**ABSTRACT**

A central problem in development is how fates of closely related cells are segregated. Lineally related motoneurons (MNs) and interneurons (INs) express many genes in common yet acquire distinct fates. For example, in mouse and chick Lhx3 plays a pivotal role in the development of both cell classes. Here, we utilize the ability to recognize individual zebrafish neurons to examine the roles of Lhx3 and its paralog Lhx4 in the development of MNs and ventral INs. We show that Lhx3 and Lhx4 are expressed by post-mitotic axial MNs derived from the MN progenitor (pMN) domain, p2 domain progenitors and by several types of INs derived from pMN and p2 domains. In the absence of Lhx3 and Lhx4, early-developing primary MNs (PMNs) adopt a hybrid fate, with morphological and molecular features of both PMNs and pMN-derived Kolmer–Agduhr (KA) INs. In addition, we show that Lhx3 and Lhx4 distinguish the fates of two pMN-derived INs. Finally, we demonstrate that Lhx3 and Lhx4 are necessary for the formation of later-developing V2a and V2b INs. In conjunction with our previous work, these data reveal that distinct transcription factor families are deployed in post-mitotic MNs to unequivocally assign MN fate and suppress the development of alternative pMN-derived IN fates.

**KEY WORDS:** Zebrafish, Motoneuron, Interneuron, Kolmer–Agduhr, V2a, V2b, Lhx3, Lhx4

**INTRODUCTION**

Progressive restriction of cell fate during development is a complex process that requires coordinated changes in gene expression. After terminal division of progenitors, unique combinations of transcription factors promote acquisition of post-mitotic characteristics that define individual cell types. However, cells closely related by lineage share a common regulatory history and express many of the same transcription factors. This is particularly true in the vertebrate ventral spinal cord, where different types of neurons derive from a single progenitor domain specified by a common set of transcription factors (Lewis, 2006).

To understand how the fates of closely related neurons are specified, we examined derivatives of the progenitor of motoneuron (pMN) domain in the zebrafish ventral spinal cord. The zebrafish pMN lineage is well-characterized and generates several alternative neuron classes (Park et al., 2004). Analysis of these neuron classes is simplified because each spinal hemisegment has only a few neuron types, the development of which can be followed in intact embryos (Fig. 1) (Lewis and Eisen, 2003; McLean and Fetcho, 2008). Between 9 and 15 h post fertilization (hpf), the pMN domain generates 3-4 early-born primary MNs (PMNs) (Myers et al., 1986), as well as two interneurons (INs), Kolmer–Agduhr (KA) and ventral-longitudinally descending (VeLD) (Park et al., 2004), within each spinal hemisegment. Both PMNs and these two INs can be identified by soma position, axon morphology and gene expression (Bernhardt et al., 1990, 1992; Kuwada et al., 1990). After 15 hpf, the pMN domain generates additional cell types: smaller, more numerous secondary MNs (SMNs); oligodendrocyte precursors; and a third class of INs, circumferentially descending (CiD) (Park et al., 2004). CiDs are morphologically and molecularly similar to excitatory V2a INs that arise from the dorsally adjacent p2 progenitor domain later in development (Batista et al., 2008; Kimura et al., 2008). At early stages these cells can be distinguished because CiDs are olig2-positive, whereas V2a INs are vglut1-positive (Kimura et al., 2006; Park et al., 2004). As in amniote vertebrates, zebrafish p2 progenitors also generate inhibitory V2b INs (Batista et al., 2008; Kimura et al., 2008).

After their terminal divisions, pMN-derived neurons express unique combinations of transcription factors from three different families. PMNs and SMNs are Islet+ Mnx+ Lhx3+; VeLDs are Islet+ Mnx+ Lhx3+; CiDs are Islet+ Mnx− Lhx3+; KA’s are Islet− Mnx− Lhx3− (Batista et al., 2008; Hutchinson and Eisen, 2006; Kimura et al., 2006; Seredick et al., 2012). Knockdown of either Islet or Mnx family members compromises aspects of MN identity, and PMNs acquire features of specific pMN-derived INs. For example, in the absence of Islet proteins, PMNs aberrantly express GABA and have ipsilaterally descending axons in the ventral longitudinal fasciculus, characteristic of VeLDs (Hutchinson and Eisen, 2006). By comparison, in the absence of Mnx, one PMN subtype aberrantly expresses vglut2 and vglut1 and often has ipsilaterally descending axons in the lateral neuropil, which are characteristics of CiDs (Seredick et al., 2012). These results suggest that different transcription factor families expressed in post-mitotic MNs are dedicated to suppressing specific alternative IN programs within MNs. This prompted us to test the hypothesis that in PMNs, Lhx family members suppress characteristics of the remaining pMN-derived IN, KA.

Lhx3 is an LIM homeodomain transcription factor that plays pivotal roles in MN and IN fates during neural development in both vertebrates and invertebrates (Thaler et al., 2002; Thor et al., 1999). In vertebrates, genome duplication created an Lhx3 paralog, Lhx4, that can substitute for Lhx3 in formation of V2a- and MN-promoting complexes (Gadd et al., 2011). Although mouse knockouts of either Lhx3 or Lhx4 are perinatally lethal (Li et al., 1994; Sheng et al., 1996), MNs are generated normally (Sharma et al., 1994) and are assigned somatic identities through a process of ‘epigenetic’ reprogramming (Sheng et al., 1996). This suggests that Lhx3 and Lhx4 redundantly specify features of both PMNs and pMN-derived Kolmer–Agduhr MNs (PMNs) adopt a hybrid fate, with morphological and molecular characteristics of both PMNs and pMN-derived Kolmer–Agduhr (KA) INs.
et al., 1998). However, at cervical levels, MN subtype identity is altered in the combined absence of Lhx3 and Lhx4. Cervical MNs, the axons of which normally exit ventrally, acquire features of spinal accessory column (SAC) MNs, and their axons exit dorsally (Sharma et al., 1998). Importantly, the fate of MNs at other axial levels remains unknown. Despite intensive investigation of Lhx3 involvement in regulatory networks that promote MN, V2a and V2b fates (Joshi et al., 2009; Lee et al., 2012, 2008, 2009; Lee and Pfaff, 2003; Thaler et al., 2002), it remains untested whether Lhx3 or Lhx4 is necessary for ventral spinal cord IN specification.

Here, we show that zebrafish Lhx4 is expressed, like Lhx3, in MNs and INs derived from both pMN and p2 domains. Morpholino oligonucleotide (MO) knockdown of Lhx3 and Lhx4 revealed that, at thoracic levels, many cells fated to become MNs aberrantly acquire characteristics of KA INs. In addition, overexpression of Lhx3 in pMN-derived neurons and progenitors causes an increase in CiDs at the expense of KA’s. Moreover, Lhx3 and Lhx4 are necessary for specification of many late-developing V2a and V2b INs. These findings provide new evidence of roles for Lhx3 and Lhx4 in the fates of specific ventral spinal cord INs and demonstrate that these Lhx proteins suppress a competing KA IN program within both trunk level axial MNs and pMN-derived CiD INs.

RESULTS

Lhx3 and Lhx4 are expressed in MNs, ventral INs and p2 progenitors

Zebrafish lhx3 is expressed in pMN-derived PMNs, SMNs, VeLDs and CiDs (Appel et al., 1995; Batista et al., 2008), whereas KA’s are not reported to express lhx3. Lhx3 and Lhx4 cooperate to promote MN identity in mouse (Sharma et al., 1998); however, Lhx4 was uncharacterized in zebrafish. Thus, we determined the lhx4 expression pattern on embryos of various stages and compared it with lhx3. lhx4 expression begins at 11 hpf, the same time as lhx3 (Fig. 2A,B). Like lhx3, lhx4 is expressed in two medial cell stripes in the region that will form the ventral spinal cord. At 24 hpf, lhx4 is expressed in fewer cells than lhx3 (Fig. 2C,D). To understand the relationship between lhx3 and lhx4 expression, we assayed them simultaneously in Tg(nrp1a:GFP) embryos that label MNs (Sato-Maeda et al., 2008). At 16 hpf, all lhx4-expressing spinal cord cells co-express lhx3, whereas some lhx3-expressing cells do not express lhx4 (Fig. 2E, arrowheads). The position of these cells relative to the nrp1a:GFP transgene suggests that they are primarily pMN-derived MNs and INs that also express lhx3 (Appel et al., 1995). At 20 hpf, lhx3 and lhx4 are co-expressed in more dorsal cells. The relative locations of these more dorsally located co-expressing cells suggest they are p2-derived INs that express lhx3 (Batista et al., 2008; Kimura et al., 2008). At 24 and 28 hpf, lhx3 and lhx4 appear to be almost entirely co-expressed (Fig. 2G,H). Thus, at these stages, lhx4, like lhx3, is expressed in MNs as well as in pMN- and p2-derived INs. In addition, lhx4 is expressed in the pituitary and in a set of dorsal INs between the eyes (supplementary material Fig. S1). lhx4 expression in the pituitary is similar to that of lhx3; however, the dorsal IN population that expresses lhx4 does not express lhx3.

To examine expression of Lhx3 and Lhx4 proteins, we generated polyclonal antibodies (see Materials and Methods; supplementary material Fig. S2). To confirm that Lhx4 is expressed in MNs, we compared expression patterns of embryos double-labeled with anti-Lhx4 and anti-Islet (Korzh et al., 1993). At 18 hpf, anti-Lhx4 antibody labeled exclusively Islet+ cells, suggesting that initially only PMNs express Lhx4 (Fig. 2I). By contrast, at 18 hpf many cells are Lhx3+Islet−, consistent with Lhx3 expression in VeLDs, CiDs and V2as (Fig. 2I, arrowhead) (Appel et al., 1995; Batista et al., 2008). By 24 hpf, Lhx3 and Lhx4 continued to be expressed in all Islet+ cells, indicating expression in all PMNs and at least some SMNs (Fig. 2K,L). At this time, anti-Lhx4 antibody labeled many Islet+ cells in the ventral spinal cord, consistent with Lhx4 expression expanding into VeLDs, CiDs and V2as. These data indicate that Lhx4, like Lhx3, is expressed in PMNs, SMNs, VeLDs, CiDs and V2as but is excluded from medially located KA’s (Fig. 2Q).

To examine whether Lhx3 and Lhx4 are expressed in spinal cord progenitors similar to mouse and chick (Sharma et al., 1998; Tsuchida et al., 1994), we co-labeled transgenic embryos expressing GFP in pMN or p2 progenitors with anti-Lhx3/4 antibodies and a mitotic marker, anti-phospho Histone H3 (P-H3). We identified pMN progenitors using Tg(olig2:GFP) embryos that express GFP in pMN progenitors and their post-mitotic derivatives (Shin et al., 2003). We found that GFP+ Lhx3/4+ cells in 12, 16 and 20 hpf Tg(olig2:GFP) embryos never co-expressed P-H3 (Fig. 2M and data not shown). We confirmed that Lhx3 and Lhx4 are excluded from pMN progenitors by labeling proliferating cells with 5-ethyl-2′-deoxyuridine (EdU) at 11 hpf, the time of peak PMN generation (Myers et al., 1986). When processed at 12 hpf, no Lhx3/4+ cells had incorporated EdU (Fig. 2N,P). We identified p2 progenitors, using Tg(wwx1:GFP) embryos that express GFP in progenitors just prior to their final division as well as in V2 INs (Kimura et al., 2008). By contrast to pMN progenitors, at 20 hpf some p2 progenitors were Lhx3/4− and P-H3+ (Fig. 2O,P). These data show that Lhx3 and Lhx4 are expressed by p2 progenitors but are absent from pMN progenitors, including KA’ progenitors.

Lhx3 and Lhx4 promote normal MN axon morphology

The altered axon projection patterns of cervical MNs in mouse embryos lacking Lhx3 and Lhx4 (Sharma et al., 1998) prompted us to examine zebrafish trunk MNs in the absence of these proteins. We labeled MO-injected embryos with zn1 and anti-Syt2b antibodies that reveal axons of two PMN subtypes, ventrally projecting caudal primary (CaP) and dorsally projecting middle primary (MiP) (Melandon et al., 1997; Myers et al., 1986;
Injection of either lhx3 or lhx4 MO alone had modest to no effect on PMN axon formation (Fig. 3A-C,E). However, co-injection of lhx3 and lhx4 MOs severely reduced PMN axon numbers (Fig. 3D,E). Reduction was sporadic along the length of the trunk and in many cases differentially affected CaPs and MiPs within a single trunk segment. SMN development was also compromised. lhx3 and lhx4 MO-injected embryos lacked Alcama (Fashena and Westerfield, 1999) expression on SMN somata and fasciculated axons (Fig. 3F,G). Live imaging of s1020t; Tg(UAS-E1b:Kaede) embryos revealed that SMN axons projecting to dorsal and ventral axial muscles adjacent to trunk somites 5-15 were variably present at 48 hpf. Motor nerves that were present were much thinner, suggesting fewer SMN axons per nerve (Fig. 3H,I). By contrast to Lhx3- and Lhx4-deficient mouse cervical MNs (Sharma et al., 1998), axons of Lhx3- and Lhx4-deficient zebrafish trunk MNs that left the spinal cord invariably exited at the ventral root.

The loss of trunk MN axons following Lhx3 and Lhx4 knockdown prompted us to test whether these MNs ever formed. Lhx3 and Lhx4 knockdown resulted in normal numbers of Islet+ trunk cells at 28 and 48 hpf, suggesting that both PMNs and SMNs are generated (Fig. 4A,C,G-J). This is similar to Lhx3- and Lhx4-expressing cells in the ventral spinal cord. (Q) Combinatorial expression of three transcription factors that uniquely label all four neuronal derivatives of the zebrafish pMN domain. Scale bars: 20 μm.

**Fig. 2.** lhx4 and lhx3 are expressed in many cells in the ventral neural tube. All images oriented with anterior to the left. (A,B) Dorsal view of 11 hpf embryos. Both lhx3 (A) and lhx4 (B) are expressed beginning at 11 hpf in two medial cell stripes. (C,D) Lateral view of spinal cord adjacent to somites 8-12 at 24 hpf embryo. Both lhx3 (C) and lhx4 (D) are expressed in the pMN domain; however, lhx4 is expressed in fewer cells than lhx3. (E-F) lhx3 and lhx4 expression in ventral neural tube of Tg(nrp1a:GFP) embryos. (E) At 16 hpf, lhx3 and lhx4 are expressed at the dorsoventral level of GFPC MNs, whereas some GFPC cells express only lhx3 (arrowheads). (F) At 20 hpf, lhx3 and lhx4 expression has expanded to include more dorsal GFP+ cells (arrowheads). (G-H) At 24 hpf and 28 hpf, lhx3 and lhx4 are broadly co-expressed in GFP+ and GFPC cells throughout the lateral half of ventral spinal cord. (I-L) Lateral views of spinal cord hemisegments adjacent to somites 8-12 co-labeled for Islet and either Lhx3 or Lhx4. Dorsal Islet+ cells are Rohon–Beard sensory neurons. Anti-Islet labels PMNs at 18 hpf, and PMNs plus a few SMNs at 24 hpf. (I) At 18 hpf, anti-Lhx3 labels all Islet+ PMNs as well as slightly more dorsal cells (arrowhead) in ventral neural tube. (J) At 18 hpf, anti-Lhx4 labels almost exclusively Islet+ PMNs. (K) By 24 hpf, anti-Lhx3 labels all Islet+ PMNs and SMNs and an increased number of cells in ventral neural tube. (L) Anti-Lhx4 also labels all Islet+ PMNs and SMNs in addition to other more dorsal cells (arrowhead) in ventral neural tube at 24 hpf. (M) Lateral view of 12 hpf Tg(olig2:GFP) embryo co-labeled with anti-Lhx3/4 and anti-phospho-Histone H3 (P-H3). (N) Dorsal view of 12 hpf Tg(olig2:GFP) embryo incubated with Edu from 11 hpf and labeled with Edu and anti-Lhx3/4. (O) Lateral view of 20 hpf Tg(vsx1:GFP) embryo co-labeled with anti-Lhx3/4 and anti-P-H3. (P) Summary of Lhx3- and Lhx4-expressing cells in the ventral spinal cord. (Q) Combinatorial expression of three transcription factors that uniquely label all four neuronal derivatives of the zebrafish pMN domain.
Lhx3 and Lhx4 prevent PMN acquisition of KA′ IN characteristics

The reduction of trunk motor projections in lhx3+/lhx4 MO-injected embryos led us to examine the phenotype of cells otherwise fated to become MNs. As the loss of Islet or Mnx results in hybrid PMNs with VeLD or CiD characteristics, respectively (Hutchinson and Eisen, 2006; Seredick et al., 2012), we hypothesized that PMNs in embryos lacking Lhx3 and Lhx4 would have characteristics of KA′ INs. To test this, we co-labeled embryos with antibodies against Islet and Gad and found that, in the absence of Lhx3 and Lhx4, many Islet+ ventral spinal cord cells co-expressed Gad (Fig. 5A,B) and GABA (data not shown). These cells also expressed chat (chata, choline O-acetyltransferase a – Zebrafish Model Organism Database), which encodes the synthetic enzyme for acetylcholine, their normal transmitter (Fig. 5C,D). These results provide evidence that, in the absence of Lhx3 and Lhx4, PMNs develop a hybrid identity in which they express both MN and IN characteristics.

To resolve the identity of MN-IN hybrids, we labeled pMN-derived cells by injecting UAS:EGFP:PAAX plasmid into s1020t embryos. This allowed us to visualize morphologies and proportions of MNs, VeLDs and KA′s. In addition to labeled MiPs, CaPs, VeLDs and KA′s seen in control embryos (Fig. 5E,F), in the absence of Lhx3 and Lhx4 we observed classes of aberrant axon projections. Notably, all aberrantly projecting neuron classes acquired ipsilaterally ascending axons, a hallmark of pMN-derived KA′s. There were two types of INs with axons that ascended through lateral spinal cord (ascending interneurons, aINs): most of these INs had a proximal axon that descended through the spinal cord before turning laterally and ascending through the lateral neuropil (Fig. 5G), although some aINs had axons that ascended through the spinal cord without a proximal descending segment (Fig. 5H). The laterally positioned somata of these neurons distinguished them from medially positioned KA′ somata. A second class of aberrant neurons had bifurcating spinal axons, one ascending and the other one descending (bIFs; Fig. 5I). A third class had MN-IN hybrid morphologies (MN-aIN; Fig. 5J). These cells had motor projections into the ventral myotome as well as ascending IN axons. The aberrant neurons with ascending axons were probably transformed MNs, as there were fewer MNs without a significant increase in MNs, VeLDs and KA′s seen in control embryos. These results provide evidence that, in the absence of Lhx3 and Lhx4, PMNs develop a hybrid identity in which they express both MN and IN characteristics.

and Lhx4 and suggest that, in zebrasfish, Islet1 expression in trunk MNs is regulated independently of Lhx3 and Lhx4. These data are consistent with our previous results showing that, in the absence of Islet1, prospective zebrafish trunk MNs retain Lhx3 expression but differentiate as INs (Hutchinson and Eisen, 2006).

To confirm the specificity of our MO phenotype, we performed a mosaic rescue experiment by injecting UAS:EGFP-P2A-Ihx3 plasmid into the s1020t enhancer trap line in which GAL4VP16 is expressed under olig2 control (Wyart et al., 2009). This allowed us to label cells overexpressing Lhx3 and to evaluate the proportion of labeled MNs. In control embryos, MNs comprised 55% of labeled neurons (Table 1), whereas in embryos injected with lhx3 and lhx4 MOs MNs comprised only 31% of labeled neurons. Co-expression of Lhx3 with EGFP restored the percentage of labeled MNs to 55%.

Lhx3 and Lhx4 are required for normal PMN axon morphology.

(A-D) Lateral views of 28 hpf embryos co-labeled with zn1 and anti-Syl2b. Control (A), lhx3 MO-injected (B) and lhx4 MO-injected (C) embryos have normal dorsal MiP axons (arrows) and ventral CaP axons (arrowheads). MiP, CaP, or MiP and CaP axons are often absent from lhx3+/lhx4 MO-injected embryos (D). Apparent variation in intraspinal labeling in B and C was a consequence of embryo orientation and does not reflect differences in IN axon labeling. (E) Quantification of MiP and CaP phenotypes in control and MO-injected embryos. (F,G) Lateral views of somites 8-12 of 72 hpf embryos labeled with anti-Alcama. Alcama labeling of SMN axons and somata was almost completely missing from embryos injected with lhx3+/lhx4 MOs. (H,I) Lateral views of 48 hpf s1020t;Tg(UAS-E1b:Kaede) embryos. Ventral nerves in lhx3+/lhx4 MO-injected embryos were thinner (arrows), yet exited through the ventral root (arrowhead); inset shows an optical cross-section. Scale bars: 20 μm.
(supplementary material Fig. S3), but far fewer KA’s expressed GABAergic properties (supplementary material Fig. S4). Because KA’s do not express lhx3 or lhx4, we hypothesized that these genes might affect KA’ differentiation cell non-autonomously. Previous studies showed that Notch promotes KA’ identity (Shin et al., 2007) and that PMNs express Notch ligands (Appel and Eisen, 1998; Hadden et al., 1998); thus, Lhx3 and Lhx4 might regulate expression or presentation of Notch ligands in PMNs to promote Gad expression in KA’s. We tested this idea by overexpressing the Notch intracellular domain (NICD) and by blocking Notch activity with DAPT. Notch overexpression resulted in supernumerary GABAergic KA’s, and DAPT treatment diminished the number of GABAergic KA’s (supplementary material Fig. S4), as observed in previous studies. Consistent with our hypothesis, overexpressing NICD in lhx3+lhx4 MO-injected embryos restored GABAergic KA’ numbers to wild-type levels. However, blocking Notch activity in lhx3+lhx4 MO-injected embryos enhanced reduction of GABAergic KA’s (supplementary material Fig. S4). Together, these results suggest that, although both Notch and Lhx3 and Lhx4 affect KA’ differentiation, they do so through parallel pathways.

### Lhx3 promotes CiD specification at the expense of KA’s

Our data suggest that Lhx3 and Lhx4 inhibit KA’ characteristics in PMNs; thus, we predicted that misexpression of lhx3 would inhibit KA’ formation. To test this hypothesis, we injected UAS: EGFP-P2A-lhx3 plasmid into s1020t embryos to drive Lhx3 expression in olig2-expressing cells (Wyart et al., 2009). In addition to the typical MNs, VeLDs and KA’s labeled in control embryos, we observed two classes of INs with aberrant morphologies. One class had an ipsilateral, descending axon that emerged from a ventromedial soma, a position characteristic of KA’s (dKA’s; Fig. 6A). The other class had an axon that emerged from a ventromedial soma and grew circumferentially before descending through the lateral neuropil. This class also had an ascending collateral that arose from the proximal portion of the main axon (CiD-KA’; Fig. 6B). This characteristic CiD axon morphology was not observed in control embryos, presumably because CiDs typically arise later than the time at which we assayed (Park et al., 2004). The aberrant INs with descending axons were probably transformed KA’s, as fewer KA’s formed without significant increases in the proportions of either MNs or VeLDs (Fig. 6C). These results demonstrate that Lhx3 is sufficient to inhibit KA’ fate and support the idea that Lhx3 specifies CiDs.

### Lhx3 and Lhx4 promote formation of late-born V2 INs

Vertebrate p2 progenitors express Lhx3 and produce immature V2 INs that differentiate into two distinct types: V2as maintain Lhx3 expression, express Delta and differentiate as Vsx2’ excitatory INs; V2bs extinguish Lhx3 expression and require Notch activity to differentiate as Tal1’ Gata3’ inhibitory INs (Batista et al., 2008; Del Barrio et al., 2007; Kimura et al., 2008; Lundfald et al., 2007; Peng et al., 2007). Lhx3 overexpression is sufficient to induce ectopic V2a formation in chick (Tanabe et al., 1998; Thaler et al., 2002), but whether Lhx3 and Lhx4 are necessary to promote V2 development remains untested.

To address this question, we knocked down Lhx3 and Lhx4 in Tg(vsx1:GFP) embryos and scored vsx2 and tal1 expression in GFP+ neurons. At 24 hpf, we observed no difference in the number of V2a and V2b INs (Fig. 7A-D, K). However, in slightly older embryos we noticed a reduced number of vsx2’ cells in the absence of Lhx3 and Lhx4 (data not shown). Thus, we examined the number of V2as and V2bs at 48 hpf, when many more are present, using a Tg(vsx2:GFP) line (Kimura et al., 2006) to score V2as, or labeling Tg(vsx1:GFP) embryos with gata3 to identify V2bs. Whereas the number of immature Tg(vsx1:GFP) V2 neurons at 48 hpf was unchanged (Fig. 7I, J, M), the number of neurons expressing differentiated V2a and V2b markers was significantly reduced (Fig. 7E-H, L) by the absence of Lhx3 and Lhx4. These results suggest that Lhx3 and Lhx4 are dispensable for the earliest-born V2 INs, but are required for differentiation of later-born V2a and V2b INs.

### DISCUSSION

Here, we have made three principal findings: first, Lhx3 and Lhx4 cooperate to inhibit a competing KA’ IN program within PMNs

Table 1. Lhx3 rescues the lhx3 lhx4 MO-induced motor axon phenotype

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<th>UAS-E1b:EGFP</th>
<th>lhx3 lhx4 MOs UAS-E1b:EGFP</th>
<th>lhx3 lhx4 MOs UAS-E1b:EGFP-P2A-lhx3</th>
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<td>Percentage of EGFP+ neurons with motor projections</td>
<td>54.6% (53/97 MNs, 15 embryos)</td>
<td>30.8% (25/81 MNs, 15 embryos)</td>
<td>55.3% (47/85 MNs, 17 embryos)</td>
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Fig. 5. Lhx3 and Lhx4 prevent PMNs from acquiring KA' IN characteristics. (A-D) Lateral views of 24 hpf embryos. (A,B) Islet and Gad are co-expressed in lhx3+/lhx4 MO-injected embryos (arrowheads). (C,D) MNs continue to express chat in lhx3+/lhx4 MO-injected embryos. (E-I) Mosasically labeled PMN derivatives in 28-32 hpf s1020 embryos. (E,F) Injection of UAS:EGFP:CaAX DNA labels PMNs, including both CaP (E) and MiP (F), as well as KA' (E) and VeLD (F) in control embryos. (G-I) Mosasically labeled PMN derivatives in 28-32 hpf s1020 embryos injected with lhx3 and lhx4 MOs. (G) Aberrant neuron with an axon that initially projects caudally before turning laterally and ascending the spinal cord (aIN; n=9). (H) Aberrant neuron with an axon that ascends without a proximal descending segment. (I) Aberrant neuron with bifurcating axons, one ascending and one descending (bIN; n=4). (J) Dye-labeled MN-IN hybrid neuron with both ascending IN and peripheral MN axons in embryo injected with lhx3+/lhx4 MOs (n=2); dye dorsal to the labeled neuron is from an adjacent cell killed during labeling. (K) Relative proportions of labeled pMN-derived neurons. Three new neuron classes appear in lhx3+/lhx4 MO-injected embryos; only MNs are under-represented relative to control embryos (P=2.2×10^-5). The percentage of labeled cells is listed for each condition. Scale bars: 20 μm.

Distinct transcription factor families suppress specific IN fates during PMN development

Lineage analysis revealed that PMNs and KA’s both derive from the pMN domain and can be siblings (Park et al., 2004). We determined that Lhx3 and Lhx4 act together to prevent PMNs from acquiring KA’ features, including transmitter expression and axonal projection.

Zebrafish MNs have an inherent propensity to acquire characteristics of pMN-derived INs. Knockdown of Islet1 results in excess VeLDs and eliminates motor projections (Hutchinson and Eisen, 2006), and knockdown of Mnx1 causes MiP PMNs to acquire CiD features (Seredick et al., 2012). These results reveal the regulatory logic by which MN and pMN-derived IN fates are segregated (Fig. 8B): one family of transcription factors blocks each of the alternative pMN-derived IN programs in post-mitotic MNs. This mechanism efficiently resolves identities of distinct neurons, the fates of which diverge only after the final progenitor division.

Notably, knockdown of Lhx3 and Lhx4 does not result in a complete MN-to-KA’ fate conversion. Resulting cells adopt a hybrid phenotype, expressing PMN markers while also acquiring KA’ characteristics, and in some cases even adopting a hybrid morphology. Formation of MN-IN hybrids is a consistent phenotype following knockdown of many zebrafish transcription factors, including those described above, as well as Nkx6.1 and Nkx6.2 (Hutchinson et al., 2007) and the Met receptor (Tallafuss and Eisen, 2006), and knockdown of Mnxs causes MiP PMNs to acquire CiD characteristics (Lee et al., 2008). These results suggest that none of these genes, with the possible exception of Islet, act as true master regulators of MN fate. Rather, each gene or gene family refines identity by suppressing the inherent potential of MNs to initiate genetic programs normally restricted to INs. Similar observations have been made in mouse. In Runx1 mutant mice, a subset of brachial level MNs co-express Islet1 and the IN marker Pax2 (Stifani et al., 2008). In Nkx6.1 Nkx6.2 double mutants, Islet1 and the IN marker Evx1 are transiently co-expressed (Vallstedt et al., 2001). In Mnx1 knockout mice, MNs transiently co-express the V2a marker, Vsx2 (aka Chx10) (Arber et al., 1999; Thaler et al., 1999). Interestingly, Vsx2 binds to an Mnx1 enhancer in vivo and blocks expression of an MN reporter in vitro (Lee et al., 2008), suggesting that a reciprocal strategy of
SAC MNs, the only class of spinal MN that normally lacks Lhx3 and Lhx4, MNs develop morphological and biochemical characteristics of KA INs. Forcing expression of Lhx3 in pMN-derived neurons results in fewer KA INs, and late p2 progenitors labeled by GFP is unaffected in lhx3+/+ MO-injected embryos. Islet was included to exclude MNs that weakly express GFP (Reimer et al., 2013). (K) Quantification of early-born V2a and V2b INs. Islet was included to exclude MNs that weakly express GFP. (Reimer et al., 2013). (L) Quantification of early-born V2a and V2b INs. Islet was included to exclude MNs that weakly express GFP. (Reimer et al., 2013). (M) Quantification of V2 INs. In control embryos, some weakly GFP+ cells co-express Islet; these cells are MNs and were not included in p2 progenitor and V2 IN counts. Scale bars: 20 μm.

Lhx3 and Lhx4 promote differentiation of late-born V2a and V2b INs. (A-D) Lateral views of 24 hpf Tg(vsx1:GFP) embryos. (A,B,K) The number of early-born V2a INs labeled with vsx2 is unchanged in lhx3+/lhx4 MO-injected embryos. (C,D,K) The number of early-born V2b INs labeled with taft is unchanged in lhx3+/lhx4 MO-injected embryos. (E-H) Lateral views of 2 dpf Tg(vsx1:GFP) embryos. (E,F) V2as labeled by GFP in 2 dpf Tg(vsx2:EGFP) embryos are reduced in lhx3+/lhx4 MO-injected embryos. (G,H) Single optical section; the number of V2bs co-labeled by gata3 and GFP in Tg(vsx1:GFP) embryos is reduced in lhx3+/lhx4 MO-injected embryos. (I,J) Cross-section through mid-trunk of 2 dpf Tg(vsx1:GFP) embryos. The number of V2s and late p2 progenitors labeled by GFP is unaffected in lhx3+/lhx4 MO-injected embryos. Islet was included to exclude MNs that weakly express GFP (Reimer et al., 2013). (K) Quantification of early-born V2a and V2b INs. Islet was included to exclude MNs that weakly express GFP. (Reimer et al., 2013). (L) Quantification of early-born V2a and V2b INs. Islet was included to exclude MNs that weakly express GFP. (Reimer et al., 2013). (M) Quantification of V2 INs. In control embryos, some weakly GFP+ cells co-express Islet; these cells are MNs and were not included in p2 progenitor and V2 IN counts. Scale bars: 20 μm.

Fig. 7. Lhx3 and Lhx4 promote differentiation of late-born V2a and V2b INs. (A-D) Lateral views of 24 hpf Tg(vsx1:GFP) embryos. (A,B,K) The number of early-born V2a INs labeled with vsx2 is unchanged in lhx3+/lhx4 MO-injected embryos. (C,D,K) The number of early-born V2b INs labeled with taft is unchanged in lhx3+/lhx4 MO-injected embryos. (E-H) Lateral views of 2 dpf Tg(vsx1:GFP) embryos. (E,F) V2as labeled by GFP in 2 dpf Tg(vsx2:EGFP) embryos are reduced in lhx3+/lhx4 MO-injected embryos. (G,H) Single optical section; the number of V2bs co-labeled by gata3 and GFP in Tg(vsx1:GFP) embryos is reduced in lhx3+/lhx4 MO-injected embryos. (I,J) Cross-section through mid-trunk of 2 dpf Tg(vsx1:GFP) embryos. The number of V2s and late p2 progenitors labeled by GFP is unaffected in lhx3+/lhx4 MO-injected embryos. Islet was included to exclude MNs that weakly express GFP. (Reimer et al., 2013). (K) Quantification of early-born V2a and V2b INs. Islet was included to exclude MNs that weakly express GFP. (Reimer et al., 2013). (L) Quantification of early-born V2a and V2b INs. Islet was included to exclude MNs that weakly express GFP. (Reimer et al., 2013). (M) Quantification of V2 INs. In control embryos, some weakly GFP+ cells co-express Islet; these cells are MNs and were not included in p2 progenitor and V2 IN counts. Scale bars: 20 μm.

Lhx3 segregates alternative fates of pMN-derived INs
Very little is known regarding fate segregation among pMN-derived INs. Our forced-expression experiments reveal that Lhx3 acts, at least in part, as a molecular switch to segregate CiD from KA INs. Our results and others (Appel et al., 1995; Batista et al., 2008) show that by 18 hpf Lhx3 is expressed in cells in addition to MNs in ventral neural tube; some of these cells are pMN-derived INs. This expression is maintained until at least 28 hpf. CiDs are derived from the pMN domain after 15 hpf, following PMN formation (Park et al., 2004). Together, these data indicate that Lhx3 is in the right place at the right time to promote CiD and inhibit KA INs. This idea is further corroborated by the differential ability of PMN derivatives, Lhx3 and Lhx4 segregate CiD from KA INs with descending axons. (B) Within MNs, Lhx3 and Lhx4 suppress acquisition of CiD characteristics. Mnx family members suppress acquisition of CiD characteristics by MIP MNs and Islet1 suppresses acquisition of VeLD characteristics. (C) Among pMN domain neuronal derivatives, Lhx3 and Lhx4 segregate CiD from KA INs, and Islets segregate MN from VeLD fates. We hypothesize that Mnx contributes to segregation of VeLD from CiD fate, based on its role in blocking MIP from acquiring CiD characteristics.

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subtypes to acquire CiD features in the absence of Mnxs (Seredick et al., 2012) as a consequence of subtle but important differences in temporal expression of Islet transcription factors. CaPs express either Islet1 or Islet2a continuously. Thus, in the absence of Mnxs, they maintain expression of Islets and Lhx5 and retain MN characteristics. By comparison, MiPs transiently downregulate Islet (Appel et al., 1995; Hutchinson et al., 2007; Hutchinson and Eisen, 2006). In the absence of Mnxs, MiPs temporarily express only Lhx during the Islet-negative period, the combinatorial code for CiDs, and can acquire a hybrid CiD phenotype.

We were surprised to find that Lhx3 and Lhx4 regulate expression of GABAergic properties in KA’s, although KA’s do not express Lhx3. This cell non-autonomous effect is not mediated via Notch signaling, the most obvious possibility (Shin et al., 2007). There are many other possible pathways, including electrical signaling through gap junctions. Most neurons within the developing zebrafish ventral spinal cord are electrically coupled through the first day of development (Saint-Amant and Drapeau, 2001; Wyart et al., 2004), and electrical activity has been shown to promote acquisition of transmitter phenotype (Spitzer, 2012). Lhx MO-injected embryos do not move properly at early developmental stages, perhaps as a result of disrupted activity of Lhx3 MN and/or INs. It would be interesting to investigate this possibility in future experiments.

We show that all Lhx3+ Lhx4+ INs in the zebrafish spinal cord have descending axons, and that the intraspinal portion of the vast majority of MNs descend to the nearest adjacent ventral root. Even when forced expression of Lhx3 fails to drive CiD axon morphology in KA’s, it is often sufficient to redirect KA’axons to extend into the caudal spinal cord. In mouse, forced expression of Lhx3 in Mnx1+ MNs re-directs many axons into axial muscle at the expense of projections to normal targets in the limb, sympathetic ganglia and intercostal muscles (Sharma et al., 2000). One apparent target of Lhx3 overexpression is Fgffr1 (Shirasaki et al., 2006). Attraction to FgfR was proposed to set the axon trajectory of the Lhx3+ medial motor column MNs that project to dermamyotome. Clearly, other signals contribute to medial motor column MN guidance at this choice point, as relatively few motor axons are misrouted in Fgffr1 conditional mutants. It is intriguing to consider that an Fgf gradient established in the neural tube (Diez del Corral et al., 2003) might contribute to the descending pathways taken by these Lhx3+ Lhx4+ neurons.

Lhx3 and Lhx4 act together to specify late-developing V2 INs

Previous work in chick demonstrated sufficiency of Lhx3 in promoting expression of Vsx2, a V2a fate marker (Tanabe et al., 1998; Thaler et al., 2002). Our observations reveal that Lhx3 and Lhx4 are necessary for differentiation of both V2a and V2b INs, without affecting the number of immature V2 INs formed. Curiously, some V2a and V2b INs continue to be generated in Lhx3- and Lhx4-deficient embryos. In zebrafish, the first-born V2a and V2b pairs form independently of Lhx3 and Lhx4, as do a significant fraction of the V2a and V2b INs generated by 48 hpf. Although we cannot rule out that incomplete knockdown contributes to persistence of V2a and V2b INs at 48 hpf, there might be parallel or redundant mechanisms that cooperate with Lhx3 and Lhx4 to promote V2 IN specification. One attractive candidate is Bhlhe22 (previously Bhlhb5), an Olig-related transcription factor, the expression of which in V2 progenitors and V2a INs overlaps with Lhx3 (Skaggs et al., 2011). Reminiscent of our results after Lhx3 and Lhx4 knockdown, loss of Bhlhe22 function in chick reduced the number of V2a and V2b INs.

Interestingly, even after Lhx3 and Lhx4 knockdown, zebrafish embryos remain touch-responsive; however, they cannot sustain swimming through 4 dpf (data not shown). This is consistent with preservation of early-born V2a INs driving the highest-frequency movements during the initial phase of the escape response, and reduced numbers of later-born V2a INs driving lower-frequency movements during subsequent phases of the escape response (McLean and Fetcho, 2009). These observations suggest the exciting possibility that Lhx3 and Lhx4 coordinate development of subclasses of V2a INs underlying the circuitry responsible for setting locomotor speed, a question that remains to be addressed in future studies.

MATERIALS AND METHODS

Zebrafish lines

Wild-type, Tg(mpr1a:GFP)jo12 (Sato-Maeda et al., 2008), Tg(olfig2:EGFP)jo12 (Shin et al., 2003), Tg(gvsx1:GFPPm15) (Kimura et al., 2008), Tg(gvsx2:EGFP)jo12 (Kimura et al., 2006) and Tg(o.6-hsp70:GAL4VP16) (hereafter referred to as s10290), Tg(UAS-E1b:Kaede)jo1000 (Wyart et al., 2009) zebrafish (Danio rerio) embryos were collected from natural crosses and staged by hours post fertilization at 28.5°C (hpf) and gross morphology (Kimmel et al., 1995). All experiments were carried out in accordance with animal welfare laws, guidelines and policies and were approved by the University of Oregon Institutional Animal Care and Use Committee.

Isolation of Lhx4

We isolated a full-length zebrafish lhx4 transcript (1171 base pairs; 390 amino acids) from 24 hpf zebrafish cDNA using the following primers: forward 5’-CACACGGCGGAAAAGACTCAG-3’; reverse 5’-TTTGCGCACCCGCAACTCG-3’. In phylogenetic analyses the protein encoded by this gene clusters with other Lhx4 proteins, not with Lhx3 proteins, indicating that it is an Lhx4 homolog (data not shown). Like other Lhx3 and Lhx4 proteins, zebrafish Lhx4 has two very highly conserved LIM domains as well as a highly conserved homeodomain.

Immunohistochemistry, RNA in situ hybridization and Edu labeling

Embryos were fixed and processed for in situ hybridization or antibody staining as previously described (Seredick et al., 2012). RNA probes include: islet2a and lhx3 (Appel et al., 1995); chat (Tallafuss and Eisen, 2008); tal1 and gata3 (Batista et al., 2008); vsx2 (Kimura et al., 2006); and lhx4. Primary antibodies include: mouse anti-Alcama (ZIRC, zn5; 1:4000); rabbit anti-GABA (Sigma, A2052; 1:1000); rabbit anti-Gad (Abcam, ab11070; 1:500); mouse anti-GFP (Clontech, 632381; 1:1000); or LifeTechnologies, A11120; 1:1000); rabbit anti-GFAP (LifeTechnologies, A11122; 1:1000); mouse anti-phospho-Histone H3 (Abcam, ab14955; 1:1000); mouse anti-Islet (DSHB, 39.4D5; 1:1000); mouse anti-Syt2b (ZIRC, formerly zm1; 1:1000); mouse zn1 (ZIRC; 1:200).

To label proliferating cells, embryos were treated with 10 mM Edu solution (Click-iT Edu Alexa Fluor 555; Invitrogen, C10338) in embryo medium plus 15% DMSO for 20 min on ice. After rinsing into embryo medium, embryos recovered for 40 min and were fixed and processed by immunohistochemistry. Incorporated Edu was detected according to manufacturer’s instructions.

Antibodies against Lhx3 and Lhx4 were generated by immunizing rabbits with truncated proteins corresponding to amino acids 237-397 of Lhx3 and 241-389 of Lhx4. We generated three antibodies: anti-Lhx3 antibody labels nuclei in ventral spinal cord and pituitary, the same cell population labeled by lhx3 RNA probe. Anti-Lhx4 antibody labels fewer nuclei in ventral spinal cord than anti-Lhx3, as well as the pituitary and a dorsal IN population in the head, the same cell populations labeled by lhx4 RNA probe. A third antibody labels many nuclei in the ventral spinal cord as anti-Lhx3, as well as the pituitary and the dorsal INs in the head labeled by anti-Lhx4; we named this antibody anti-Lhx3/4.
Morpholino injections
One-to-two cell stage embryos were injected with 2.5 nl of translation-blocking MOs (GeneTools) targeting lhx3 MO, 5′-GGTTTACCACTACCCCAAGGAAGATGGGA-3′ (125 μM), and/or lhx4 MO, 5′-GGCACGCAGCCCGCTTTACG-3′ (350 μM). lhx3 and lhx4 MO injection specifically eliminated expression of their respective proteins (supplementary material Fig. S2). MO specificity was confirmed using mis-match control MOs [lhx3 MO 5-mis (5′-GGTTTAAAGACCTGGGACCTTTCA-3′) and lhx4 MO 5-mis (5′-GGACCCACCCCGGA-ACTTCCATGT-3′)] and rescue of the MN phenotype by mosaic overexpression of lhx3. MO-injected embryos were morphologically normal and lacked elevated cell death assessed by Acridine Orange staining.

Mosaic labeling and Lhx3 overexpression within pMN-derived cells

Statistical analysis
Comparisons of means between groups were performed using two-sample unequal variance t-tests. An exact binomial test was used to assess changes in proportions of labeled MNs. Multiple comparisons were performed using a one-way ANOVA, followed by a post hoc Tukey-Kramer test. Analyses were performed using JMP 10 and Microsoft Excel. All data are reported as means±s.d.

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Competing interests
The authors declare no competing financial interests.

Author contributions
S.S., S.A.H. and J.S.E. designed the study and wrote the draft manuscript, with contributions from L.V.R. and J.C.T. S.S., S.A.H., L.V.R., J.C.T. and J.S.E. all performed experiments for the study.

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Supplementary material
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Del Barrio, M. G., Taveira-Marques, R., Murayama, Y., Yuk, D.-I., Li, S., Wines-Sassensfe In the lhx3 MO-injected embryos with a Zeiss Pascal confocal microscope. Adobe Photoshop was used to adjust image brightness and contrast.


