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

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SUMMARY

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 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 have 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 *islet1* expression which is required both to promote the MiP subtype and to suppress interneuron development.

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). *islet* gene expression is dynamic in these cells (Figure 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 down-regulate *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 down-regulate expression of *islet1* (Appel et al., 1995; Inoue et al., 1994; Korzh

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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 the alternating pattern of *islet2* and *islet1* 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 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 negatively-regulated by Nkx6.1. In the absence of Nkx6.1 function, *Nkx6.2* expression spreads ventrally into the pMN domain and partially substitutes for Nkx6.1 during motoneuron formation (Vallstedt et al., 2001). Mice deficient for both Nkx6.1 and Nkx6.2 lose nearly all 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-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.

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’ CGG CTT CAA GGC TCA TTC 3’) and a reverse homeobox primer (5’ CCA TTT AGT TCT TCT GTT CTG 3’) isolated a full-length cDNA clone from a 14−19 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

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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 5' TCCACTCATACCCTCCATC 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 in 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., 2000) 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), monoclonal mouse anti-Islet (1:200; antibody recognizes both Islet1 and Islet2 proteins (Korzh 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; Clonetech, 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 hrs in 4% paraformaldehyde (PFA) and 1 × Fix Buffer (Westerfield, 2000) at 4°C, blocked in 1 × PBS/ 5%NGS/4 mg/ml BSA/0.5% Triton x-100 for 1 hour at room temperature, incubated in primary antibody diluted in block overnight at 4°C, washed at room temperature for 1.5 hours in PBS/ 0.1% Tween-20, incubated in secondary antibody diluted in block for 4 hrs at room temperature, and then washed for 1.5 hrs at room temperature in PBS/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’-GGTGCGCCGGAGCCACAGGACAAAC-3’) on the assumption it would interfere with initiation of translation at either start site. We also utilized a splice blocking MO (5’-CGCGCAAAACTCACCCGCACAGGGA-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 5mg/ml *nkx6.2* MO diluted in 0.2M KCl and phenol red was injected into the yolk cell of 2 cell-stage embryos; for double MO injections we added 2.5mg/ml *nkx6.1* MO (described in Cheesman et al., 2004). All our MO injections worked efficiently since 84.3% (27/32) of *nkx6.1* MO-injected, 90.9% (30/33) of *nkx6.2* MO-injected embryos, and 76.1% (35/46) of *nkx6* double MO-injected embryos had a MiP axon phenotype.

Microscopy

Images of zebrafish embryos were captured on a Zeiss Axioplan equipped with a digital camera, a Bio-Rad Radiance, or a Zeiss Pascal confocal microscope. In a few cases an Olympus IX81

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microscope with an FV300 confocal was used to capture images. The brightness and contrast of images was adjusted in Adobe Photoshop.

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 in Eisen et al. (1989). GABA-positive interneurons were counted as described in 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 to 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 which was then followed by Velocity image arithmetic to identify co-labeled cells. The co-labeled cells were then pseudo-colored blue.

RESULTS AND DISCUSSION

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

Studies implicating two Nkx6 homologues 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 zebrafish *nkx6* genes are most closely related to other vertebrate *nkx6* genes, distinct from *nkx2* family members (Figure S1A). 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 (Figure S1B). Similar to mouse *Nkx6.3* (Alanentalo et al., 2006), zebrafish *nkx6.3* expression is restricted to a group of cells in the hindbrain and is not found in the spinal cord (Figure S1C), thus it cannot be involved in PMN development.

nkx6.1 and *nkx6.2* are dynamically expressed in the zebrafish pMN domain. 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 is approximately the same as the number of *olig2*-expressing cell rows dorsal to the floor plate (see Park et al., 2002), showing that like *olig2*, *nkx6.1* is expressed in the pMN domain (Cheesman et al., 2004). At 10 hpf, just before PMN progenitors begin leaving the cell cycle, *nkx6.1* is expressed in more pMN domain cells than *nkx6.2* (Figure 1B,C). At 14 hpf, *nkx6.1* and *nkx6.2* expression are almost indistinguishable and cross-sections reveal that both genes are confined to neurectoderm (data not shown). At 18 hpf, *nkx6.1* and *nkx6.2* expression is no longer apparent in many ventrally-located cells (Figure 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

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extinguished from PMNs by 17−19 hpf, the time they undergo axogenesis. Both *nkx6.1* and *nkx6.2* continue to be expressed in the ventral spinal cord through at least 24 hpf (Figure S2).

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 (Korzh et al., 1993) and an Nkx6 polyclonal antibody that recognizes both Nkx6.1 (Cheesman et al., 2004) and Nkx6.2 (Figure S2). At 14 hpf, both CaPs and MiPs express Islet and Nkx6 (Figure 1F). By 16 hpf, CaPs have extinguished expression of Nkx6 and express only Islet (Figure 1G-I). In contrast, MiPs have downregulated expression of Islet1 protein, similar to their downregulation of *islet1* mRNA (Appel et al., 1995) but continue to express Nkx6 (Figure 1G). By 17 hpf, MiPs have initiated a second phase of Islet1 protein expression and continue to express Nkx6 (Figure 1H). MiPs express Nkx6 until 18 hpf, after which time they downregulate it (Figure 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 may 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 learn whether PMNs form in the absence of Nkx6.2 or in the absence of both Nkx6 proteins, 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 (Figure 2A-D) suggesting 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

CaPs and MiPs continue to express Nkx6 proteins after subtype commitment, thus 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 (Figure 2E-H) and developed normal ventrally-projecting axons (Figure 3A-D). Thus, Nkx6 proteins appear unnecessary for CaP subtype specification or axon pathfinding.

In contrast to CaPs, MiPs were severely affected by 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 (Figure 2I-K). In the absence of both Nkx6 proteins, very few MiPs expressed *islet1* at 18 hpf (Figure 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 (Figure 3A-C). MiP axons were present in only 61% of segments in *nkx6.1* MO-injected embryos as compared to 87% of segments in *nkx6.2* MO-injected embryos and 98% of segments in control embryos (n=83 segments in 9 *nkx6.1* MO-injected embryos; n=168 segments in 17 *nkx6.2* MO-injected embryos; n=100 segments in 10 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 segments in 24 *nkx6* MO-injected embryos). The MiP axons that remained in *nkx6* MO-injected embryos were severely truncated (Figure 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 with *islet1* RNA (Figure 3E,F). MiP axons were present in 98% of segments in control embryos (n=100 segments in 10 embryos), 40% of segments in *nkx6* double MO-injected embryos (n=240 segments in 24 embryos), and 74% of segments in

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embryos co-injected with *islet1* RNA and *nkx6* MOs (n=270 segments in 27 embryos). These data provide evidence that Islet1 is sufficient for 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*.

In the absence of Nkx6 proteins MiPs become more interneuron-like

The failure to maintain *islet1* expression and 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). To test this hypothesis, 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 (Figure 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 (Figure 4C,D), but often failed to retract it. Some MiPs had a dorsal axon, but it was frequently excessively-branched (Figure 4B) or truncated (Figure 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 (Figure 4D). Finally, some MiPs did not project either ventral or dorsal motor axons, but only extended an interneuron-like axon within the spinal cord (Figure 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 axons as revealed by zn1 and znp1 Ab labeling (Figure 4G). Our results show that in the absence of Nkx6 proteins, MiPs form but they fail to extend their normal dorsal axon and instead they adopt a more interneuron-like morphology, often developing a “hybrid” phenotype in which they display morphological characteristics of both motoneurons and interneurons.

To learn 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 (Figure 5A,B,G) and labeled MiPs were not GABA positive (Figure 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 normal in *nkx6* double MO-injected embryos (data not shown). In 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), is downregulated in embryos lacking Nkx6 (Figure 5C,D,G), whereas expression of *eng1b*, which is specifically in CiA interneurons (Higashijima et al., 2004), is expanded in *nkx6* double MO-injected embryos (Figure 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-

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injected embryos did not express either one 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.

CONCLUSIONS

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 to 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 have used (Cheesman et al., 2004) recognizes both Nkx6.1 and Nkx6.2 proteins (Figure S2), however, we cannot eliminate the possibility of additional *nkx6* orthologs until the zebrafish genome sequence is completed. 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 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 promotes PMN formation and inhibits interneuron development (Hutchinson and Eisen, 2006). Here we suggest that the second phase of Islet1 expression promotes 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 colleague reported that in the absence of Nkx6 proteins, motoneurons transiently expressed *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, in 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.

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It will be important to determine whether interneuron specification similarly requires continuous suppression of motoneuron development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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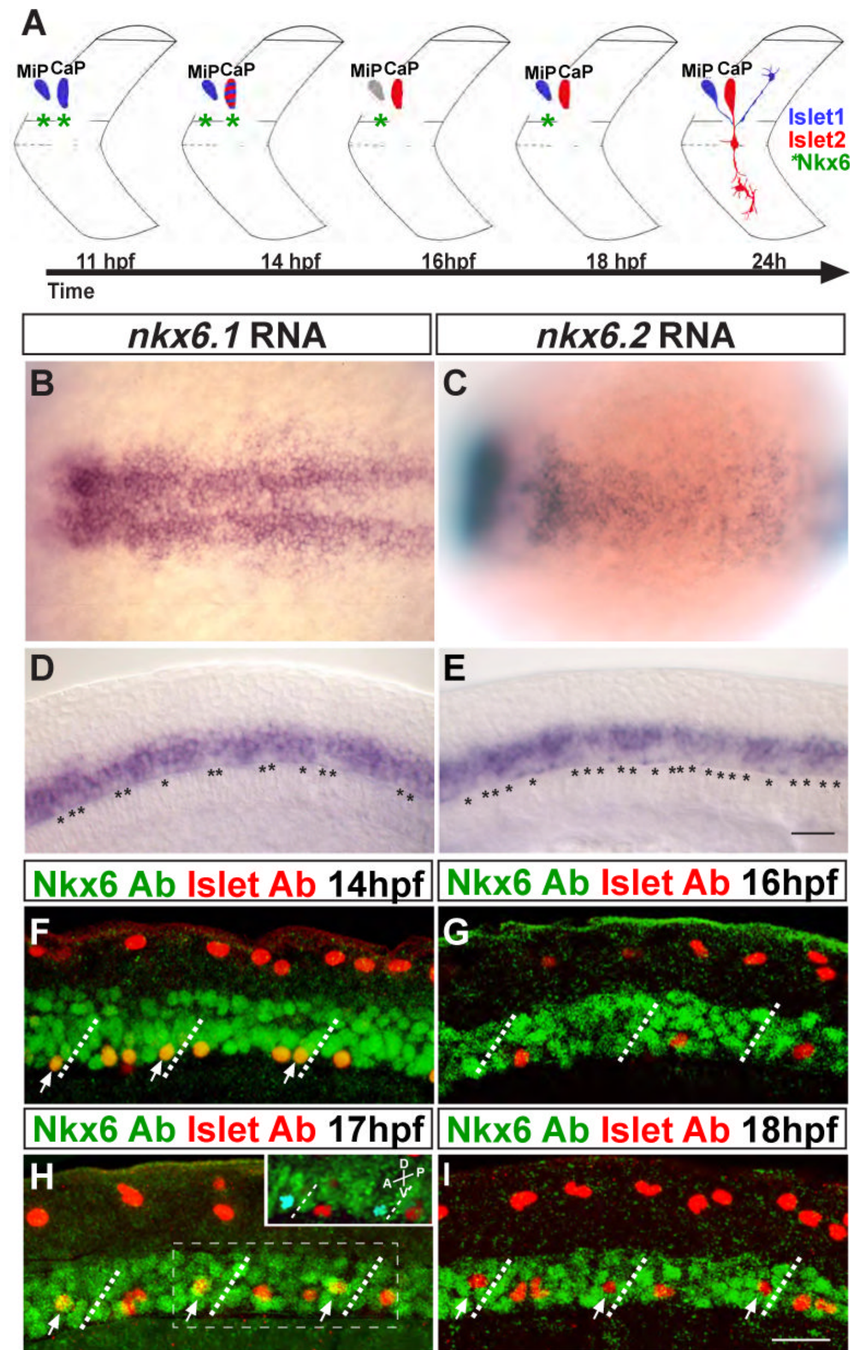

Figure 1. Two Nkx6 proteins are dynamically expressed in PMNs

(A) Schematic of Islet and Nkx6 protein dynamics during PMN subtype specification at the somite level 8–12. 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 neurectodermal 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 are expressed in the ventral spinal cord, however, some of the ventral-most cells are *nkx6*-negative (marked by asterisks). (F-I) Lateral views of Islet and Nkx6 co-expression. MiP and CaP were identified by soma position relative to somite boundaries (denoted by dotted lines). (F) Single optical slice showing 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 (denoted by arrows in H) were identified by the Velocity software program (see Methods) and are pseudo-colored blue. (I) By 19 hpf MiPs (arrows) have also downregulated Nkx6. Scale bar, B-E 50μM; F-I 20μM.

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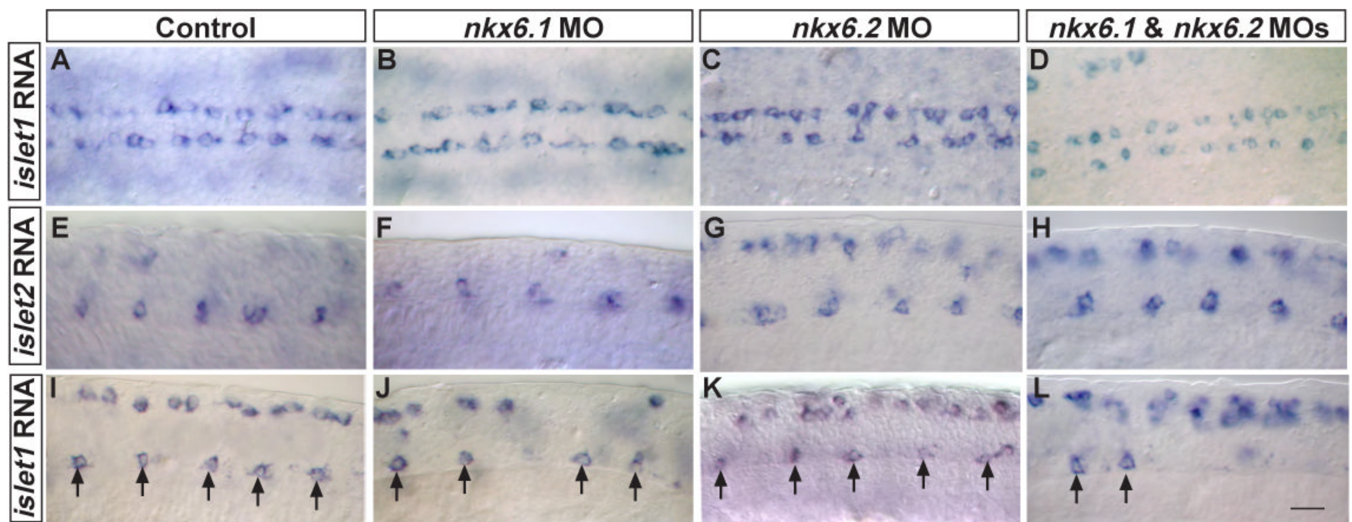

Figure 2. Nkx6 proteins regulate late *islet1* expression

(A-D) Dorsal views at 11−12 hpf. (A) *islet1* mRNA expression in PMNs of control embryos. (B-D) *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 (J) there are fewer *islet1*-positive cells (*islet1*-positive cells present in 74.4% of segments; n=133 segments in 14 embryos) as compared to 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 may be an overestimate in some cases, because of *islet1* RNA expression in RoP motoneurons (Appel et al., 1995). In some of these figures 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, 20μm.

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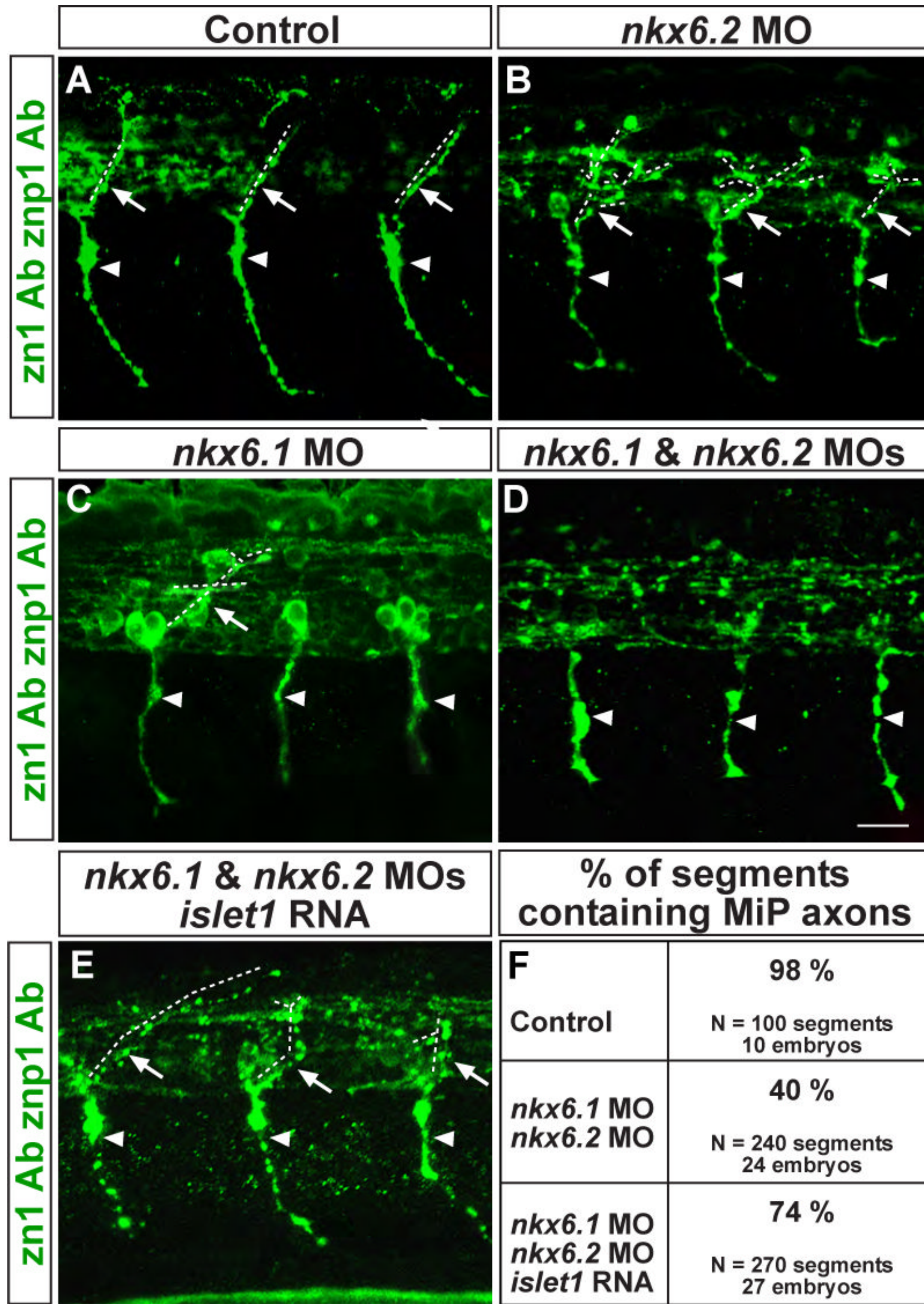

Figure 3. MiP axons are absent from embryos lacking Nkx6 proteins

(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. (B,C) Embryos lacking Nkx6.2 or Nkx6.1 have normal CaP axons but MiP axons were absent, truncated or excessively branched compared to 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, 20μM.

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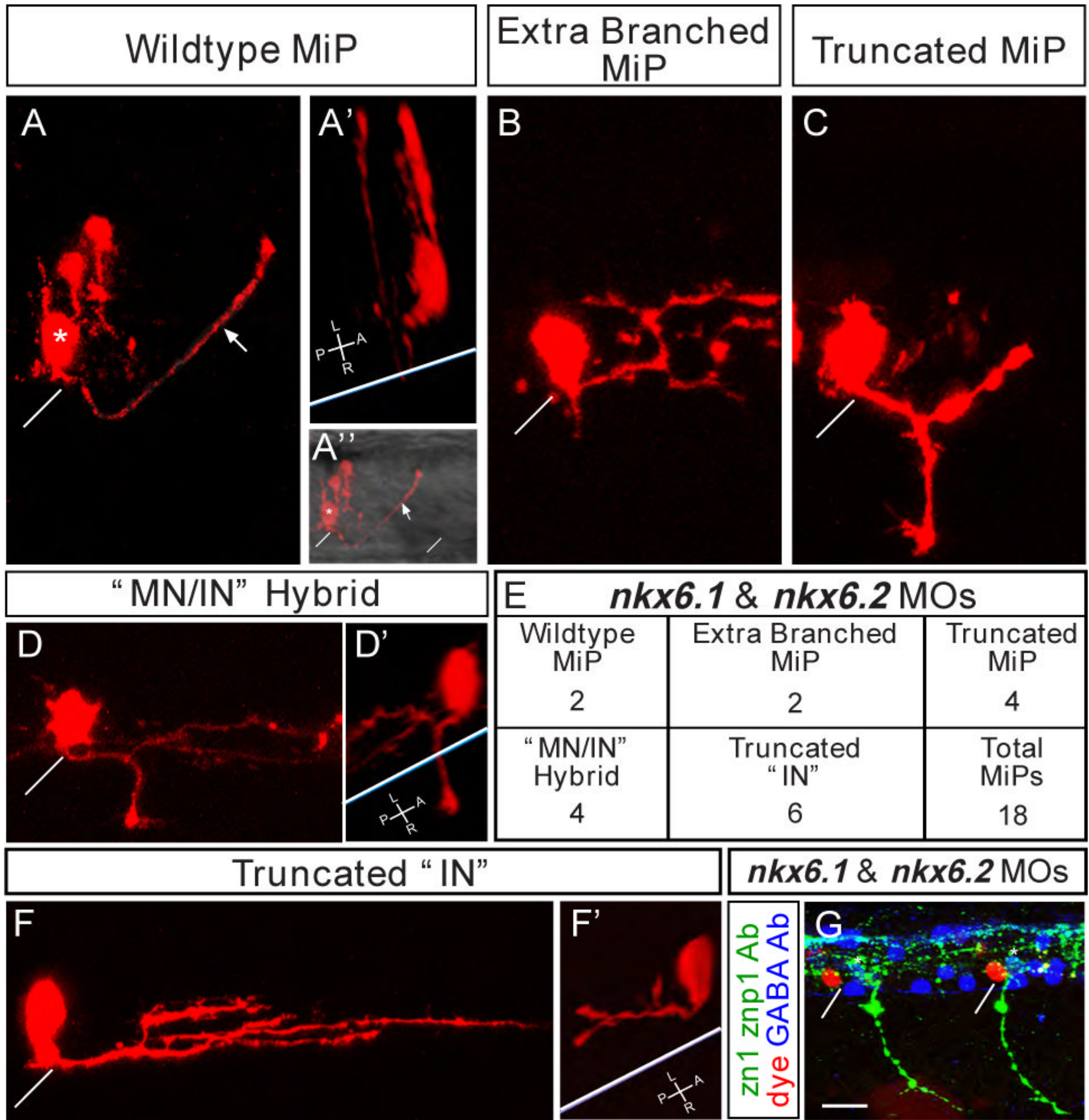

Figure 4. MiPs become more interneuron-like in the absence of Nkx6 proteins

(A-E) Single cells in the MiP position were identified and labeled at 24 hpf as described in 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 here and in panels A”, B, C, D, F, G. The asterisk shows the MiP cell body. Two other cells were also labeled during micropipette penetration of the spinal cord; these are located just dorsal of the MiP cell body. A’ shows a 3D rotation of the confocal image. The white line here, and in panels D’ and F’, indicates the ventral aspect of the spinal cord. Note that the MiP dorsal axon loops around this line, showing that it extends out of the spinal cord. A” shows 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

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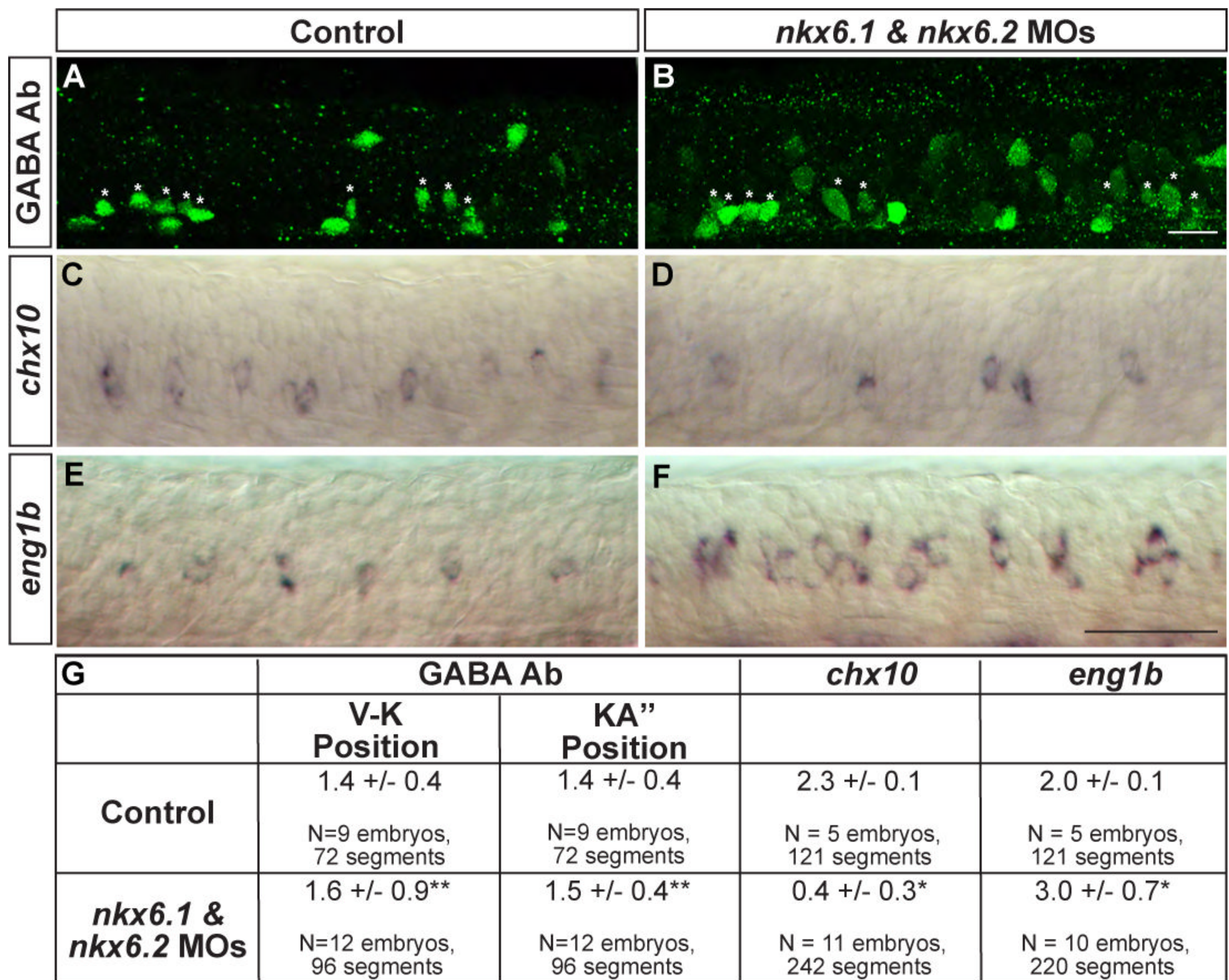
the dorsal axon is truncated and excessively branched. (C) This cell has abnormally retained the ventral axon and the dorsal axon is truncated. (D) This cell has abnormally retained the ventral axon; the 3D rotation in D' shows that it extends out of the spinal cord. The cell has not developed a dorsal 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. (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 postion), and are in segments with normal CaP axons but lacking MiP axons as revealed by labeling with zn1 and znp1 Abs (green). Scale bar A-F, 10μM; G, 20μM.



	GABA Ab		chx10	eng1b
	V-K Position	KA" Position		
Control	1.4 +/- 0.4 N=9 embryos, 72 segments	1.4 +/- 0.4 N=9 embryos, 72 segments	2.3 +/- 0.1 N = 5 embryos, 121 segments	2.0 +/- 0.1 N = 5 embryos, 121 segments
nkx6.1 & nkx6.2 MOs	1.6 +/- 0.9** N=12 embryos, 96 segments	1.5 +/- 0.4** N=12 embryos, 96 segments	0.4 +/- 0.3* N = 11 embryos, 242 segments	3.0 +/- 0.7* N = 10 embryos, 220 segments

Average number of interneuons per segment
* p-value < 0.000003
**p-value >0.45

Figure 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 bar A-B, 20μm; C-F, 50μm.

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