine kinases (Tallafuss and Eisen, 2008), and genes responsible for axon pathfinding (Eisen and Pike, 1991; Beattie et al., 2000; Gray et al., 2001), how these genes interact with one another during MN specification remains largely unknown. To uncover additional genes that are involved in MN development, including both transcription factors and genes that may be acting downstream of them, I performed a microarray screen. Here I show that *inab*, a gene uncovered in my screen, is expressed in a subset of zebrafish primary MNs and an identified IN, and potentially has a role in axon outgrowth.

CHAPTER IV

IDENTIFICATION AND CHARACTERIZATION OF A MOTONEURON SUBTYPE-SPECIFIC GENE

INTRODUCTION

For correct locomotor circuitry to form in a developing vertebrate embryo, motoneuron axons must contact the appropriate muscle target. Motoneurons (MNs) are classified into subtypes that are dependent in part on their axon projection and morphology, a hallmark of appropriate MN subtype specification. MN specification is a continual process mediated by the expression of genes that both promote MN characteristics - such as an axon that leaves the spinal cord to innervate muscle - and repress interneuron characteristics - such as an axon that remains within the spinal cord to synapse onto other neurons. Although a number of genes have been identified as being involved in MN specification, many more remain unknown.

The zebrafish spinal cord is an ideal model in which to study questions of cell fate and specification, as there are a small number of individually identifiable neurons that can be observed in live animals over the course of development (Lewis and Eisen, 2003). The earliest-developing MNs in the zebrafish spinal cord are referred to as primary MNs (PMNs); there are also later-developing MNs referred to as secondary MNs (Myers, 1985). PMNs are especially amenable to study, as they have distinct subtypes, each of which projects an axon to a subtype-specific region in the overlying muscle (Eisen et al., 1986; Myers et al., 1986) and expresses a number of genes differentially (Hutchinson and Eisen, 2006; Van Ryswyk et al., *in preparation*). Not only can the mechanisms of PMN subtype specification be addressed genetically in the zebrafish, but the genes known to be involved in zebrafish MN specification are conserved across vertebrates.

To uncover additional genes that promote MN development, I performed a microarray screen. By comparing the transcriptome of embryonic zebrafish spinal cords manipulated to have excess MNs to that of spinal cords manipulated to have decreased numbers of MNs, I was able to select a number of candidate genes. These candidates were regulated in the same direction in each condition as control genes

known to be expressed in zebrafish MNs. One of these candidate genes is *inab*, a neuronal intermediate filament.

Neuronal intermediate filaments are one family of proteins thought to be involved in the differentiation of neurons as well as the acquisition of axonal morphology (Lariviere and Julien, 2004). Upregulation of a number of neuronal intermediate filament proteins has been correlated with the alteration in cellular morphology that accompanies differentiation (Cochard and Paulin, 1984; Chang and Goldman, 2004). The genes that encode some of these proteins, such as *nestin* and *alpha-internexin* (*ina*), are expressed in mammalian neuroblasts at the end of their migration and beginning of their differentiation (Pachter and Liem, 1985; Kaplan et al., 1990; Lendahl et al., 1990; Wang et al., 2006). *nestin* is a well-characterized marker of neural progenitors (Lendahl et al., 1990; Michalczyk and Ziman, 2005), and both it and *ina* are thought to be involved in the differentiation of neural progenitor cells and the accompanying acquisition of proper axonal morphology (Nixon and Shea, 1992; Ching et al., 1999; Chang and Goldman, 2004; Lariviere and Julien, 2004; Park et al., 2010).

The homolog of *ina* in fish and frog, also named *ina*, has been shown to be crucial in axon outgrowth and is upregulated in retinal ganglion cell axons after crush of the optic nerve (Glasgow et al., 1994; Asch et al., 1998; Niloff et al., 1998). Interestingly, different intermediate filament genes are expressed in different subpopulations of neurons as they acquire distinct morphologies (Chang and Goldman, 2004). Therefore, it seems likely that the particular intermediate filament protein present in a neuron plays a role in its differentiation and acquisition of axon morphology, and therefore could be important for subtype specification.

In addition to its well-characterized role in the optic nerve, *ina* is known to be expressed in the spinal MNs of goldfish and frog, although its role in cells other than retinal ganglion cells has not yet been elucidated (Glasgow et al., 1994; Zhao and Szaro, 1997). Zebrafish have two paralogs of *ina* - *inaa* and *inab* - of which *inab* has been shown to be expressed in zebrafish MNs (Asch et al., 1998; Leake et al., 1999). Its presence there means that it could be involved in MN subtype specification.

Here I focus on one subtype of zebrafish PMN, CaP/VaP. CaP and VaP are initially equivalent MNs, referred to as CaP/VaPs, that go on to acquire different fates (Eisen et al., 1990; Eisen, 1992). CaP is present in all spinal hemisegments, and can be identified by a long ventrally-extending axon. VaP is only present in approximately half of the spinal hemisegments, projects a short ventrally-extending axon, and typically dies during embryonic development. I also focus on an identified IN that can be a sibling to MNs, VeLD. There are up to two VeLDs on each side of each spinal hemisegment; the VeLD cell body is located just dorsal to the PMNs (Bernhardt et al., 1990). VeLD can be identified by its ventral descending axon, which projects caudally for up to 10 segments (Kuwada et al., 1990).

I demonstrate that zebrafish *inab* is dynamically expressed in CaP and VaP PMNs in the spinal cord, and in VeLD interneurons. Although *inab* does not appear to be necessary for MN specification, the axon morphology of CaP MNs is disrupted when it is misspliced. This work suggests that neuronal intermediate filament proteins are necessary for proper axonal outgrowth, which may in turn be related to proper MN subtype specification.

METHODS
Zebrafish

Wild-type (AB), *smu^b641* (Varga et al., 2001), *Tg(nrp1a:GFP)^jsl2* (Sato-Maeda et al., 2008), *Tg(mnx1:GFP)^ml2* (Flanagan-Steet et al., 2005), and *Tg(vsx1:GFP)^nns5* (Kimura et al., 2008) zebrafish were maintained in a laboratory breeding colony according to established protocols (Westerfield, 2007). Embryos collected from natural crosses were allowed to develop at 28.5ºC and staged according to morphological criteria (Kimmel et al., 1995).

RNA synthesis and injections

Capped dominant negative Suppressor of Hairless RNA was synthesized and injected as previously described (Cornell and Eisen, 2000). This was sufficient to generate ectopic motoneurons as assayed by *in situ* hybridization with *islet2a* probe (Appel et al., 1995).

Spinal cord dissociations

Embryonic dissociation protocol was adapted from one previously used in the lab (Beattie and Eisen, 1997). Embryos were grown up to 20 hpf and were anesthetized using Finquel (MS-222) (Argent Laboratories; Redmond, WA, USA). To obtain isolated spinal cords, embryos were incubated in 7.5x pancreatin (MP Biomedicals; Irvine, CA, USA) in zebrafish Ringer's until tissues began to separate (about 1 minute). Embryos were then triturated with Pasteur pipettes of decreasing size. Isolated spinal cords were then transferred to Leibowitz's L15 medium (Gibco/Life Technologies; Grand Island, NY, USA) containing 10% fetal bovine serum for 1-2 minutes to inactivate the pancreatin, after which they were stored in TriReagent (Molecular Research Center, Inc.; Cincinnati, OH, USA) at -80ºC.

Microarray sample preparation

Isolated spinal cords in TriReagent were pooled and homogenized. RNA extraction was performed according to standard protocols (Chomczynski and Sacchi, 2006). The extracted RNA was then amplified using a MessageAmp II aRNA kit (Ambien/Life Technologies; Grand Island, NY, USA) according to the supplied instructions. Amplified RNA was shipped to NimbleGen (Roche NimbleGen Inc.; Madison, WI, USA) where cDNA probes were synthesized and hybridized to Zebrafish Gene Expression 385K Arrays.

Microarray data analysis

Data analysis was carried out using ArrayStar software (DNASTAR Inc.; Madison, WI, USA). Candidate genes were selected by relative transcript abundance across conditions and gene expression pattern criteria.

RNA probe generation

Full-length cDNA sequences containing the genes of interest were obtained from Open Biosystems (Thermo Fischer Scientific, Lafayette, CO, USA). The *ccdc85al* cDNA was amplified with the following primers: forward 5'-TGTACGGAAGTGTTACT TCTGCTC-3' and reverse 5'-GGATCCATTAACCCTCACTAAAGGGAAGGCCGCGACCTGC

AGCTC-3'. The *nr2f1b* cDNA was amplified with the following primers: forward 5'-A ACAGCTATGACCATGATTAC-3' and reverse 5'-GTAAAACGACGGCCAGT-3'. The *inab* cDNA was amplified with the following primers: forward 5'-TGGATAACCGTATTACC GCC-3' and reverse 5'-CGCGCAATTAACCCTCACTAAATCACTAGTCATACCAGGATC-3'. For all genes, T3 RNA Polymerase (Roche Applied Sciences; Indianapolis, IN, USA) was used to make probe for RNA *in situ* hybridization according to standard protocols (Thisse and Thisse, 2008).

Fluorescent RNA *in situ* hybridization

RNA *in situ* hybridization was performed according to standard protocols (Thisse and Thisse, 2008), with the following modifications: For 2-color fluorescent *in situ* hybridization, anti-sense probes were labeled with digoxygenin-UTP (Roche Applied Sciences; Indianapolis, IN, USA) and dinitrophenol-UTP (Perkin-Elmer; Waltham, MA, USA). Following overnight hybridization, unbound probe was removed with three 30-minute washes at 67ºC in 50% formamide, 5x SSC, and 0.1% SDS, followed by stringent washes in 50% formamide, 2x SSC, and 0.1% Tween-20. Labeled probes were detected with HRP-conjugated anti-DIG (1:1000; Jackson ImmunoResearch; West Grove, PA, USA) or HRP-conjugated anti-DNP (1:1000, Perkin-Elmer), and stained with fluorescein or Cy-3 -tyramide (1:50; Perkin-Elmer) for 1-10 minutes.

Probes used include *ccdc85al*, *nr2f1b*, and *inab*; *islet1* and *islet2a* (Appel et al., 1995); *gad1b* and *gad2* (collectively referred to as *gad*), *slc17a6a*, *slc17a6b*, and *slc17a7* (collectively referred to as *vglut*), *slc6a9* and *slc6a5* (collectively referred to as *glyt*) (Higashijima et al., 2004b); and *vsx2* (Kimura et al., 2006).

Morpholino injections

Approximately 2μL of 300μM splice-blocking morpholinos (Gene Tools, LLC; Philomath, OR, USA) against *inab* (a combination of SB2: 5'-GGAATCCTAGATGACGT GATAATTC-3' and SB3: 5'-CAGTGATGGTTTATTACCTGTAAGC-3') were injected into

1 to 2-cell stage embryos. This was sufficient to cause missplicing as assayed by primers designed to flank the splice sites (forward: 5'-CCTGGAGAAAAAGGTCGAATCC-3' and reverse: 5'-GCTATTTTCTATGTCAAGCGCC-3') (Figure 21C).
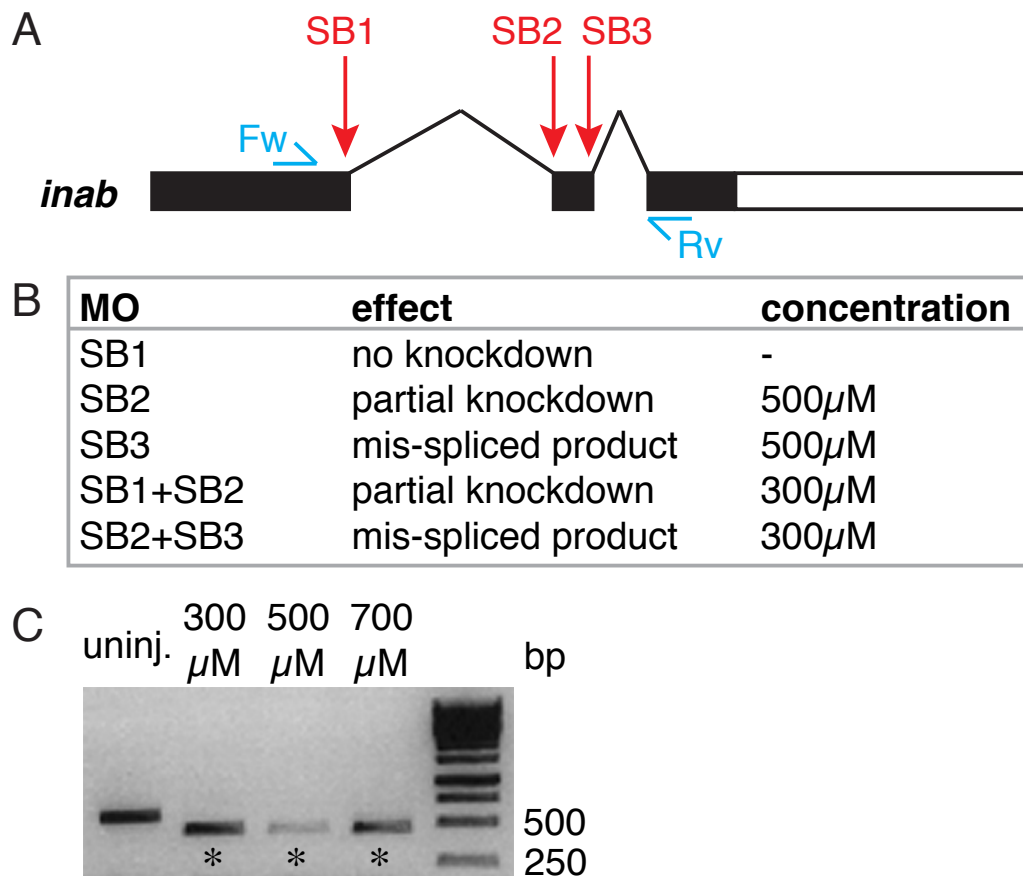
Figure 21. Splice-blocking morpholinos cause missplicing of *inab* mRNA
(**A**) Schematic of the *inab* gene. *inab* has three exons and two introns. Splice-blocking MOs (SB) target various splice sites in the gene - SB1 targets the exon 1/intron1 boundary, SB2 targets the intron 1/exon 2 boundary, and SB3 targets the exon 2/intron 2 boundary. Forward and reverse primers sit in the first and third exons, respectively. (**B**) Table of MO combinations, the effect they have on *inab* mRNA splicing, and the lowest concentration of MO at which the effect is seen. (**C**) PCR results confirming that uninjected embryos have wild type *inab* (580 bp band), and embryos injected with 300μM, 500μM, or 700μM of both SB2 and SB3 MOs have misspliced *inab* (asterisks, 490 bp band).

Image acquisition

      All images were acquired on a Zeiss Pascal confocal microscope using either a 20x objective or a 40x water-immersion objective. The brightness and contrast of images was adjusted using Photoshop CS5 (Version 12.0, Adobe Systems, Inc.; San Jose, CA, USA).

Image quantification

      Image analysis was carried out using the Bonfire program - a series of custom scripts for Matlab (MathWorks; Natick, MA, USA) (Langhammer et al., 2010). Images were scored blind, and resulting data were transferred to Excel for statistical analysis. Significance was determined using a two-tailed Student's T-test, with a p-value of less than 0.05 being considered significant.

RESULTS

Expression profiling of zebrafish spinal cord using NimbleGen microarrays

      In my microarray screen, I compared the spinal cords of embryonic zebrafish with excess MNs to spinal cords with a decreased number of MNs. To create embryos with more MNs than normal, I injected wild-type embryos with mRNA encoding a dominant negative Suppressor of Hairless [dnSu(H)] protein. This dominant negative protein interferes with Delta/Notch signaling in the embryo, which creates excess early-born neurons and increases the number of MNs (Cornell and Eisen, 2000). To create embryos with fewer than normal numbers of MNs, I incrossed the *smoothened* mutant line *smu^b641^*. The Smoothened protein plays a crucial role in Hedgehog signaling, which is required for normal MN development (Lewis and Eisen, 2001). Homozygous *smu^b641^* mutants have disrupted Hedgehog signaling, and consequently almost completely lack MNs (Lewis and Eisen, 2001; Varga et al., 2001).

      To cut down on background from other tissues, I separated the spinal cords from the rest of the fish using a dissociation procedure developed in the lab (Beattie and Eisen, 1997). After collecting a sufficient number of spinal cords (~200 per condition), I extracted the RNA from each sample and amplified it. To confirm that

spinal cords were present in each case, I used tissue-specific primers to check for the presence of spinal cord, as well as the absence of contaminating hindbrain and muscle (data not shown). I then prepared these amplified RNA samples and sent them to NimbleGen, where they were made into cDNA probes, hybridized to a chip, and processed as three single-channel microarrays. The data from these microarrays were normalized and sent back to me.

I then selected a list of genes that were at least two times upregulated in the excess MN condition and two times downregulated in the decreased MN condition, as compared to wild-type spinal cords (the magnitude of which corresponds to the approximate regulation of verified MN-specific genes in the microarrays). I narrowed the list down by excluding "housekeeping" and hypothetical genes, those expressed outside of the central nervous system, and those initially expressed earlier than the birth of MNs or significantly after their maturation. To verify and further investigate the expression patterns of the candidate genes I chose, I obtained a clone of each gene from Open Biosystems and synthesized RNA probes for each of them.

One of the candidate genes, *ccdc85al*, is predicted to be a transcription factor, and is expressed in a population of neurons just dorsal to the PMNs in the zebrafish spinal cord (Figure 22A). Another one of the candidate genes, *nr2f1b*, is a transcription factor-encoding gene that is expressed in MNs, although it is also broadly expressed in the spinal cord (Figure 22B). Here I describe in depth both the
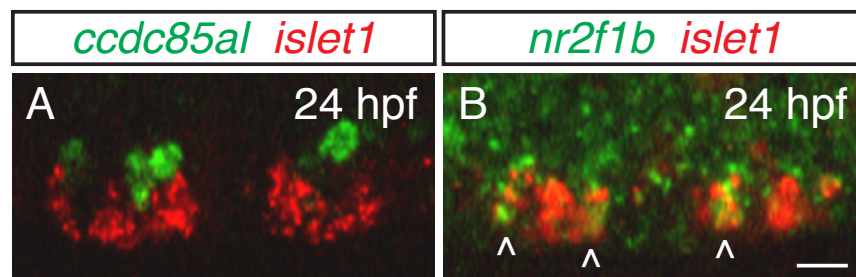

Figure 22. *ccdc85al* and *nr2f1b* are expressed in the zebrafish spinal cord
(**A-B**) Single confocal slices of 24 hpf embryos. *ccdc85al* is expressed in a population of cells just dorsal to the *islet1*+ MNs (A). *nr2f1b* is expressed broadly throughout the spinal cord, including expression in *islet1*+ MNs (B). Scale bar: 15μm in A-B.

expression and functional analysis of my third candidate gene, the intermediate filament-encoding gene *inab*.

inab is dynamically expressed in a subset of primary motoneurons and in VeLD interneurons

I characterized the expression of *inab* in the zebrafish spinal cord using RNA *in situ* hybridization. To determine if PMNs express *inab*, I labeled embryos for *islet1* mRNA, which all PMNs express before 14 hours postfertilization (hpf) (Appel et al., 1995), and *inab*. In embryos older than 14 hpf, I labeled with both *islet1* mRNA (which after 14 hpf is expressed in PMN subtypes MiP and RoP) and *inab*, or *islet2a* mRNA (which after 14 hpf is expressed in PMN subtype CaP/VaP) (Appel et al., 1995) and *inab*. *inab* is expressed in CaP and VaP between 16 and 24 hpf (Figure 23, A-H). *inab* mRNA could not be detected before 14 hpf, and its expression was downregulated in CaP and VaP by 24 hpf.

In addition to its expression in a subset of PMNs, *inab* is also expressed in cells that are slightly more dorsal than the PMNs. To determine the identity of these cells, I labeled transgenic *mnx1:GFP* embryos with *inab* riboprobe. The *mnx1:GFP* transgene is expressed in all PMN subtypes and the interneuron VeLD before 24 hpf (Van Ryswyk et al., *in preparation*). VeLD can also be identified by its expression of the neurotransmitter GABA (Bernhardt et al., 1992). The *inab*[+] IN is labeled by both the *mnx1* transgene and an mRNA probe against the GABA synthetic enzymes, leading me to conclude that it is VeLD (Figure 23, I&J). *inab* expression in VeLD is

Figure 23 (next page). *inab* **is dynamically expressed in a subset of primary motoneurons and VeLD interneurons**
(**A-J**) Single confocal slices of embryos labeled with *inab* and *islet* riboprobes. At 14 hpf, *inab* is coexpressed with neither *islet1* (A) nor *islet2a* (B). Between 16 and 24 hpf, *inab* is coexpressed with *islet2a*[+] MNs (open arrowheads in D, F, H) but not *islet1*[+] MNs (C, E, G). *inab* is expressed in the VeLD IN, as determined by its coexpression with both GFP in the *mnx1:GFP* transgenic line (solid arrowhead in I) and *gad* mRNA (solid arrowhead in J). (**K**) Schematic of *inab* mRNA dynamics during early development. Between 14 and 18 hpf, *inab* expression is initiated in both CaP and VaP MNs and the VeLD IN. By 24 hpf, *inab* expression in CaP and VaP is downregulated, although it persists in the VeLD IN and an additional *vglut*[+] IN. Scale bar: 20μm in A-H, 15μm in I, and 20μm in J.
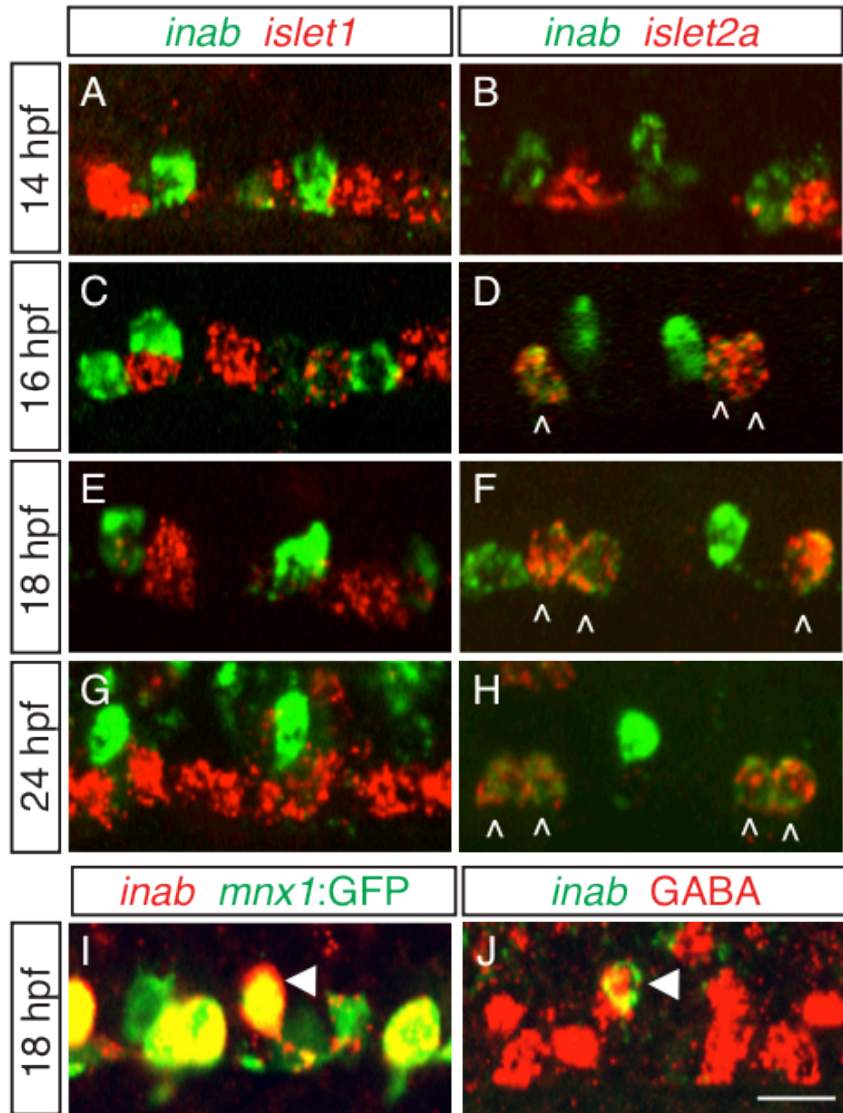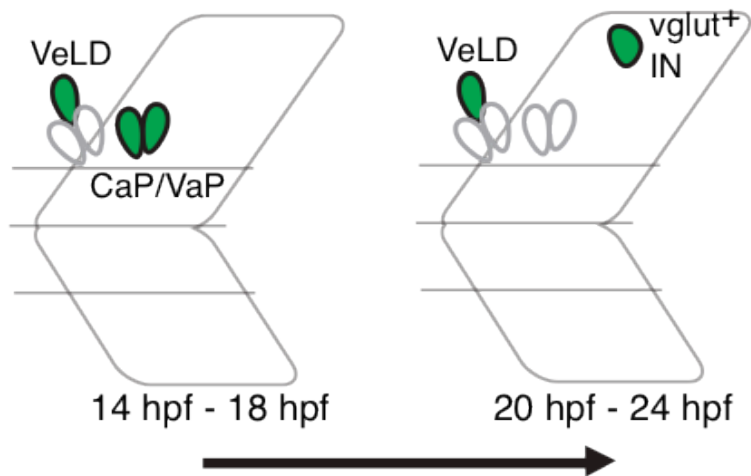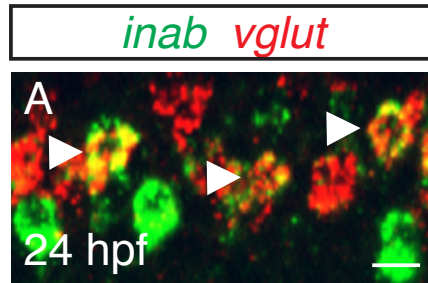
Figure 24. *inab* is expressed in a glutamatergic dorsal interneuron
At 24 hpf, *inab* is coexpressed with *vglut* in cell dorsal to the VeLD interneuron
(arrowheads, A). Scale bar: 10μm.

initiated at 14 hpf and persists through 24 hpf.

       inab is also expressed in a more dorsal, glutamatergic population of INs, with

expression initiating at 18 hpf (Figure 24), but I excluded them from this analysis.

inab may be required for proper development of PMN axons

       To test the function of *inab* in PMN development, I used splice-blocking

morpholinos (MOs) to prevent proper splicing of *inab* mRNA. I designed MOs to

target the exon 1/intron 1 boundary (SB1), the intron 1/exon 2 boundary (SB2),

and the exon 2/intron 2 boundary (SB3) (Figure 21A). On its own, injection of SB2

decreased the amount of correctly-spliced *inab* (Figure 21B), and injection of SB3

alone increased the amount of incorrectly-spliced *inab* (Figure 21B). Co-injection of

SB2 and SB3 also increased the amount of incorrectly-spliced *inab*, and was able to

do so at a lower concentration than that of injecting SB3 alone (Figure 21C). Because

I was not able to fully knock down *inab*, what follows is an analysis of the result of

missplicing of *inab* caused by co-injection of the SB2 and SB3 MOs.

       inab is expressed in CaP/VaP and VeLD, and so I hypothesized that its

knockdown would have an effect on the axons of these particular cells. To assay any

changes in axon morphology, I co-injected SB2 and SB3 MOs into both the *nrp1a:GFP*

transgenic line, in which GFP is expressed in CaP/VaPs exclusively at 18 hpf (Sato-

Maeda et al., 2008), and the *mnx1:GFP* transgenic line, in which GFP is expressed in

all PMNs and VeLD at from 14 hpf on (Van Ryswyk et al., *in preparation*).

CaP/VaPs begin projecting an axon ventrally around 17 hpf (Eisen et al., 1986; Myers et al., 1986). This axon is clearly visible by 20 hpf in both the *mnx1:GFP* and *nrp1a:GFP* transgenics (Flanagan-Steet et al., 2005; Sato-Maeda et al., 2006; Sato-Maeda et al., 2008). VeLD also extends its axon posteriorly between 18 and 20 hpf (Bernhardt et al., 1990), and it too can be clearly identified in the *mnx1:GFP* transgenic by 20 hpf (Van Ryswyk et al., *in preparation*). When *inab* is misspliced, the CaP and VeLD axons are both present and have a similar overall morphology to those of control embryos in the *mnx1:GFP* line at 22 hpf (Figure 25A, B). However, MO-injected embryos appear to have slightly misshapen CaP axons as compared to controls (Figure 25A, B).

To further examine the CaP axon morphology, I looked at a later time point in the MO-injected *nrp1a:GFP* embryos. By 24 hpf the CaP and VaP axons in the MO-injected embryos appear to have more branching along their length than in control embryos (Figure 25C, D). To attempt to quantify this difference, I used Bonfire analysis to examine the branching patterns of CaP axons in control and MO-injected *nrp1a:GFP* embryos. The Bonfire program uses spatial information about the position of neurites to quantify characteristics such as the number of branch points along the length of an axon, or the relative connectivity of the neurites

Figure 25 (next page). *inab* may be required for proper development of some PMN axons
(**A-D**) Z-projections of confocal images of control and MO-injected transgenic embryos. At 20 hpf, CaP axons extend ventrally in both control and MO-injected *mnx1:GFP* embryos (solid arrowheads in A, B). Descending VeLD axons are also present in both control and MO-injected *mnx1:GFP* embryos (open arrowheads in A, B). At 24 hpf, ventral CaP axons are present in both control and MO-injected *nrp1a:GFP* embryos (C, D), but MO-injected embryos appear to have more branching along the length of the axon (arrows, D). (**E-F**) Graphs representing quantification of axon branching. There are both significantly more branch points (p=0.004) and more terminal points (p=0.004) in MO-injected embryos (n=33 cells) than in control embryos (n=24 cells) (asterisks, E). Overall, there are significantly more total processes (p=0.004) in MO-injected embryos (n=33 cells) than in control embryos (n=24 cells) (asterisks, F). The number of primary processes is the same in both conditions, but MO-injected embryos have significantly more secondary (p=0.004) and tertiary (p=0.004) processes than control embryos (asterisks, F). All error bars represent standard error of the mean. Scale bar: 20µm in A-D.

control   *inab* SB2 & SB3 MOs

mnx1:GFP   22 hpf

A   B

control   *inab* SB2 & SB3 MOs

nrp1a:GFP   24 hpf

C   D

E

average number of points

□ control n=24

■ MO-injected n=33

* p<0.05

branch   terminal

F

average number of processes

□ control n=24

■ MO-injected n=33

* p<0.05

total   primary   secondary   tertiary
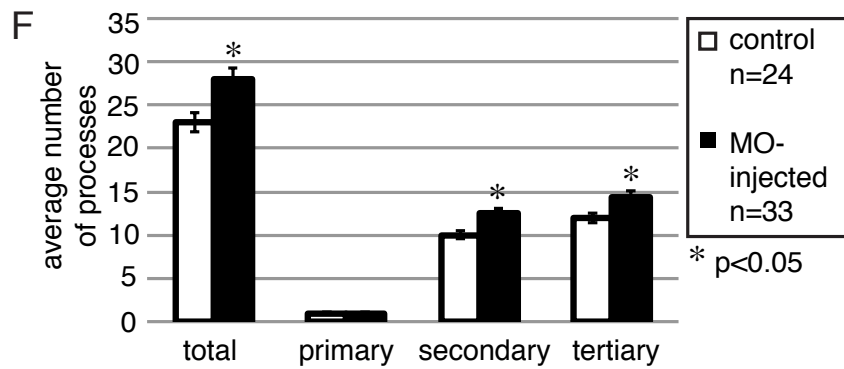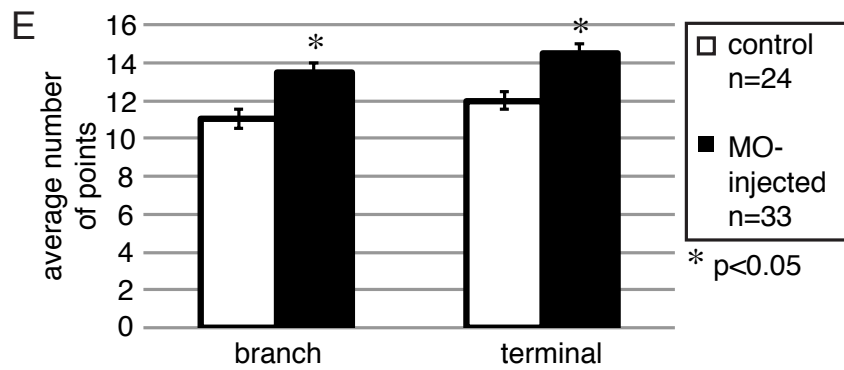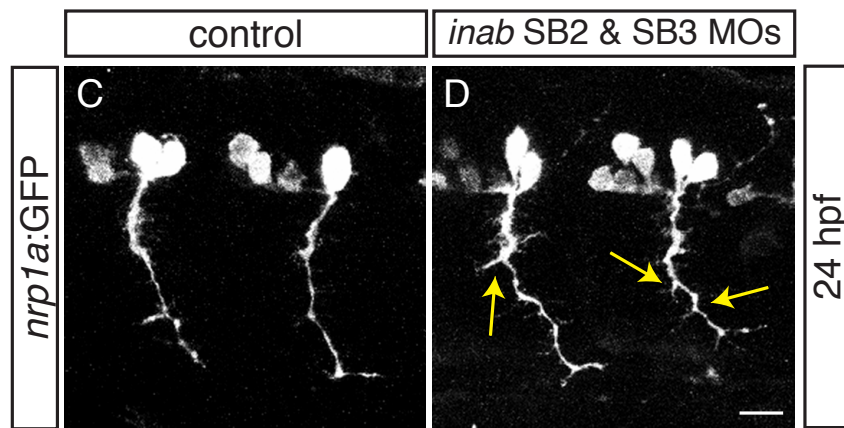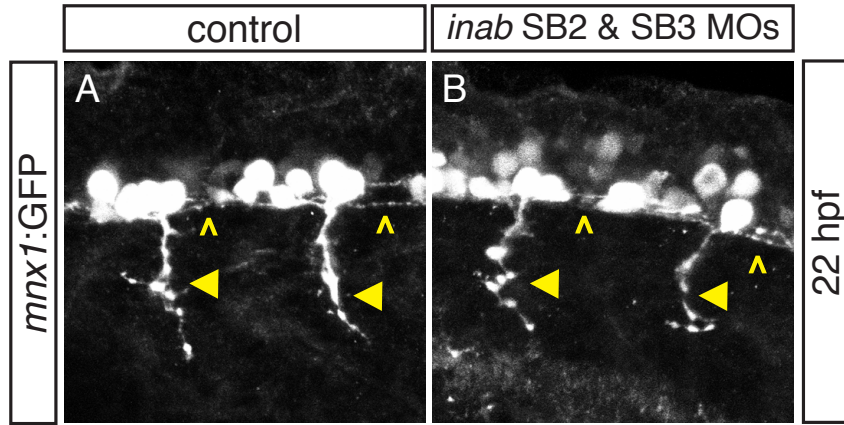
(Langhammer et al., 2010).

In this analysis, a branch point is the region at which a neurite splits into two smaller neurites, and a terminal point is where a neurite ends. A process is an uninterrupted stretch of neurite that begins either at the soma of the cell or a previous branch point, and ends at the next branch point or a terminal point. A primary process begins at the soma, a secondary process begins at the branch point of a primary process, and a tertiary process begins at the branch point of a secondary process. I found that MO-injected embryos have significantly more branch points along the length of the CaP axon, as well as significantly more terminal points, than do control embryos ($p < 0.05$, Figure 25E). As an increase in branch points would suggest, MO-injected embryos also have a significantly higher number of total processes along their CaP axon than control embryos, and the increase comes from a larger number of secondary and tertiary processes ($p < 0.05$, Figure 25F). These small but significant differences suggest that while the overall morphology of CaP axons is not changed by the presence of misspliced *inab*, the branching along the length of the axon is increased.

DISCUSSION

Expression patterns of identified candidate genes in the zebrafish spinal cord

Here I have identified three candidate genes that could be involved in zebrafish spinal cord development. I show that one of my candidate genes, the predicted transcription factor-encoding gene *ccdc85al*, is expressed in a population of cells just dorsal to the PMNs. Although it is not expressed in MNs, it may be in closely-related INs that are generated from the same progenitor domain as MNs (Park et al., 2004). Investigation of *ccdc85al* could potentially be interesting, as much remains to be learned about the INs that are generated from the MN progenitor domain.

I also show that another of my candidate genes, the transcription factor-encoding gene *nr2f1b*, is expressed broadly throughout the zebrafish spinal cord, including expression in PMNs. Based on its expression pattern, this gene may be less informative about mechanisms of MN specification than a gene with a cell type-

69

specific expression pattern. The presence of *nr2f1b* in the spinal cord, however, merits further investigation.

Dynamic expression of *inab* in identified zebrafish spinal neurons

I show that the neuronal intermediate filament gene *inab* is expressed in a subset of zebrafish PMNs - CaP and VaP - and a closely-related IN - VeLD. The expression of *inab* in such a small number of cell types lends credence to the hypothesis that differential expression of intermediate filament proteins is in part responsible for the diversity in morphology across different types of neurons (Chang and Goldman, 2004). The CaP axon morphology is distinct from that of the other zebrafish PMNs, and the VeLD axon morphology is distinct from that of other ventral INs. Specific expression of a particular intermediate filament, in this case *inab*, may contribute to the acquisition of distinct axon morphologies.

In addition to being expressed in such a specific subset of cells, the timing of *inab* expression also supports the concept that this gene may be involved in directed axon outgrowth and the acquisition of axon morphology. Both CaP and VeLD neurons begin extending their axons around 17 hpf (Eisen et al., 1986; Myers et al., 1986; Bernhardt et al., 1990). *inab* is first expressed in these cells at 16 hpf, just prior to axogenesis. *inab* expression is also only maintained in CaP through 24 hpf, after the CaP axon has projected ventrally towards its muscle target (Myers et al., 1986). The specific temporal expression of *inab* immediately before and during axogenesis in both CaP and VeLD places it in the right position to be involved in directed axon outgrowth.

In mice, expression of the *inab* homolog *ina* is restricted to a large number of postmitotic cells in the central and peripheral nervous system, including "spinal nerves" (Wang et al, 2006), although this is the highest resolution currently available. The ability to distinguish individual cells in the zebrafish spinal cord lends remarkable power to correlate gene expression with potential function in particular cells. Expression of *inab* in CaP and VeLD could be a mechanism by which these neuronal subtypes acquire their distinct axon morphologies. It will be interesting to learn whether other neuronal intermediate filament genes are expressed in the

70

other subtypes of zebrafish PMNs, as well as other INs that are generated from the MN progenitor domain.

In addition to *inab*, zebrafish also have another paralog of *ina* - named *inaa* - about which only its sequence is known. *inaa* could potentially be expressed in the same subset of neurons as *inab*, a different subset of PMNs, in a different set of spinal neurons altogether, or not expressed within the spinal cord. I am working to characterize the expression of *inaa* using RNA *in situ* hybridization. If *inaa* expression is identical to *inab* expression, it would suggest that it is playing a redundant or supporting role in CaP and VeLD. However, *inaa* expression might be slightly or completely different than that of *inab*, suggestive of a divergence in function. It will be exciting to learn whether *inab* and *inaa* have similar or distinct functions in the zebrafish spinal cord.

Potential function of *inab* in outgrowth of a zebrafish PMN subtype

Although I was unable to fully investigate the role of *inab* in zebrafish spinal MNs, my results lend some insight into its possible function. *inab* has been shown to be involved in directed axon outgrowth in the retinal ganglion cells of fish and frogs (Glasgow et al., 1994; Asch et al., 1998; Niloff et al., 1998), but a role in spinal MNs has not yet been demonstrated in either of these models. My results are the first indication that *inab* may be involved in directed axon outgrowth in the vertebrate spinal cord. The forced missplicing of *inab* through the use of splice-blocking MOs demonstrated that *inab* is potentially involved in the outgrowth of MN axons and is necessary to prevent excess branching. This phenotype is specific to CaP, as *inab* is expressed only in a subset of PMNs and its missplicing does not appear to affect other PMN axons. And though I was initially unable to see a phenotype for the VeLD axon when *inab* is misspliced, this does not rule out a role for *inab* in VeLD. Further investigation of this particular IN subtype will be necessary to determine if intermediate filaments are involved in its axon outgrowth.

Several possibilities exist that could explain the subtle phenotype that missplicing of *inab* produces. First, it may be that the increased branching of CaP axons is only a partial phenotype because Inab protein was not fully knocked down.

Translation-blocking MOs do not currently exist for this gene, so the option of fully eliminating Inab protein with MOs cannot be pursued at this time. No complete knockdown of *inab* has been done in either fish or frog models, so it is difficult to infer what a complete loss-of-function phenotype would be.

Secondly, the situation may be more complicated due to the potential presence of *inaa*, the *inab* paralog. Nothing is known about this gene other than its sequence. Depending on its expression pattern, *inaa* could be acting with *inab* during the outgrowth of PMN axons. If *inab* and *inaa* are acting redundantly, it may be necessary to knock them both down in order to see a full phenotype. If the functions of *inaa* and *inab* have diverged, the possibility exists that both are acting on CaP axon outgrowth in different ways, and eliminating both proteins would produce a more severe phenotype.

Finally, it is possible that the *inab* missplicing phenotype is subtle but complete. Mouse models lacking *ina* show no requirement for the gene in either the overall development of the nervous system or the radial growth of axons (Levavasseur et al., 1999). This indicates that *inab* may not be necessary for directed axon outgrowth of spinal neurons. Interestingly, overexpression of *ina* causes both abnormal filamentous accumulations in cerebellar neuron axons and related motor coordination deficits (Ching et al., 1999). Although the motor coordination in zebrafish lacking *inab* has not been investigated, it is worth noting that gain-of-function of *ina*, rather than loss-of-function, creates this motor phenotype in another vertebrate. Additionally, a mouse model of a hereditary neuropathy exhibits a related phenotype in that *ina* is abnormally accumulated in MN axons, causing swelling (Tseng et al., 2008). This misregulation in the MN axons could be related to the branching phenotype I observed in zebrafish MN axons.

These results - even if the phenotype is subtle or incomplete - are motivating because they are the first demonstration of a role for *inab* in spinal MNs. Further research will be necessary to fully resolve the function of *inab*, and possibly *inaa*, in the zebrafish spinal cord. It is hard to imagine that this intermediate filament with such specific expression is not involved in the axonal development of CaP and VeLD neurons.

CHAPTER V

CONCLUSION

The specification of vertebrate motoneurons is an excellent model for how, over the course of development, a single cell can emerge from a group of progenitors and develop as a distinct, individual cell with a specific function. This process relies on the correct spatial and temporal expression of genes, from morphogens and transcription factors to downstream elements such as receptors, ion channels, and various types of structural proteins. These genes work together, acting with one another in networks or acting in parallel pathways, to guide a single cell from progenitor to postmitotic motoneuron (MN) to specific MN subtype. To address the underlying question of what distinguishes a MN from closely-related interneurons (INs), an analysis of the individual genes involved is often necessary. Here, I have used the zebrafish model to examine roles for specific genes in the specification of both MNs and MN subtypes, as well as investigating the roles of specific genes in preventing MNs from developing as INs.

The transcription factor-encoding genes *lhx3* and *lhx4* have been implicated in MN specification in both the mouse and chick models (Sharma et al., 1998; Shirasaki and Pfaff, 2002; Thaler et al., 2002). Previous work has shown that zebrafish *lhx3* is expressed in both MNs and ventral INs (Appel et al., 1995). In this dissertation, I provided evidence that zebrafish *lhx3* and *lhx4* are differentially expressed in specific MNs and INs, and that both genes are necessary for normal MN and ventral IN development. I showed that both *lhx* genes are necessary to prevent MNs from expressing IN-specific characteristics and developing as MN/IN hybrids, suggesting that the two genes have at least some redundant functions. I also showed that both *lhx3* and *lhx4* are necessary for the formation of some INs derived from the progenitor of MN (pMN) domain. This work has provided a finer analysis of what distinguishes MNs and closely-related INs from one another, and has shown both MN- and IN-specific functions for the *lhx* genes in zebrafish.

The transcription factor-encoding gene *mnx1* and its homolog *MNR2* have also been implicated in MN specification in both mouse and chick (Tanabe et al.,

1998; Arber et al., 1999; Thaler et al., 1999; William et al., 2003). In this dissertation, I have shown that all three zebrafish *mnx* genes - *mnx1*, and the two *MNR2* paralogs *mnx2a* and *mnx2b* - are dynamically expressed in early-developing zebrafish primary MNs and an identified IN that is sibling to the primary MNs. This is different than in other vertebrate models, where *mnx1* and *MNR2* were thought to be restricted to MNs only during early development (Tanabe et al., 1998; Arber et al., 1999; Thaler et al., 1999), although now it is known that *mnx1* is expressed in a specific set of INs of unknown origin (Hinckley et al., 2005; Wilson et al., 2005). I provided evidence that the zebrafish *mnx* genes are necessary both to promote the acquisition of some MN subtype-specific characteristics and to prevent the acquisition of some IN-specific characteristics. The *mnx* genes also appear to be involved in regulating MN specification in part through interactions with the transcription factor-encoding gene *islet1*. This work has not only provided a finer analysis of what distinguishes MNs from their sibling INs, but has also highlighted the complexities of the gene networks specifying MN fate.

The intermediate filament protein-encoding gene *ina* has been implicated in axon outgrowth in various vertebrate models (Glasgow et al., 1994; Asch et al., 1998; Niloff et al., 1998; Chang and Goldman, 2004). I identified the zebrafish homolog of this gene, *inab*, as important for zebrafish MNs in a microarray screen. Axon outgrowth is an important part of MN subtype specification, and *inab* is a good candidate to be involved in axon outgrowth of specific MN subtypes. In this dissertation I have shown that zebrafish *inab* is dynamically expressed in a subset of primary MNs and an identified IN that is sibling to the primary MNs. I have demonstrated that axon morphology is abnormal when *inab* is misspliced, potentially indicating that *inab* is necessary for proper axon outgrowth of a subtype of MNs. Further work will be necessary to determine the specific function that *inab* has in the zebrafish spinal cord, but a role in MN subtype specification seems likely.

With this work, I have resolved with finer detail some of the genetic components of MN and IN specification by taking advantage of the ability to follow development of individually identified neurons at high resolution and in living embryos. There are a number of genes, expressed in both MNs and often closely-

74

related INs, that are necessary both to promote the acquisition of MN-specific characteristics and to suppress the acquisition of IN-specific characteristics. Although MN specification has been intensively studied, it is a dynamic, complicated process, and many details of the underlying mechanisms are yet to be clarified. Further research will uncover exactly how these genes are interacting with one another to promote MN development in vertebrate embryos.

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