

# Nkx6 proteins specify one zebrafish primary motoneuron subtype by regulating late *islet1* expression

Sarah A. Hutchinson\*, Sarah E. Cheesman\*, Laura A. Hale, Jason Q. Boone and Judith S. Eisen†

The ability of animals to carry out their normal behavioral repertoires requires exquisitely precise matching between specific motoneuron subtypes and the muscles they innervate. However, the molecular mechanisms that regulate motoneuron subtype specification remain unclear. Here, we use individually identified zebrafish primary motoneurons to describe a novel role for Nkx6 and *Islet1* proteins in the specification of vertebrate motoneuron subtypes. We show that zebrafish primary motoneurons express two related Nkx6 transcription factors. In the absence of both Nkx6 proteins, the CaP motoneuron subtype develops normally, whereas the MiP motoneuron subtype develops a more interneuron-like morphology. In the absence of Nkx6 function, MiPs exhibit normal early expression of *islet1*, which is required for motoneuron formation; however, they fail to maintain *islet1* expression. Misexpression of *islet1* RNA can compensate for loss of Nkx6 function, providing evidence that *Islet1* acts downstream of Nkx6. We suggest that Nkx6 proteins regulate MiP development at least in part by maintaining the *islet1* expression that is required both to promote the MiP subtype and to suppress interneuron development.

**KEY WORDS:** Zebrafish, Motoneuron, *Islet1*, Nkx6, Interneuron

## INTRODUCTION

Motoneurons of the vertebrate central nervous system have precise subtype identities characterized by soma location, axon trajectory, target muscle innervation and combinatorial transcription factor expression (Lewis and Eisen, 2003; Shirasaki and Pfaff, 2002). Specification of motoneuron subtypes has been well-studied in mouse and chick embryos (Jurata et al., 2000; Pfaff and Kintner, 1998; Tsalik et al., 2003); however, nothing is currently known about the molecular mechanisms that establish very fine-grained motoneuron patterning, such as the segmentally reiterated, individually identified primary motoneurons (PMNs) of the zebrafish spinal cord.

We focus our attention on two PMN subtypes, CaP and MiP, that are present in a segmentally repeated, alternating pattern (Lewis and Eisen, 2003). CaPs project axons that innervate ventral muscle and express the LIM homeobox gene *islet2*, whereas MiPs project axons that innervate dorsal muscle and express the LIM homeobox gene *islet1* (Lewis and Eisen, 2003). The expression of *islet* genes is dynamic in these cells (Fig. 1A); both CaP and MiP express *islet1* around the time they exit the cell cycle (Appel et al., 1995; Inoue et al., 1994; Korzh et al., 1993; Tokumoto et al., 1995). MiPs then transiently downregulate *islet1* expression and reinitiate it prior to axogenesis (Appel et al., 1995). Thus, MiPs express *islet1* in two distinct phases – an early phase and a late phase. In contrast to MiPs, CaPs initiate expression of *islet2* while they still express *islet1*, and then downregulate expression of *islet1* (Appel et al., 1995; Inoue et al., 1994; Korzh et al., 1993; Tokumoto et al., 1995). Thus, CaPs have an early phase of *islet1* expression and a later phase of *islet2* expression. The end result of these dynamic changes in *islet* gene expression is that by the time of axon extension, MiPs express exclusively *islet1* and CaPs express exclusively *islet2*.

Transplantation of single CaPs and MiPs revealed that their subtypes are initially labile and responsive to environmental signals, but become committed shortly before axogenesis (Eisen, 1991), around the time that the alternating pattern of *islet2* and *islet1* expression is established (Appel et al., 1995). Thus, it was surprising to learn that either *Islet1* or *Islet2* protein is sufficient to specify both CaP and MiP subtypes, suggesting that the differences between these PMNs might be regulated by factors upstream of the *islet* genes (Hutchinson and Eisen, 2006). Homeodomain transcription factors expressed in the motoneuron progenitor (pMN) domain, such as Nkx6.1 (Cheesman et al., 2004), are good candidates for performing this function.

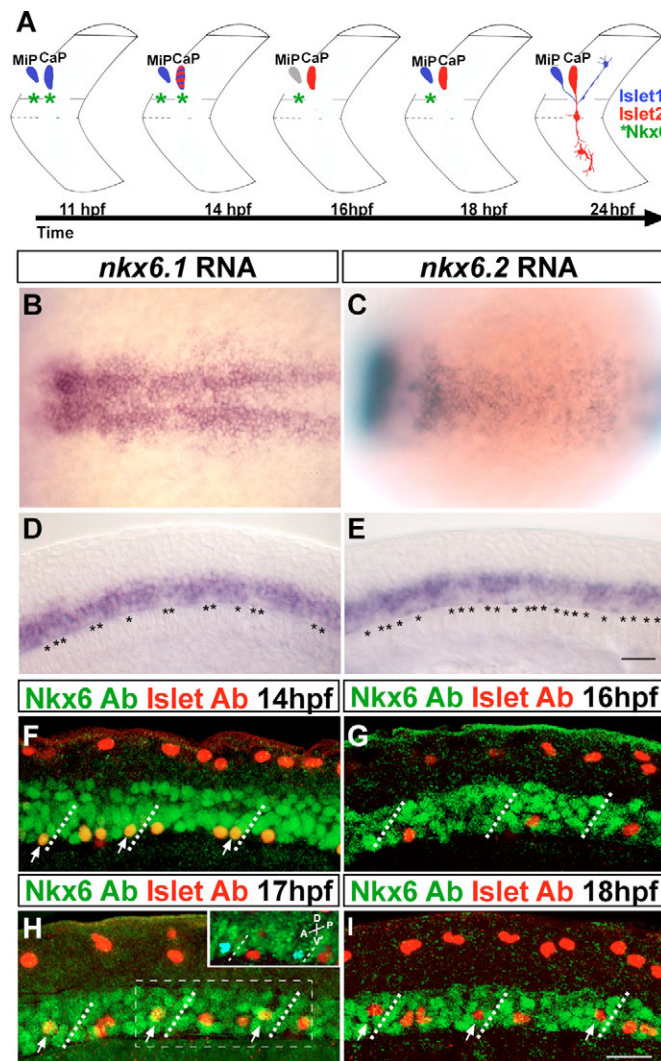
The Nkx6 transcription factor family is important in formation of mouse spinal motoneurons. One family member, *Nkx6.1*, is expressed in the spinal cord pMN domain and is required for formation of a proportion of all spinal motoneuron subtypes (Sander et al., 2000). Another family member, *Nkx6.2*, is expressed dorsal to the pMN domain and is negatively regulated by Nkx6.1. In the absence of Nkx6.1 function, *Nkx6.2* expression spreads ventrally into the pMN domain and Nkx6.2 partially substitutes for Nkx6.1 during motoneuron formation (Vallstedt et al., 2001). Mice deficient in both Nkx6.1 and Nkx6.2 lose nearly all their spinal motoneurons (Vallstedt et al., 2001). As in mouse, zebrafish Nkx6.1 is expressed in the pMN domain and is also important for formation of some zebrafish motoneurons (Cheesman et al., 2004). Nkx6.1-deficient zebrafish lack secondary motoneurons (SMNs), a type of spinal motoneuron that develops later than PMNs (Myers, 1985), but have normal PMNs, raising the possibility that additional Nkx6 proteins might regulate zebrafish PMN formation.

Here, we provide evidence for a novel role of Nkx6 proteins in motoneuron subtype specification. We show that zebrafish have at least three *nkx6* genes, two of which are expressed in the pMN domain and transiently in postmitotic PMNs. Nkx6 proteins are not required for PMN formation because early *islet1* expression is normal in embryos lacking Nkx6 proteins. In Nkx6-deficient embryos, CaPs have normal *islet2* expression and project normal ventral axons out of the spinal cord; however, MiPs fail to initiate the second phase of *islet1* expression and do not form their subtype-

Institute of Neuroscience, 1254 University of Oregon, Eugene OR 97403, USA.

\*These authors contributed equally to this work

†Author for correspondence (e-mail: eisen@uoneuro.uoregon.edu)



specific, dorsal peripheral axons, instead adopting a more interneuron-like morphology by projecting an axon within the spinal cord. We suggest that Nkx6 proteins control MiP subtype specification at least in part by regulating the second phase of *islet1* expression and that this late expression of *islet1* is required to promote the MiP subtype and to suppress interneuron development.

## MATERIALS AND METHODS

### Animals

Wild-type (AB) and *Tg(islet:GFP)* (Higashijima et al., 2000) embryos were reared and staged by hours post-fertilization at 28.5°C (hpf), and gross morphology (Kimmel et al., 1995).

### Cloning

A search of the zebrafish genome revealed an *nkx6.2* gene highly similar to zebrafish *nkx6.1* (Cheesman et al., 2004). A forward primer to an *nkx6.2*-specific region (5'-CGGCTCAAGGCTCATTC-3') and a reverse homeobox primer (5'-CCATTAGTTCTTCTGTTCTG-3') isolated a full-length cDNA clone from a 14- to 19-hpf library (Appel and Eisen, 1998). These primers did not amplify control *nkx6.1* cDNA. The isolated clone contains an open reading frame and UTR sequences. The DNA sequence predicts a 279 amino acid protein containing both a homeodomain and an NK decapeptide, which defines the nkx gene family. The GenBank accession number for the complete zebrafish *nkx6.2* mRNA is DQ416765. We isolated a fragment of *nkx6.3* from first-strand cDNA (forward primer, 5'-AGTCCAACATCTCAGGATCC-3'; reverse primer,

## Fig. 1. Two Nkx6 proteins are dynamically expressed in PMNs.

(A) Schematic of Islet and Nkx6 protein dynamics during PMN subtype specification at the 8- to 12-somite level. At 11 hpf, MiP and CaP both express Islet1 (blue) and Nkx6 (green asterisks). At 14 hpf, CaP and MiP maintain Nkx6 and Islet1 expression and CaP has initiated expression of Islet2 (red). At 16 hpf, Islet1 is downregulated in MiP (gray); CaP has downregulated expression of Nkx6 and Islet1, but continues to express Islet2. By 18 hpf, MiP has reinitiated expression of Islet1. At 24 hpf, neither MiP nor CaP expresses Nkx6; MiP still expresses Islet1, and CaP still expresses Islet2. (B,C) Dorsal views of 10-hpf embryos. (B) *nkx6.1* mRNA is expressed in many medial neuroectodermal cells in the pMN domain. (C) *nkx6.2* mRNA is expressed in the same domain as *nkx6.1*. (D,E) Lateral views of 18-hpf embryos showing that (D) *nkx6.1* and (E) *nkx6.2* mRNA is expressed in the ventral spinal cord; however, some of the ventral-most cells are *nkx6*-negative (asterisks). (F-I) Lateral views of Islet and Nkx6 co-expression. MiP and CaP were identified by soma position relative to somite boundaries (dotted lines). (F) Single optical slice showing that all ventrally located PMNs express Islet and Nkx6 proteins at 14 hpf, whereas dorsally located Rohon-Beard neurons express only Islet. (G-I) Projected z-stacks showing localization of Nkx6 and Islet Abs in the ventral neural tube. (G) By 16 hpf, CaPs no longer express Nkx6. (H) At 17 hpf, MiPs (arrows) express Nkx6, whereas CaPs are Nkx6-negative. Inset is a 3D rendering of the boxed area; cells co-expressing Nkx6 and Islet (arrows in H) were identified by the Velocity software program (see Materials and methods) and are pseudo-colored blue. The slight differences between the boxed area in H and the inset are due to the differences in angle of visualization: H is a direct lateral view, whereas the inset is rotated (see axes in inset). (I) By 19 hpf, MiPs (arrows) have also downregulated Nkx6. Scale bars: 50  $\mu$ m in B-E; 20  $\mu$ m in F-I.

5'-TCCAATCATACCCTCCATC-3') and based on this clone (GenBank accession DQ415639) and genomic clones, compiled a putative full-length sequence.

### In situ RNA hybridization

Zebrafish RNA in situ hybridization was performed as described previously (Appel and Eisen, 1998). *nkx6.1*, *islet1* and *islet2* mRNA probes were described previously (Appel et al., 1995; Cheesman et al., 2004), as were *pax2a* (Thaeron et al., 2000), *evx1* (Thaeron et al., 2000), *eng1b* (Higashijima et al., 2004) and *chx10* (Kimura et al., 2006). The *nkx6.2* mRNA probe was made from full-length *nkx6.2* DNA. *nkx6.3* expression was determined using a probe against a portion of the *nkx6.3* gene that excluded the homeodomain. In the *nkx6.3* anti-GFP double-label experiment, the RNA in situ hybridization was performed first, followed by antibody detection.

### Immunohistochemistry

The following primary antibodies (Abs) were used: polyclonal rabbit anti-Nkx6.1 (1:1200; gift of O. Madsen, Hagedorn Research Institute, Gentofte, Denmark), monoclonal mouse anti-Islet [1:200; antibody recognizes both Islet1 and Islet2 proteins (Korzhan et al., 1993); 39.4D5, Developmental Studies Hybridoma Bank], monoclonal mouse zn1 (Trevarrow et al., 1990), monoclonal mouse znp1 (Trevarrow et al., 1990), monoclonal anti-GFP (1:200; Clontech, JL-8.), and polyclonal rabbit anti-GABA (1:1000; Sigma). Secondary antibodies from Molecular Probes were used: goat anti-mouse Alexa-488 (1:1000) and goat anti-rabbit Alexa-568 (1:1000). One secondary antibody from Jackson Labs was also used: goat anti-rabbit Cy5 (1:200). Embryos were fixed for 3.5-4 hours in 4% paraformaldehyde (PFA) and 1 $\times$ Fix Buffer (Westerfield, 2000) at 4°C, blocked in 1 $\times$ PBS/5% NGS/4 mg/ml BSA/0.5% Triton X-100 for 1 hour at room temperature, and incubated in primary antibody (diluted in blocking solution) overnight at 4°C. Embryos were then washed at room temperature for 1.5 hours in PBS containing 0.1% Tween 20, incubated in secondary antibody (diluted in blocking solution) for 4 hours at room temperature, and then washed for 1.5 hours at room temperature in PBS containing 0.1% Tween 20.

### Morpholino injections

The Nkx6.2 conceptual amino acid translation revealed two potential start methionines; we do not know if one or both of them are functional start sites. As they are close together, GeneTools (Corvallis, Oregon) designed a translation-blocking MO just upstream of the first methionine (5'-GGTGC GCCGGAGCCACAGGACAAAC-3') on the premise that it would interfere with initiation of translation at either start site. We also utilized a splice-blocking MO (5'-CGCGAAAAC TACCCGCACAGGGA-3') that began at position 386 of the *nkx6.2* open reading frame and ended in the first intron, blocking the splice-donor site of exon 1. Both *nkx6.2* MOs produced similar phenotypes. Several nanoliters of 5 mg/ml *nkx6.2* MO, diluted in 0.2 M KCl, were injected with Phenol Red into the yolk cell of two-cell-stage embryos; for double MO injections, we added 2.5 mg/ml *nkx6.1* MO (see Cheesman et al., 2004). All our MO injections worked efficiently, with 84.3% (27/32) of *nkx6.1* MO-injected, 90.9% (30/33) of *nkx6.2* MO-injected, and 76.1% (35/46) of *nkx6* double MO-injected embryos exhibiting a MiP axon phenotype.

### Microscopy

Images of zebrafish embryos were captured on a Zeiss Axioplan equipped with a digital camera, on a Bio-Rad Radiance, or on a Zeiss Pascal confocal microscope. In a few cases, an Olympus IX81 microscope with an FV300 confocal was used to capture images. The brightness and contrast of images was adjusted in Photoshop (Adobe).

### Motoneuron observation and quantification

All observations of CaP and MiP motoneurons were made in the spinal cord adjacent to somites 8-12. To quantify MiP axons, we counted the number of MiP axons in 28-hpf control, *nkx6* single and double MO-injected embryos, as well as *nkx6* MOs and *islet1* RNA co-injected embryos labeled with zn1 and *znp1* Abs. Axons were scored as MiPs if they projected posterior and dorsal to the zn1-labeled CaP soma. The percentage of MiP axons remaining in experimental embryos was calculated relative to controls. The percentage of segments with cells labeled with *islet1* RNA in the MiP position was calculated from cells counted in segments 8-12 of 18-hpf control, *nkx6.1*, *nkx6.2* and *nkx6* double MO-injected embryos. For experiments in which

individual MiPs were dye-labeled, we recognized these cells by their position and used protocols described by Eisen et al. (Eisen et al., 1989). GABA-positive interneurons were counted as described by Hutchinson and Eisen (Hutchinson and Eisen, 2006). The number of cells labeled with *chx10* or *eng1b* riboprobes was counted in segments 1-11 on each side of 24-hpf control and *nkx6* double MO-injected embryos. The average number of positive cells per segment in experimental embryos was compared with that in control embryos.

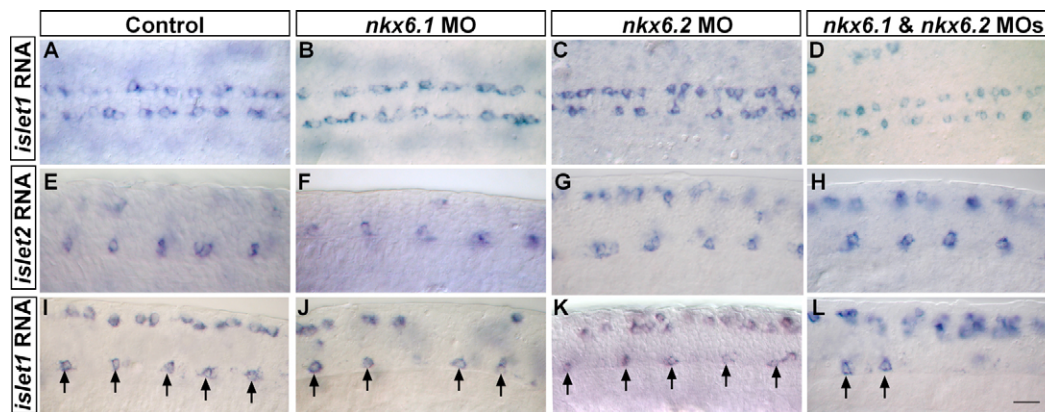
### 3D analysis of confocal images

Velocity software was used to generate 3D images of individually labeled MiPs. A velocity classifier based on intensity was used to generate a threshold for red and green images of Nkx6 and Islet Abs, followed by Velocity image arithmetic to identify co-labeled cells. The co-labeled cells were then pseudo-colored blue.

## RESULTS

### Two *nkx6* genes are dynamically expressed in zebrafish primary motoneurons

Studies implicating two Nkx6 homologs in mouse motoneuron formation (Vallstedt et al., 2001) and showing that zebrafish Nkx6.1 is unnecessary for PMN formation (Cheesman et al., 2004) raised the possibility of additional *nkx6* genes in zebrafish. We isolated zebrafish *nkx6.2* and *nkx6.3* based on publicly available genomic DNA sequences. Phylogenetic analysis indicated that zebrafish *nkx6* genes are most closely related to other vertebrate *nkx6* genes, distinct from *nkx2* family members (see Fig. S1A in the supplementary material). Alignment of the three zebrafish Nkx6 predicted proteins revealed that Nkx6.1 and Nkx6.2 are more similar to each other than to Nkx6.3 (see Fig. S1B in the supplementary material). Similar to mouse Nkx6.3 (Alanentalo et al., 2006), zebrafish *nkx6.3* expression is restricted to a group of



**Fig. 2. Nkx6 proteins regulate late *islet1* expression.** (A-D) Dorsal views at 11-12 hpf. (A) *islet1* mRNA expression in PMNs of control embryos. *islet1* mRNA expression is unaffected by *nkx6.1* (B), *nkx6.2* (C) or double *nkx6* (D) MO-injection. (E-L) Lateral views of axial level 8-12 at 18 hpf. (E-H) *islet2* mRNA expression is normal in control and *nkx6* MO-injected embryos. (I-L) *islet1* mRNA expression is confined to MiPs (arrows). In *nkx6.1* MO-injected embryos, there are fewer *islet1*-positive cells (J; *islet1*-positive cells present in 74.4% of segments;  $n=133$  segments in 14 embryos) as compared with controls (I; *islet1*-positive cells present in 82.4% of segments;  $n=97$  segments in 12 embryos). The faint cell in J in the segment lacking an arrow is on the other side of the embryo; as far as we can tell the distribution of segments lacking *islet1*-positive MiPs is random and not correlated between the two sides of the embryo. (K) *nkx6.2* MO-injected embryos also had a decrease in *islet1* expression (*islet1*-positive cells present in 67.7% of segments;  $n=99$  segments in 10 embryos). (L) The most dramatic loss of *islet1* expression was in double *nkx6* MO-injected embryos (*islet1*-positive cells present in 58.7% of segments;  $n=126$  segments in 13 embryos). The number of *islet1*-positive cells might be an overestimate in some cases, because of *islet1* RNA expression in RoP motoneurons (Appel et al., 1995). In some of these images, there appear to be abnormal numbers of Rohon-Beard (RB) spinal sensory neurons (*islet1*-positive cells in the dorsal spinal cord). This is an artifact of the way we mount the embryos to visualize PMNs in focus in many adjacent segments, which often requires tilting the embryos. The number of RBs appears within the normal range in *nkx6* MO-injected embryos. Scale bar: 20  $\mu\text{m}$ .

cells in the hindbrain and is not found in the spinal cord (see Fig. S1C in the supplementary material), and thus it cannot be involved in PMN development.

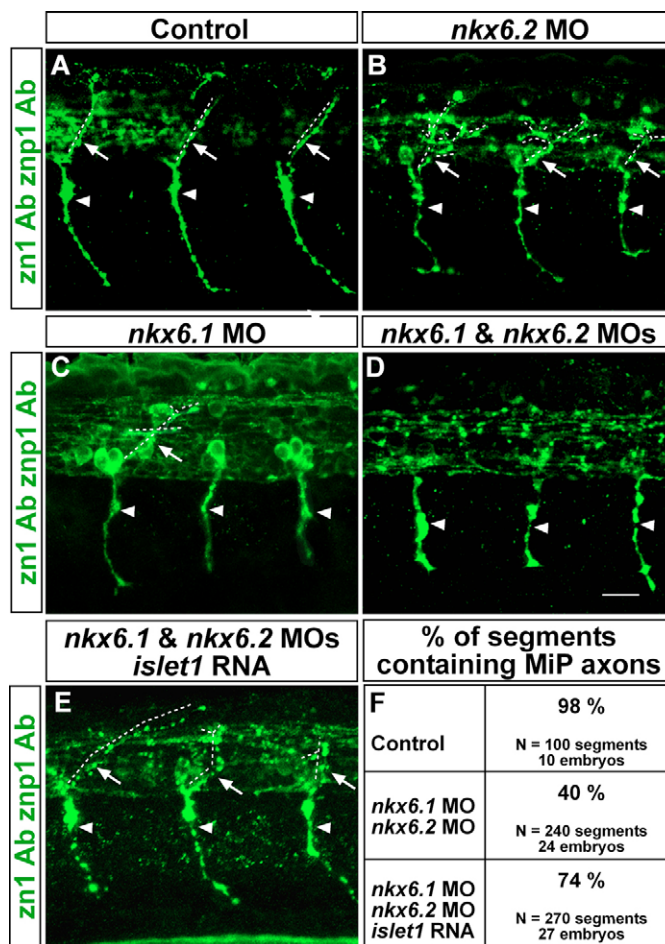
*nkx6.1* and *nkx6.2* are dynamically expressed in the zebrafish ventral spinal cord. We previously showed that *nkx6.1* is expressed in the ventral spinal cord, both in the floor plate and in several longitudinal rows of cells dorsal to the floor plate (Cheesman et al., 2004). The number of *nkx6.1*-expressing cell rows dorsal to the floor plate (Cheesman et al., 2004) is approximately the same as the number of *olig2*-expressing cell rows dorsal to the floor plate (see Park et al., 2002). Thus, like *olig2*, *nkx6.1* is expressed in the pMN domain (Cheesman et al., 2004). However, it is also expressed in additional cells ventral to the pMN domain, and might also be in some cells dorsal to the pMN domain. At 10 hpf, just before PMN progenitors begin leaving the cell cycle, *nkx6.1* was expressed in more pMN domain cells than *nkx6.2* (Fig. 1B,C). At 14 hpf, expression of *nkx6.1* and *nkx6.2* were almost indistinguishable and cross-sections revealed that both genes were confined to neuroectoderm (data not shown). At 18 hpf, *nkx6.1* and *nkx6.2* expression was no longer apparent in many of the ventrally located cells (Fig. 1D,E). We previously showed that the ventral cells lacking *nkx6.1* at this stage are PMNs (Cheesman et al., 2004), suggesting that expression of both *nkx6* genes is extinguished from PMNs by 17–19 hpf, the time when they undergo axogenesis. Both *nkx6.1* and *nkx6.2* continued to be expressed in the ventral spinal cord through at least 24 hpf (see Fig. S2 in the supplementary material).

Nkx6 proteins are differentially expressed in CaPs and MiPs, as shown by our double-label experiments with an Islet monoclonal antibody that recognizes both Islet1 and Islet2 proteins (Korzhan et al., 1993), and an Nkx6 polyclonal antibody that recognizes both Nkx6.1 (Cheesman et al., 2004) and Nkx6.2 (see Fig. S2 in the supplementary material). At 14 hpf, both CaPs and MiPs expressed Islet and Nkx6 (Fig. 1F). By 16 hpf, CaPs had extinguished expression of Nkx6 and expressed only Islet (Fig. 1G–I). By contrast, MiPs downregulated expression of Islet1 protein, similar to their downregulation of *islet1* mRNA (Appel et al., 1995), but continued to express Nkx6 (Fig. 1G). By 17 hpf, MiPs initiated a second phase of Islet1 protein expression and continued to express Nkx6 (Fig. 1H). MiPs expressed Nkx6 until 18 hpf, after which time it was downregulated (Fig. 1I). Nkx6 proteins are expressed in PMN progenitors (Cheesman et al., 2004), thus these proteins are present at the right time to be involved in PMN formation. The differential expression of Nkx6 proteins in postmitotic MiPs and CaPs suggests that Nkx6 might also participate in specification of MiP and CaP subtypes.

### Nkx6 proteins are not required for primary motoneuron formation

We previously showed that Nkx6.1 is unnecessary for PMN formation (Cheesman et al., 2004). To ascertain whether PMNs form in the absence of Nkx6.2, or in the absence of both Nkx6.1 and Nkx6.2, we injected embryos with morpholino antisense oligonucleotides (MOs). Early expression of *islet1* was normal in the absence of Nkx6.1, Nkx6.2, or both Nkx6 proteins (Fig. 2A–D), suggesting that PMNs formed normally. Thus, although Nkx6 proteins are expressed in the pMN domain, they appear unnecessary for PMN formation.

### The requirement for Nkx6 proteins distinguishes CaP and MiP subtypes



**Fig. 3. MiP axons are absent from embryos lacking Nkx6 proteins.** (A–D) Lateral views at axial level 8–12 of 28-hpf embryos labeled with zn1 and znp1 Abs. Arrowheads point to CaP axons; arrows point to MiP axons; dashed lines indicate MiP axon trajectories. Embryos lacking Nkx6.2 or Nkx6.1 (B,C) have normal CaP axons, but MiP axons were absent, truncated or excessively branched compared with control embryos (A). (D) Loss of MiP axons is most severe in double *nkx6* MO-injected embryos, but CaP axons are normal. (E) Loss of MiP axons in embryos lacking Nkx6.1 and Nkx6.2 is restored by co-injection of *islet1* mRNA with the *nkx6* MOs. (F) Quantification of segments lacking MiP axons in control, *nkx6* MO-injected, and *nkx6* MO plus *islet1* mRNA-injected embryos. Scale bar: 20  $\mu$ m.

Because CaPs and MiPs continue to express Nkx6 proteins after subtype commitment, we asked whether Nkx6 proteins have a role in subtype specification. In the absence of Nkx6.1, Nkx6.2, or both Nkx6 proteins, CaPs expressed *islet2* normally (Fig. 2E–H) and developed normal, ventrally projecting axons (Fig. 3A–D). Thus, Nkx6 proteins appear unnecessary for CaP subtype specification or axon pathfinding.

In contrast to CaPs, MiPs were severely affected by the lack of Nkx6 proteins. In the absence of either Nkx6.1 or Nkx6.2, some MiPs failed to initiate the late phase of *islet1* expression (Fig. 2I–K). In the absence of both Nkx6 proteins, very few MiPs expressed *islet1* at 18 hpf (Fig. 2I,L). In single *nkx6* MO-injected embryos, many MiPs had dorsal axons that were truncated or excessively branched and some MiPs failed to form dorsal axons (Fig. 3A–C). MiP axons were present in only 61% of segments in *nkx6.1* MO-

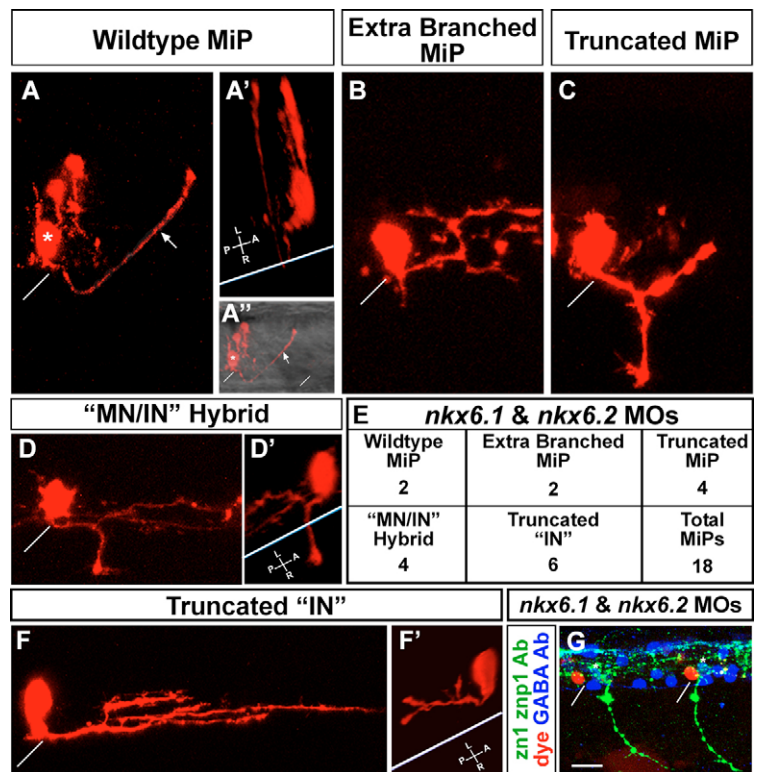
injected embryos, as compared with 87% of segments in *nkx6.2* MO-injected embryos and 98% of segments in control embryos ( $n=83$  segments in nine *nkx6.1* MO-injected embryos;  $n=168$  in 17 *nkx6.2* MO-injected embryos;  $n=100$  in ten control embryos). MiP axons were most severely affected in embryos injected with both *nkx6.1* and *nkx6.2* MOs, as indicated by the presence of MiP dorsal axons in only 40% of segments ( $n=240$  in 24 *nkx6* MO-injected embryos). The MiP axons that remained in *nkx6* MO-injected embryos were severely truncated (Fig. 3D). Thus, Nkx6 proteins are required for proper MiP subtype specification and axon pathfinding.

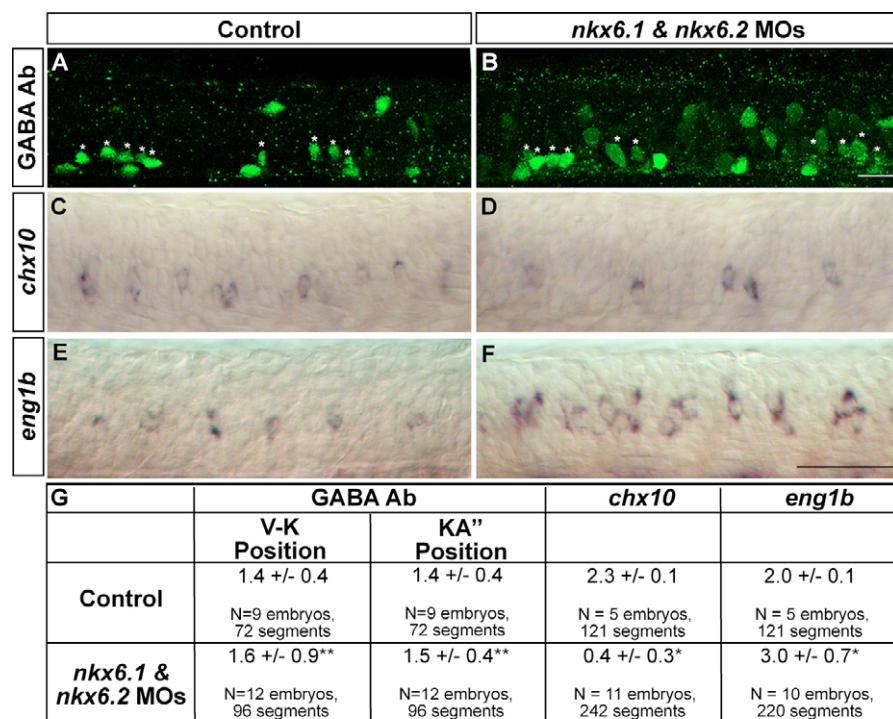
The loss of MiP axons in embryos lacking both Nkx6 proteins correlated with the failure of MiPs to initiate the late phase of *islet1* expression. Therefore, we reasoned that MiPs did not form normal axons because this process requires Islet1 protein. To test this hypothesis, we co-injected *nkx6.1* and *nkx6.2* MOs together with *islet1* RNA (Fig. 3E,F). MiP axons were present in 98% of segments in control embryos ( $n=100$  in ten embryos), in 40% of segments in *nkx6* double MO-injected embryos ( $n=240$  in 24 embryos), and in 74% of segments in embryos co-injected with *islet1* RNA and *nkx6* MOs ( $n=270$  in 27 embryos). These data provide evidence that Islet1 is sufficient for the projection of normal MiP axons even in the absence of Nkx6 proteins, and suggest that Nkx6 proteins regulate MiP specification at least in part by regulating expression of *islet1*.

### MiPs become more interneuron-like in the absence of Nkx6 proteins

**Fig. 4. MiPs become more interneuron-like in the absence of Nkx6 proteins.** (A-F') Single cells in the MiP position were identified and labeled at 24 hpf as described by Eisen et al. (Eisen et al., 1989); images were captured at 28 hpf. (A) Wild-type MiP, with its characteristic dorsal axon. The diagonal white line shows the location of the overlying segment boundary (likewise in A', B, C, D, F, G). The asterisk shows the MiP cell body. The arrows (A, A') indicate the MiP dorsal axon. Two other cells were also labeled during micropipette penetration of the spinal cord; these are located just dorsal of the MiP cell body. (A') 3D rotation of the confocal image. The white line indicates the ventral aspect of the spinal cord (likewise in D' and F'). Note that the MiP dorsal axon loops around this line, showing that it extends out of the spinal cord. (A'') The same view as A, but the fluorescent image is merged with a brightfield image to show the ventral aspect of the spinal cord and the overlying segment boundaries. (B-E) Range of phenotypes of MiPs in embryos lacking Nkx6 proteins. (B) This cell has a normal ventral axon remnant, but the dorsal axon is truncated and excessively branched. (C) This cell has abnormally retained the ventral axon and the dorsal axon is truncated. (D) This cell has abnormally retained the ventral axon; the 3D rotation in D' shows that it extends out of the spinal cord. The cell has not developed a dorsal axon, but instead has an interneuron-like axon within the spinal cord, as shown in the rotation in D'. (E) Quantification of phenotypes shown in B-D and F. IN, interneuron; MN, motoneuron. (F, F') This cell has neither a ventral nor a dorsal axon, but has an excessively branched interneuron-like axon that is truncated relative to those of wild-type interneurons. The 3D rotation in F' shows that this interneuron-like axon is entirely within the spinal cord. (G) Triple label showing that dye-labeled MiPs (red) are located in the normal MiP position, are adjacent to GABA-positive interneurons (blue) in the VeLD, KA' and KA'' positions (white asterisks above cells in the VeLD position), and are in segments with normal CaP axons but lacking MiP axons as revealed by labeling with zn1 and znp1 Abs (green). Scale bar: 10  $\mu\text{m}$  in A-D', F, F'; 20  $\mu\text{m}$  in G.

The failure to maintain *islet1* expression and the absence of dorsal axons in MiPs lacking Nkx6 proteins led us to consider whether MiPs developed as interneurons in the absence of late *islet1* expression, as they do in the absence of early *islet1* expression (Hutchinson and Eisen, 2006). We labeled individual cells in the MiP position in *nkx6* double MO-injected embryos by intracellular dye iontophoresis (Eisen et al., 1989). As a negative control, we labeled VeLD interneurons, which are located adjacent to MiPs (Eisen, 1991), and found that VeLDs are normal in *nkx6* double MO-injected embryos (12 VeLDs in 11 embryos; data not shown). Dye-labeling of MiPs in *nkx6* double MO-injected embryos revealed that these cells had a range of phenotypes (Fig. 4B-F). MiPs normally extend a short ventral axon out of the spinal cord before they form their subtype-specific dorsal axon; the ventral axon is later retracted (Eisen et al., 1989). In the absence of Nkx6 proteins, many MiPs projected a normal ventral axon (Fig. 4C,D), but often failed to retract it. Some MiPs had a dorsal axon, but often it was excessively branched (Fig. 4B) or truncated (Fig. 4C). Surprisingly, many MiPs initiated motoneuron development by projecting a normal ventral axon, but then projected an interneuron-like axon within the spinal cord instead of a dorsal motor axon (Fig. 4D). Finally, some MiPs did not project either ventral or dorsal motor axons, but only extended an interneuron-like axon within the spinal cord (Fig. 4F). However, this interneuron-like axon was excessively branched compared with axons of any of the previously described types of ventral spinal interneurons (Lewis and Eisen, 2003). As an additional control, we antibody-stained embryos in which we had individually dye-labeled MiPs to verify that dye-labeled MiPs lacking dorsal axons correlated with segments lacking dorsal MiP





Average number of interneurons per segment

\* p-value < 0.000003

\*\*p-value >0.45

**Fig. 5. Nkx6 proteins regulate *eng1b* and *chx10* expression.** (A-F) Lateral views of segments 8-12. (A,B) 28-hpf embryos labeled with GABA Ab. GABA-positive cells in the VeLD and KA' positions (denoted V-K) (see Hutchinson and Eisen, 2006) are labeled with white asterisks. *nkx6* MO-injected embryos (B) have a similar number of ventral GABA-positive interneurons as control embryos (A). (C,D) 24-hpf embryos labeled with *chx10* riboprobe. *nkx6* double MO-injected embryos (D) have fewer *chx10*-positive cells than control embryos (C). (E,F) 24-hpf embryos labeled with *eng1b* riboprobe. *nkx6* double MO-injected embryos (F) have more *eng1b*-positive cells than control embryos (E). (G) Quantification of GABA-positive, *chx10*-positive, and *eng1b*-positive cells in control and *nkx6* double MO-injected embryos. Scale bars: 20  $\mu$ m in A,B; 50  $\mu$ m in C-F.

axons as revealed by *zn1* and *znp1* Ab labeling (Fig. 4G). The results showed that in the absence of Nkx6 proteins, MiPs formed but they failed to extend their normal dorsal axon and instead adopted a more interneuron-like morphology, often developing a 'hybrid' phenotype in which they displayed morphological characteristics of both motoneurons and interneurons.

To ascertain whether the absence of late *Islet1* expression causes MiPs to take on the molecular characteristics of one or more specific types of interneurons, we characterized the expression of several different interneuron markers in *nkx6* double MO-injected embryos and assayed whether individual MiPs labeled with fluorescent dye expressed any of these markers. The GABA Ab labels several types of ventral interneurons that are derived from the pMN domain (see Hutchinson and Eisen, 2006), but the number of GABA-positive cells did not increase in *nkx6* double MO-injected embryos (Fig. 5A,B,G), and labeled MiPs were not GABA positive (Fig. 4G). Thus, we next examined the expression of several transcription factors that are expressed by interneurons in specific dorsoventral spinal cord positions. *pax2a* and *evx1* (Thaeron et al., 2000) are both expressed in interneurons in the dorsal and midregion of the spinal cord; the *evx1* expression domain is just ventral to the *pax2a* expression domain. Expression of both *pax2a* and *evx1* were was in *nkx6* double MO-injected embryos (data not shown). By contrast, *chx10* and *eng1b*, which are both expressed more ventrally than *pax2a* and *evx1*, had altered expression patterns in *nkx6* double MO-injected embryos. Expression of *chx10*, which is probably in CiD interneurons (Kimura et al., 2006), was downregulated in embryos lacking Nkx6 (Fig. 5C,D,G), whereas expression of *eng1b*, which is specifically in CiA interneurons (Higashijima et al., 2004), was expanded in *nkx6* double MO-injected embryos (Fig. 5E-G). These results are similar to the expansion of *En1* (an *eng1b* ortholog) and reduction of *Chx10* in mice lacking Nkx6.1 and Nkx6.2 (Vallstedt et al., 2001). Despite the changes in *eng1b* and *chx10* expression,

dye-labeled MiPs in *nkx6* double MO-injected embryos did not express either of these genes at 28 hpf (data not shown). Therefore, until additional markers of specific types of interneurons become available, we will remain unable to ascribe a specific type of interneuron identity to MiPs that develop in the absence of Nkx6 proteins.

## DISCUSSION

Our studies reveal dynamic differences in Nkx6 expression and function in zebrafish PMN subtypes and provide three key findings. First, we show that although two zebrafish Nkx6 proteins are expressed in PMN progenitors, they are unnecessary for PMN formation. This contrasts with mouse, in which Nkx6 proteins are required for formation of nearly all spinal motoneurons (Vallstedt et al., 2001). It is possible that in zebrafish there are additional *nkx6.1* or *nkx6.2* orthologs that we have not discovered. This seems unlikely as the Nkx6.1 antibody we used (Cheesman et al., 2004) recognizes both Nkx6.1 and Nkx6.2 proteins (see Fig. S2 in the supplementary material); however, we cannot eliminate the possibility of additional *nkx6* orthologs until the complete sequence of the zebrafish genome is available. The few spinal motoneurons that form in Nkx6 double-mutant mice appear to develop early, similar to zebrafish PMNs. Thus, it would be interesting to learn whether early-born motoneurons in mouse have other features that resemble zebrafish PMNs and distinguish them from later-developing motoneurons.

Second, we show that the MiP subtype requires Nkx6 proteins, whereas they appear dispensable for the CaP subtype. In mouse, Nkx6 proteins are required for formation of most motoneurons (Vallstedt et al., 2001) (but see above), but differential function of these proteins in distinct spinal motoneuron subtypes has not been reported. It will be important in future studies to identify the factors

that regulate CaP subtype specification, as well as to learn whether Nkx6 proteins have any role in the specification of mammalian motoneuron subtypes.

Finally, we show that Nkx6 proteins prevent MiPs from developing an interneuron-like axon by regulating late *islet1* expression. We have previously shown that the early phase of Islet1 expression promotes PMN formation and inhibits interneuron development (Hutchinson and Eisen, 2006). Here, we suggest that the second phase of Islet1 expression promotes the MiP subtype and also inhibits interneuron axon development. However, in the absence of only the late phase of Islet1 expression, MiPs often adopt a hybrid motoneuron/interneuron identity, rather than developing as interneurons, as they do in the absence of both phases of Islet1 expression.

The ability of zebrafish Nkx6 proteins to prevent MiPs from developing an interneuron-like axon is reminiscent of the ability of mouse Nkx6 proteins to inhibit pMN-domain cells from expressing interneuron-specific transcription factors (Vallstedt et al., 2001). Interestingly, Vallestedt and colleagues reported that in the absence of Nkx6 proteins, motoneurons transiently express *Evx1*, although whether these cells developed interneuron-like axons was not reported. We did not observe ectopic *evx1* expression; however, it is possible that *evx1* might be expressed early and transiently in MiPs in the absence of Nkx6 proteins. Mouse Nkx6 proteins apparently act within motoneuron progenitors. By contrast, our data raises the possibility that in MiPs, Nkx6 proteins function later, perhaps after the cell has become postmitotic. It will be important to address the timing of Nkx6 function further in future studies.

It is interesting that in the absence of Nkx6 proteins, MiPs become more interneuron-like, rather than becoming more like CaP. MiPs can develop as CaPs when they are transplanted to the CaP spinal cord position several hours before axogenesis (Appel et al., 1995; Eisen, 1991). However, when MiPs are in their normal spinal cord position, they are unlikely to transform into another PMN subtype because specification of MiP and CaP subtypes requires positional signals derived from the overlying somites (Lewis and Eisen, 2004). Our data support a model in which interneuron formation is continuously suppressed during motoneuron development. It will be important to determine whether interneuron specification similarly requires continuous suppression of motoneuron development.

We thank Bret Pearson, Chris Doe and Monte Westerfield for comments on earlier versions of the manuscript; members of the Eisen laboratory for their support; Richard Dorsky for help with spinal interneuron markers; Ole Madsen for the Nkx6 antibody; The University of Oregon Zebrafish Facility staff for animal husbandry; and Chris Rodesch and the Core Imaging Facility at The University of Utah for help with confocal microscopy. The Islet monoclonal antibody developed by Thomas Jessell and colleagues was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242. Supported by NIH grants NS23915, HD22486 and GM007413, and NSF grant DGE9972830.

#### Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/134/9/1671/DC1>

#### References

- Alanentalo, T., Chatonnet, F., Karlen, M., Sulniute, R., Ericson, J., Andersson, E. and Ahlgren, U. (2006). Cloning and analysis of Nkx6.3 during CNS and gastrointestinal development. *Gene Expr. Patterns* **6**, 162-170.
- Appel, B. and Eisen, J. S. (1998). Regulation of neuronal specification in the zebrafish spinal cord by Delta function. *Development* **125**, 371-380.
- Appel, B., Korzh, V., Glasgow, E., Thor, S., Edlund, T., Dawid, I. B. and Eisen, J. S. (1995). Motoneuron fate specification revealed by patterned LIM homeobox gene expression in embryonic zebrafish. *Development* **121**, 4117-4125.
- Cheesman, S. E., Layden, M. J., Von Ohlen, T., Doe, C. Q. and Eisen, J. S. (2004). Zebrafish and fly Nkx6 proteins have similar CNS expression patterns and regulate motoneuron formation. *Development* **131**, 5221-5232.
- Eisen, J. S. (1991). Determination of primary motoneuron identity in developing zebrafish embryos. *Science* **252**, 569-572.
- Eisen, J. S., Pike, S. H. and Debu, B. (1989). The growth cones of identified motoneurons in embryonic zebrafish select appropriate pathways in the absence of specific cellular interactions. *Neuron* **2**, 1097-1104.
- Higashijima, S., Hotta, Y. and Okamoto, H. (2000). Visualization of cranial motor neurons in live transgenic zebrafish expressing green fluorescent protein under the control of the *islet-1* promoter/enhancer. *J. Neurosci.* **20**, 206-218.
- Higashijima, S., Masino, M. A., Mandel, G. and Fetcho, J. R. (2004). Engrailed-1 expression marks a primitive class of inhibitory spinal interneuron. *J. Neurosci.* **24**, 5827-5839.
- Hutchinson, S. A. and Eisen, J. S. (2006). Islet1 and Islet2 have equivalent abilities to promote motoneuron formation and to specify motoneuron subtype identity. *Development* **133**, 2137-2147.
- Inoue, A., Takahashi, M., Hatta, K., Hotta, Y. and Okamoto, H. (1994). Developmental regulation of *islet-1* mRNA expression during neuronal differentiation in embryonic zebrafish. *Dev. Dyn.* **199**, 1-11.
- Jurata, L. W., Thomas, J. B. and Pfaff, S. L. (2000). Transcriptional mechanisms in the development of motor control. *Curr. Opin. Neurobiol.* **10**, 72-79.
- Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B. and Schilling, T. F. (1995). Stages of embryonic development of the zebrafish. *Dev. Dyn.* **203**, 253-310.
- Kimura, Y., Okamura, Y. and Higashijima, S. (2006). *alx*, a zebrafish homolog of Chx10, marks ipsilateral descending excitatory interneurons that participate in the regulation of spinal locomotor circuits. *J. Neurosci.* **26**, 5684-5697.
- Korzh, V., Edlund, T. and Thor, S. (1993). Zebrafish primary neurons initiate expression of the LIM homeodomain protein *Isl-1* at the end of gastrulation. *Development* **118**, 417-425.
- Lewis, K. E. and Eisen, J. S. (2003). From cells to circuits: development of the zebrafish spinal cord. *Prog. Neurobiol.* **69**, 419-449.
- Lewis, K. E. and Eisen, J. S. (2004). Paraxial mesoderm specifies zebrafish primary motoneuron subtype identity. *Development* **131**, 891-902.
- Myers, P. Z. (1985). Spinal motoneurons of the larval zebrafish. *J. Comp. Neurol.* **236**, 555-561.
- Park, H. C., Mehta, A., Richardson, J. S. and Appel, B. (2002). *olig2* is required for zebrafish primary motor neuron and oligodendrocyte development. *Dev. Biol.* **248**, 356-368.
- Pfaff, S. and Kintner, C. (1998). Neuronal diversification: development of motor neuron subtypes. *Curr. Opin. Neurobiol.* **8**, 27-36.
- Sander, M., Paydar, S., Ericson, J., Briscoe, J., Berber, E., German, M., Jessell, T. M. and Rubenstein, J. L. (2000). Ventral neural patterning by Nkx homeobox genes: Nkx6.1 controls somatic motor neuron and ventral interneuron fates. *Genes Dev.* **14**, 2134-2139.
- Shirasaki, R. and Pfaff, S. L. (2002). Transcriptional codes and the control of neuronal identity. *Annu. Rev. Neurosci.* **25**, 251-281.
- Thaeron, C., Avaron, F., Casane, D., Borday, V., Thisse, B., Thisse, C., Boulekbache, H. and Laurenti, P. (2000). Zebrafish *evx1* is dynamically expressed during embryogenesis in subsets of interneurons, posterior gut and urogenital system. *Mech. Dev.* **99**, 167-172.
- Tokumoto, M., Gong, Z., Tsubokawa, T., Hew, C. L., Uyemura, K., Hotta, Y. and Okamoto, H. (1995). Molecular heterogeneity among primary motoneurons and within myotomes revealed by the differential mRNA expression of novel *islet-1* homologs in embryonic zebrafish. *Dev. Biol.* **171**, 578-589.
- Trevarrow, B., Marks, D. L. and Kimmel, C. B. (1990). Organization of hindbrain segments in the zebrafish embryo. *Neuron* **4**, 669-679.
- Tsalik, E. L., Niacaris, T., Wenick, A. S., Pau, K., Avery, L. and Hobert, O. (2003). LIM homeobox gene-dependent expression of biogenic amine receptors in restricted regions of the *C. elegans* nervous system. *Dev. Biol.* **263**, 81-102.
- Vallstedt, A., Muhr, J., Pattyn, A., Pierani, A., Mendelsohn, M., Sander, M., Jessell, T. M. and Ericson, J. (2001). Different levels of repressor activity assign redundant and specific roles to Nkx6 genes in motor neuron and interneuron specification. *Neuron* **31**, 743-755.