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Islet1 and Islet2 have equivalent abilities to promote motoneuron formation and to specify motoneuron subtype identity

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The expression of LIM homeobox genes *islet1* and *islet2* is tightly regulated during development of zebrafish primary motoneurons. All primary motoneurons express *islet1* around the time they exit the cell cycle. By the time primary motoneurons undergo axogenesis, specific subtypes express *islet1*, whereas other subtypes express *islet2*, suggesting that these two genes have different functions. Here, we show that Islet1 is required for formation of zebrafish primary motoneurons; in the absence of Islet1, primary motoneurons are missing and there is an apparent increase in some types of ventral interneurons. We also provide evidence that Islet2 can substitute for Islet1 during primary motoneuron formation. Surprisingly, our results demonstrate that despite the motoneuron subtype-specific expression patterns of Islet1 and Islet2, the differences between the Islet1 and Islet2 proteins are not important for specification of the different primary motoneuron subtypes. Thus, primary motoneuron subtypes are likely to be specified by factors that act in parallel to or upstream of *islet1* and *islet2*.

KEY WORDS: Primary motoneuron, Secondary motoneuron, LIM homeodomain, Interneuron, Spinal motoneuron, pMN domain, Zebrafish

INTRODUCTION

Vertebrate motoneurons innervate muscles in an exquisitely precise pattern. Studies of motoneurons have been instrumental in revealing the processes by which progenitor cells generate different types of neurons, e.g. motoneurons or interneurons, as well as the processes by which each type of neuron adopts a specific subtype identity (Curtiss and Heilig, 1998; Edlund and Jessell, 1999; Eisen, 1999; Jurata et al., 2000; Lee and Pfaff, 2001; Shirasaki and Pfaff, 2002; Sockanathan, 2003; Tanabe and Jessell, 1996). The subtype identity of a motoneuron is defined by its axonal projection out of the central nervous system (CNS) and the specific muscle it innervates (Eisen, 1999; Pfaff and Kintner, 1998; Tosney et al., 1995). Although many proteins have been implicated in motoneuron formation and subtype specification, the precise roles of some of these proteins remain unresolved. In this paper, we focus on the roles of two related proteins, Islet1 and Islet2, in formation and specification of subtype identity of spinal motoneurons in zebrafish.

Zebrafish have two types of spinal motoneurons, primary motoneurons and secondary motoneurons (Myers, 1985), both of which are derived from the spinal cord motoneuron progenitor (pMN) domain (Kimmel et al., 1994; Park et al., 2004). Primary motoneurons (PMNs) are born early in development, around the end of gastrulation. Each PMN is individually identifiable based on its cell body position, axonal trajectory and the muscle region it innervates, providing the opportunity to study vertebrate motoneuron formation at the single cell level (Eisen et al., 1986; Myers et al., 1986; Westerfield et al., 1986). Here, we focus on two of the PMNs, MiP, which has a dorsally projecting axon, and CaP, which has a ventrally projecting axon. In contrast to PMNs, secondary motoneurons (SMNs) arise later in development and are more numerous than PMNs (Myers, 1985; Myers et al., 1986). Although some SMNs project dorsally and others project ventrally (Myers et al., 1986; Westerfield et al., 1986), it is currently unclear whether SMNs also develop individually identifiable subtypes. Because PMNs have thus far only been described in anamniote vertebrates such as fish and frogs (Eisen, 1994), it is thought that SMNs more closely resemble the motoneurons described in amniote vertebrates (Kimmel and Westerfield, 1990).

LIM homeodomain (LIM-HD) protein family members are expressed by all vertebrate motoneurons studied to date and play a prominent role in several aspects of motoneuron development, including initial specification and adoption of a particular subtype identity (Curtiss and Heilig, 1998; Eisen, 1999; Jurata et al., 2000; Lee and Pfaff, 2001; Pfaff and Kintner, 1998; Shirasaki and Pfaff, 2002; Sockanathan, 2003; Tanabe and Jessell, 1996). LIM-HD proteins have two N-terminal protein binding LIM domains and one C-terminal DNA binding homeodomain (Bach, 2000; Curtiss and Heilig, 1998). In mouse and chick, the LIM-HD protein Islet1 appears to be pan-motoneuronal around the time motoneurons exit the cell cycle (Ericson et al., 1992; Tsuchida et al., 1994). Slightly later, motoneurons express a related LIM-HD protein, Islet2 (Thaler et al., 2004; Tsuchida et al., 1994). Studies in mouse demonstrated that Islet1 is required for motoneuron formation; in the absence of Islet1, there is widespread cell death in the ventral spinal cord (Pfaff et al., 1996). By contrast, Islet2 is only required for formation of visceral motoneurons, although it is expressed at least transiently in all mouse spinal motoneurons (Thaler et al., 2004).

As in other vertebrates, zebrafish motoneurons express Islet1 and Islet2 (Appel et al., 1995; Inoue et al., 1994; Korzh et al., 1993; Tokumoto et al., 1995). SMNs express both of these proteins, but it is still unclear whether they are co-expressed or are in distinct SMN populations (Appel et al., 1995; Inoue et al., 1994). Unlike SMNs, the expression patterns of *islet1* and *islet2* have been studied in great detail in PMNs (Fig. 1A). *islet1* is expressed in all PMNs 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 transiently downregulate and then reinitiate *islet1* expression prior to axogenesis; these cells do not express *islet2* (Appel et al., 1995). By

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contrast, prior to axogenesis, CaPs initiate expression of *islet2* and then downregulate expression of *islet1* (Appel et al., 1995; Inoue et al., 1994; Korzh et al., 1993; Tokumoto et al., 1995). The end result of this dynamic pattern of *islet* gene expression is that by the time of axon extension, MiPs express exclusively *islet1* and CaPs express exclusively *islet2*. This expression pattern leads to the hypothesis that differential expression of Islet proteins specifies PMN subtype. Consistent with this idea, previous work suggested that Islet2 is required for CaP formation (Segawa et al., 2001). However, the role of Islet1 in formation of zebrafish PMNs and MiP subtype specification has not been previously explored.

We used morpholino antisense oligonucleotides (Nasevicius and Ekker, 2000) and mRNA misexpression to investigate the roles of Islet1 and Islet2 in formation of zebrafish motoneurons. Here, we provide evidence that in zebrafish, as in mouse (Pfaff et al., 1996), Islet1 is required for PMN and SMN formation. However, instead of apparently dying like mouse motoneurons that lack Islet1 (Pfaff et al., 1996), zebrafish PMNs appear to change fate and develop as interneurons in the absence of Islet1. Surprisingly, despite the highly regulated expression patterns of Islet1 and Islet2 (Fig. 1A), our results suggest that these proteins have redundant functions. We provide evidence that Islet2 can substitute for Islet1 early on (during motoneuron formation) and later on (during specification of MiP subtype identity). Our results are consistent with a model in which upstream factors that regulate the differential expression of Islet proteins or factors that act in parallel to them establish the differences between PMN subtypes.

MATERIALS AND METHODS

Embryos

Zebrafish (*Danio rerio*) embryos were collected from natural crosses of wild-type (AB) adults, raised at 28.5°C, and staged by hours post fertilization at 28.5°C (hpf) and gross morphology (Kimmel et al., 1995).

RNA in situ hybridization

RNA in situ hybridization was performed as described by Appel et al (Appel et al., 1995). RNA probes include *islet1* and *islet2* (Appel et al., 1995).

Immunohistochemistry and TUNEL labeling

The following primary antibodies (Abs) were used: monoclonal anti-Islet (Korzh et al., 1993), which recognizes Islet1 and Islet2 proteins (1:200; 39.4D5 Developmental Studies Hybridoma Bank); polyclonal anti-GABA (1:1000, Sigma); monoclonal zn1 (1:200) (Trevarrow et al., 1990); monoclonal znp1 (1:1000) (Melancon et al., 1997; Trevarrow et al., 1990); polyclonal anti-Lhx4 (1:500; S.A.H. and J.S.E., unpublished); and polyclonal anti-Lhx3 (1:500; S.A.H. and J.S.E., unpublished). The following secondary antibodies from Molecular Probes were used: goat anti-mouse Alexa-488 (1:1000), goat anti-mouse IgG_1 Alexa-488 (1:500), goat anti-mouse IgG_{2a} Alexa-488 (1:500), goat anti-mouse IgG_{2b} Alexa-546 (1:500), goat anti-rabbit Alexa-546 (1:1000) and goat anti-rabbit Alexa-488 (1:1000). Goat anti-mouse Cy5 (1:200) from Jackson Laboratories was also used. Embryos were fixed for 3.5-4.0 hours in 4% paraformaldehyde (PFA) and 1×Fix Buffer (Westerfield, 1995) 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 hours at room temperature; and then washed for 1.5 hours at room temperature in PBS + 0.1% Tween-20. Embryos were stored in 4% paraformaldehyde until analyzed.

For triple labeling with Islet Ab, Lhx4 Ab and TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling), embryos were first labeled with Islet and Lhx4 Abs. Embryos were then post-fixed for 20 minutes at room temperature in 4% PFA. After post-fixation, embryos were washed three times for 5 minutes with PBST (1×PBS + 0.1% Tween-20). Proteinase K (10 mg/ml) diluted 1:5000 in water was used to permeabilize embryos for 1.5 minutes at room temperature. Embryos were fixed for 15 minutes at room temperature in 4% PFA and washed out of fix with PBST. Embryos were incubated for 1 hour in the dark on ice followed by one hour in the dark at 37°C in TUNEL solution (Roche). After incubation, embryos were washed four times for 10 minutes in PBST; the last wash was overnight at 4°C.

Microscopy

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

RNA and morpholino injections

islet1 RNA (Appel et al., 1995; Inoue et al., 1994; Tokumoto et al., 1995) and *islet2* RNA (Appel et al., 1995; Tokumoto et al., 1995) were transcribed using the mMessage Machine (Ambion) according to instructions. One-cell stage embryos were injected with several nanoliters of 56 ng/μl *islet1* RNA or 61 ng/μl *islet2* RNA for overexpression experiments. *islet1* RNA was reduced to 28 ng/μl for rescue experiments, but *islet2* RNA remained at 61 ng/μl.

To create embryos with reduced Islet1, two splice-blocking morpholinos were designed by Gene Tools (Corvallis, Oregon) to the splice donor sites after the second and third exons of *islet1*. *islet1E2* began at position 208 of *islet1* cDNA at the end of exon 2 (5′-TTAATCTGCGTTACCTGATGTA-GTC-3′) and ended in the second intron of *islet1* genomic DNA. *islet1E3* began at position 472 of *islet1* cDNA at the end of exon 3 (5′-GAATGCAATGCCTACCTGCCATTTG-3′) and ended in the third intron of *islet1* genomic DNA. The sequence for *islet1E2* MO differs from the corresponding sequence in *islet2* genomic DNA at the end of exon 2 (5′-GATTACGTACGGTACGAGCAACTAT-3′) by 13 bp. The end of exon 3 in *islet2* genomic DNA sequence (5′-ATCCCAGGTAGTAGTAAAAATA-ATA-3′) is different from *islet1E3* MO sequence by 18 bp. Several nanoliters of 1 mg/ml *islet1E2* and 1 mg/ml *islet1E3* were co-injected into one-cell stage embryos as described previously (Lewis and Eisen, 2001). Embryos looked generally healthy and had little or no Islet1 protein remaining. The same phenotype was observed when an *islet1* translation blocking MO beginning at position –25 in the 5′UTR (5′-CCCATGTCAAGAAAGTA-AGGCGGTG-3′) was injected into one-cell stage embryos.

To create embryos with reduced Islet2, a translation blocking morpholino was designed by Gene Tools to the translation start site. *islet2* MO began at position –2 of *islet2* 5′UTR (5′-GGATGCGGTAGAATATCCACCATAC-3′) and was tagged with fluorescein. Several nanoliters of 5 mg/ml were injected into one-cell stage embryos as describe previously (Lewis and Eisen, 2001). A second morpholino also designed to the translation start site of *islet2* gave the same phenotype as the first *islet2* MO. The second *islet2* MO began at position –9 of the *islet2* 5′UTR (5′-GTAGAAT-ATCCACCATACAGGAGGG-3′). Several nanoliters of 1 mg/ml *islet1E2,* 1 mg/ml *islet1E3* and 5 mg/ml *islet2* MOs were co-injected into one-cell stage embryos to eliminate both Islet1 and Islet2 proteins.

Quantitation

We quantified the efficiency of our splice-blocking MOs by counting the number of cell nuclei labeled with *islet1* RNA adjacent to somites 8-11 and calculating the percentage of cells with nuclear *islet1* RNA labeling in *islet1* MO-injected versus control (uninjected) embryos at 20-21 hpf. We also counted the number of cells in the pMN domain adjacent to somites 8-11 labeled with Islet Ab at 28 hpf and calculated the percentage of Islet-positive cells in *islet1* MO-injected embryos versus control embryos.

PMN axons stained with zn1 and znp1 Abs were counted in embryos at 24 or 28 hpf. Axons were counted as belonging to MiPs if they extended caudal and dorsal to the CaP cell body. Axons were counted as long CaP axons if they projected ventrally of the horizontal myoseptum. Axons were counted as short CaP axons if they exited the spinal cord, but did not project ventral of the horizontal myoseptum. To represent the number of axons in uninjected control versus injected embryos, we calculated the percentage of axons remaining in segments 8-12.

Islet Ab-labeled cells in the pMN domain adjacent to somites 8-10 were counted at 72 hpf from confocal microscopy images. We calculated the percentage of Islet-positive cells in MO-injected embryos in comparison with controls.

To count interneurons, we stained embryos with GABA Ab at 24 and 28 hpf and imaged them by confocal microscopy as described above. Cells were counted as in the KA″ position if they were in the medioventral spinal cord directly lateral to the floor plate. Cells were counted as in the V-K position if they were within three cell diameters dorsal of the floor plate. The average number of interneurons in the neural tube adjacent to segments 8-11 was compared between control and injected embryos.

To examine cell death, the number of cells co-expressing TUNEL and Lhx4 was counted in the ventral spinal cord adjacent to somites 8-11.

RESULTS

Islet1 protein is required for motoneuron formation

We tested the role of Islet1 in motoneuron formation by injecting one-cell stage embryos with morpholino antisense oligonucleotides (MOs) designed to block the splice donor sites at the ends of the second and third exons of *islet1* genomic DNA. *islet1* mRNA was localized in the nucleus of PMNs in *islet1* MO-injected embryos (Fig. 1B,C), indicating that the MO blocked *islet1* splicing (Yan et al., 2002). We also stained embryos at 28 hpf with an Islet Ab that recognizes both Islet1 and Islet2 proteins (Korzh et al., 1993; Thor et al., 1991). Islet Ab staining was absent from *islet1* MO-injected embryos, indicating that Islet1 and Islet2 proteins were both significantly reduced (Fig. 1D,E). The reduction in Islet2 protein could result from *islet1* MOs affecting the splicing of *islet2* RNA. However, this is extremely unlikely because the splice site sequences are very different (see Materials and methods). In addition, *islet2* mRNA was absent from *islet1* MO-injected embryos (Fig. 2A,B). This contrasts with the nuclear localization of *islet1* mRNA in these embryos. These results are consistent with the idea that our *islet1* MOs are specific for *islet1*, and raise the possibility that Islet1 regulates *islet2* expression or that PMNs are not specified in the absence of Islet1 and thus do not express later PMN markers, such as *islet2*.

To examine the role of Islet1 in PMN formation, we looked for defects in motor axon outgrowth at 24 and 28 hpf in *islet1* MO-injected embryos using zn1 and znp1 Abs, which recognize motoneurons (Melancon et al., 1997; Trevarrow et al., 1990). We assayed the number of motor axons in segments 8-12 and found that both dorsally projecting MiP and ventrally projecting CaP axons were significantly reduced in *islet1* MO-injected embryos (Fig. 2D,E; Table 1). *islet1* MO-injected embryos had a few truncated CaP axons, something never seen in control embryos (Table 1; data not shown). However, the number of truncated CaP axons in *islet1* MO-injected embryos was significantly fewer than the number of normal CaP axons in control embryos. We examined the specificity of our *islet1* MOs by co-injection of *islet1* RNA and *islet1* MO, and found that PMN axons were restored and appeared normal (Fig. 2F; Table 1). Thus, we conclude that Islet1 is required for zebrafish PMN formation. Interestingly, we also found that, in most cases, injection of *islet1* RNA did not restore *islet2* mRNA expression (Fig. 2C). These data suggest that that Islet1 does not regulate *islet2* mRNA expression and support the hypothesis that in the absence of Islet1 PMNs are not specified, and thus do not express later markers, such as *islet2*.

Having found that Islet1 is required for formation of zebrafish PMNs, we asked whether it is also required for formation of SMNs. We assessed the role of Islet1 protein on SMN formation at 72 hpf by staining embryos with Neurolin Ab, which recognizes SMNs but not PMNs (Fashena and Westerfield, 1999). Essentially all SMN dorsally projecting and ventrally projecting axons were absent from embryos injected with *islet1* MO (Fig. 3A,B), and the number of Islet-positive cells in the ventral spinal cord was decreased by 52% (Fig. 3A′,B′). The presence of Islet-positive cells in the ventral spinal cord of *islet1* MO-injected embryos might result from reduction of MO efficacy as the embryos developed over several days. Alternatively, it might be due to the presence of Islet2 protein. We did two experiments to distinguish between these possibilities. First, we injected one-cell stage embryos with a MO designed to block the *islet2* translation start site (*islet2* MO) and found that this caused a 10% reduction in the number of Islet-positive cells in the ventral spinal cord (Fig. 3A,A′,C,C′), an almost total loss of dorsally projecting SMN axons

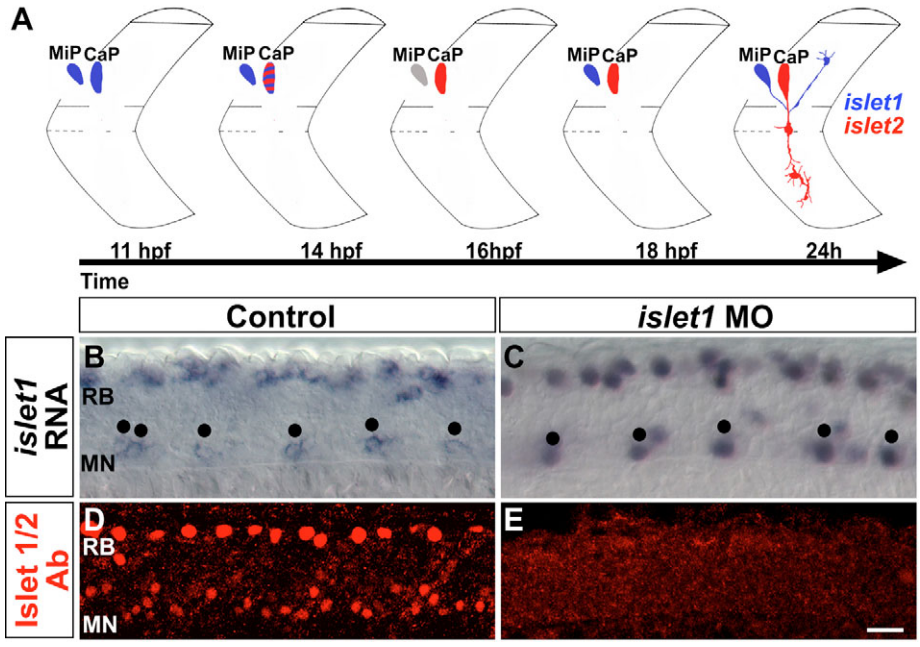


Fig. 1. Islet1 regulates Islet2 expression. In this and subsequent figures, all photographs show 8-12 segment region of whole-mount embryos with rostral towards the left and dorsal towards the top, unless otherwise noted. (**A**) Schematic showing expression of *islet1* (blue) and *islet2* (red) in CaP and MiP between 11 and 24 hpf. Blue and red stripes indicate co-expression of *islet1* and *islet2*; grey indicates downregulation of *islet1*. (**B**) At 20 hpf, *islet1* RNA is expressed in dorsal Rohon-Beard sensory neurons (RB) and MiPs in control embryos. MN designates the row containing MiPs; individual MiPs are marked by black dots. In the segment farthest to the left, both MiP and RoP are indicated by black dots. RoPs are PMNs that express *islet1* later than MiPs (Appel et al., 1995), and thus are absent from most of our figures. (**C**) *islet1* MO-injected embryos express *islet1* in RBs and MiPs, but the RNA is nuclear instead of cytoplasmic as it is in controls (47% of PMNs have nuclear *islet1* RNA staining in *islet1* MO-injected embryos when compared with 1% in controls*; *n*=7 *islet1* MO-injected embryos; *n*=8 control embryos); black dots indicate MiPs. (**D**) At 28 hpf, Islet Ab (red) labels RBs and motoneurons (MN) in control embryos. (**E**) Islet Ab labeling is absent from *islet1* MO-injected embryos (92% fewer cells labeled with Islet Ab in the pMN domain*; *n*=22 *islet1* MO-injected embryos; *n*=26 control embryos). *P<0.001. Scale bar: 20 μm.

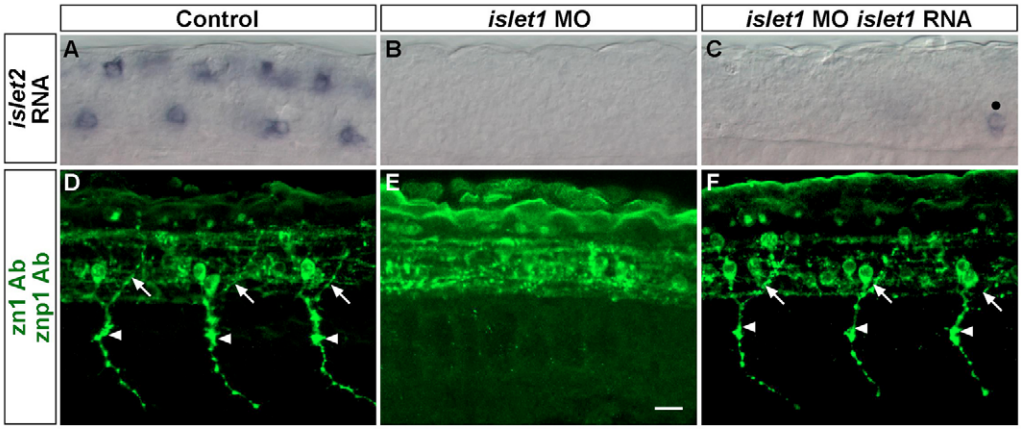


Fig. 2. Islet1 is required for PMN formation. (A-C) 20 hpf embryos stained with *islet2* riboprobe. Control embryos (**A**) express *islet2* in RBs (dorsally located cells) and CaPs (ventrally located cells). *islet1* MO-injected embryos (**B**) lack *islet2* expression. *islet1* MO-injected embryos co-injected with *islet1* RNA (**C**) also lack most *islet2* expression; one *islet2*-positive PMN is indicated by a black dot. (D-F) 28 hpf embryos stained with zn1 and znp1 Abs (green). Control embryos (**D**) have dorsally projecting MiP axons (arrows) and ventrally projecting CaP axons (arrowheads). *islet1* MO-injected embryos (**E**) lack both MiP and CaP axons. Co-injection of *islet1* MO and *islet1* RNA (**F**) restored both MiP and CaP axons. Scale bar: 20 μm.

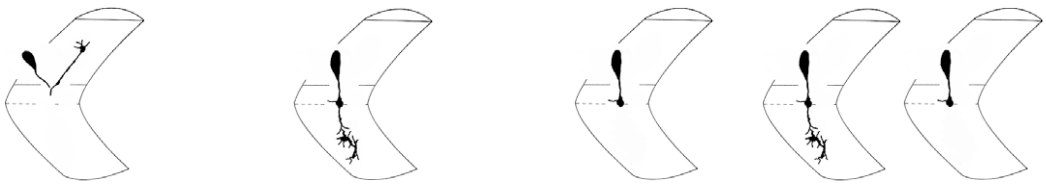
and a partial loss and disorganization of ventrally projecting SMN axons (Fig. 3C). Second, we co-injected *islet1* and *islet2* MOs and found that this caused a nearly complete loss of Islet-positive cells from the ventral spinal cord (Fig. 3D,D′). *islet1* MO-injected embryos had many more Islet-positive cells in the ventral spinal cord than did embryos injected with both *islet1* and *islet2* MOs; however, the severe reduction of SMN axon projections out of the spinal cord was similar in both cases (Fig. 3B,D). These data provide evidence that the Islet Ab labeling of SMN cell bodies in *islet1* MO-injected embryos was due to the presence of Islet2 protein. They also support the idea that Islet1 protein is required for SMN formation and Islet2 protein is required for proper SMN axon outgrowth.

Islet1 is required to specify PMN fate

In mouse, loss of Islet1 leads to absence of motoneurons and widespread cell death in the ventral spinal cord (Pfaff et al., 1996). By contrast, our analysis of *islet1* RNA expression in *islet1* MO-injected embryos revealed that PMN cell bodies were still present in the absence of Islet1 (Fig. 1B,C). To confirm this, we examined cell death in the ventral spinal cord at 28 hpf by co-labeling embryos with TUNEL and Lhx4 Ab, which labels PMNs as well as some other neurons in the ventral spinal cord (S.A.H. and J.S.E., unpublished). The number of Lhx4-positive cells was the same in *islet1* MO-injected and control embryos, and we observed no increase in the number of cells positive for TUNEL in experimental embryos, indicating that PMNs did not die in the absence of Islet1 (Table 2).

Although PMN cell bodies were present in the absence of Islet1, these cells did not project axons out of the spinal cord, thus they did not meet the criteria to be defined as motoneurons. The zebrafish pMN domain generates several types of interneurons in addition to PMNs and SMNs (Kimmel et al., 1994; Park et al., 2004), raising the possibility that PMNs might develop as interneurons in the absence of Islet1. We tested this hypothesis by examining co-expression of Lhx3 and zn1. In control embryos, PMNs are the

Table 1. Islet1 is required for PMN axon outgrowth



	MiP axons		CaP axons		Short CaP axons		Total CaP axons	
	24 hpf	28 hpf	24 hpf	28 hpf	24 hpf	28 hpf	24 hpf	28 hpf
Control	74.8% (*n*=127)	99.1% (*n*=320)	100% (*n*=127)	100% (*n*=320)	0% (*n*=127)	0% (*n*=320)	100% (*n*=127)	100% (*n*=320)
islet1 MO	11.1%* (*n*=270)	12.2%* (*n*=450)	14.8%* (*n*=270)	3.8%* (*n*=450)	35.9%* (*n*=270)	12.7%* (*n*=450)	50.7%* (*n*=270)	16.4%* (*n*=450)
islet1 MO *islet1* RNA	N/A	79.6% (*n*=250)	N/A	69.2% (*n*=250)	N/A	21.6% (*n*=250)	N/A	90.8% (*n*=250)
islet1 MO *islet2* RNA	N/A	57.8% (*n*=185)	N/A	71.9% (*n*=185)	N/A	6.5% (*n*=185)	N/A	78.4% (*n*=185)

Percentage of spinal segments with axons at axial level 8-12.
*P<0.00001 in comparison with controls.
n, number of segments.
N/A, not available.

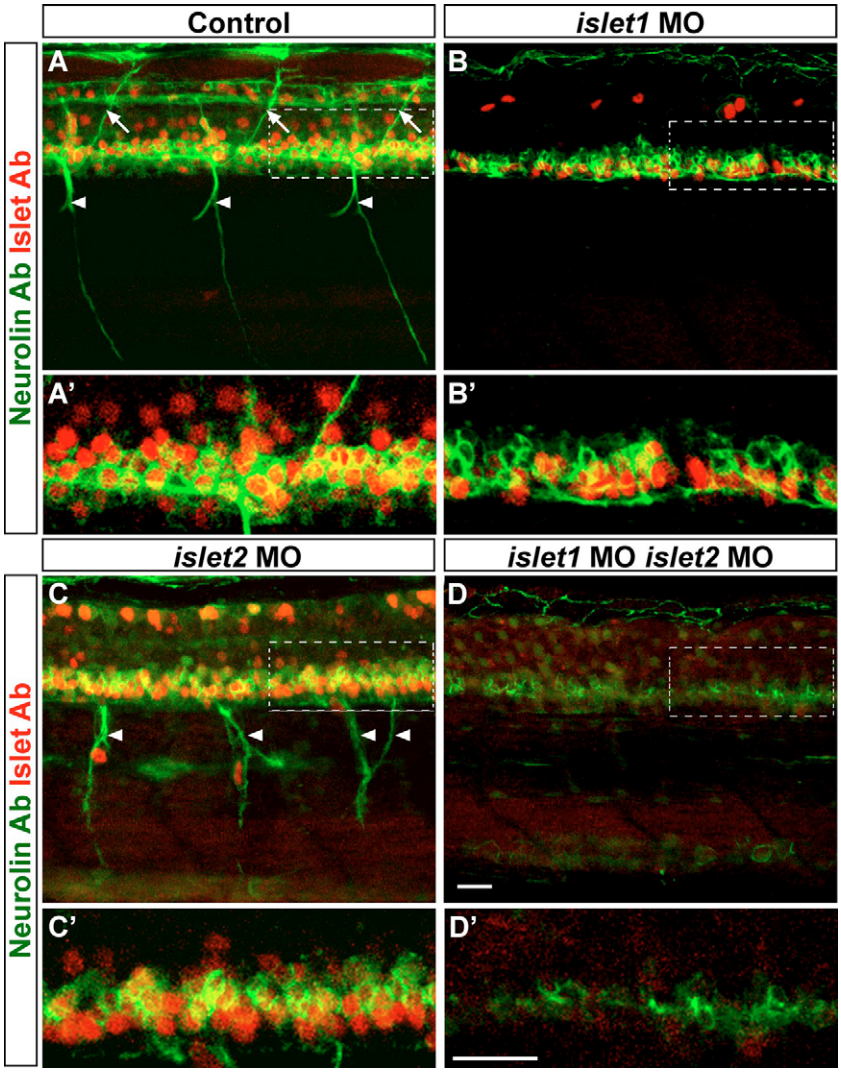


Fig. 3. Islet proteins are required for SMN formation. (A-D) 72 hpf embryos stained with Neurolin (green) and Islet (red) Abs. For each panel, one segment (outlined) is magnified and shown below (A′-D′). Control embryos (**A**) had dorsally projecting (arrows) and ventrally projecting (arrowheads) Neurolin-positive SMNs and many Islet-positive cells. *islet1* MO-injected embryos (**B**) lacked SMN axons and had 52% fewer Islet-positive cells (**P*<0.01, 18 segments of three embryos). *islet2* MO-injected embryos (**C**) had 10% fewer SMN cell bodies (**P*<0.01, 24 segments of four embryos). These embryos entirely lacked dorsally projecting SMN axons and ventrally projecting SMN axons (arrowheads) were disorganized. The cells labeled with Islet Ab outside the neural tube next to the ventrally projecting axons are most likely dorsal root ganglion cells that are out of position. Embryos co-injected with *islet1* and *islet2* MOs (**D**) lacked all SMNs and 99.5% of Islet-positive cells were absent (**P*<0.01, 18 segments of three embryos), although Neurolin-positive floor plate was still present. Scale bars: 20 μm.

predominant cell type co-labeled by Lhx3 and zn1 antibodies (Fig. 4A); however, both of these antigens are also expressed by VeLD interneurons (Appel et al., 1995) that are derived from the pMN domain (Park et al., 2004). *islet1* MO-injected and control embryos had approximately the same number and distribution of cells co-labeled with Lhx3 and zn1 Abs. In control embryos most Lhx3$^+$, zn1$^+$ cells projected axons out of the spinal cord (Fig. 4A), consistent with their PMN identity. By contrast, in *islet1* MO-injected embryos Lhx3$^+$, zn1$^+$ cells did not project axons out of the spinal cord, instead projecting axons within the spinal cord (Fig. 4B), similar to interneurons, and consistent with the hypothesis that in the absence of Islet1, PMNs developed as interneurons.

To further test the idea that in the absence of Islet1 PMNs develop as interneurons, we stained embryos with an antibody to the neurotransmitter GABA which is expressed in several types of pMN domain-derived interneurons, including VeLD, KA′ and KA″ (Bernhardt et al., 1992; Park et al., 2004). We counted the number of cells in the VeLD, KA′ and KA″ positions in the spinal segments adjacent to somites 8-11. Cells were considered to be in the KA″ position if they were located immediately lateral to the floor plate. The number of cells in the KA″ position was similar in control and *islet1* MO-injected embryos (Fig. 4C,D; Table 3). Cells in the VeLD and KA′ positions were counted together because, although both cell types are located dorsal to KA″ neurons (Park et al., 2004) and within three cell diameters dorsal of the floor plate, they are hard to distinguish without additional markers. Thus, we refer to cells in this location as V-K. There were significantly more cells in the V-K position of *islet1* MO-injected embryos than in controls (Fig. 4C,D; Table 3). Interestingly, although an average of 24 PMNs were lost from the spinal cord adjacent to somites 8-11 of *islet1* MO-injected

Table 2. PMNs do not die in *islet1* MO-injected embryos

	Number of Lhx4$^+$ cells		Number of Lhx4$^+$/TUNEL$^+$ cells	
	20 hpf	28 hpf	20 hpf	28 hpf
Control	49.1±9.5 (*n*=27)	99.3±12.3 (*n*=15)	0 (*n*=27)	0 (*n*=15)
islet1 MO	51.4±10.5* (*n*=28)	93.2±15.5* (*n*=11)	0 (*n*=28)	0 (*n*=11)

Number of Lhx4$^+$ cells in the ventral spinal cord at axial level 8-12.
Values given ±s.d.
**P*>0.05; *n*, number of embryos.

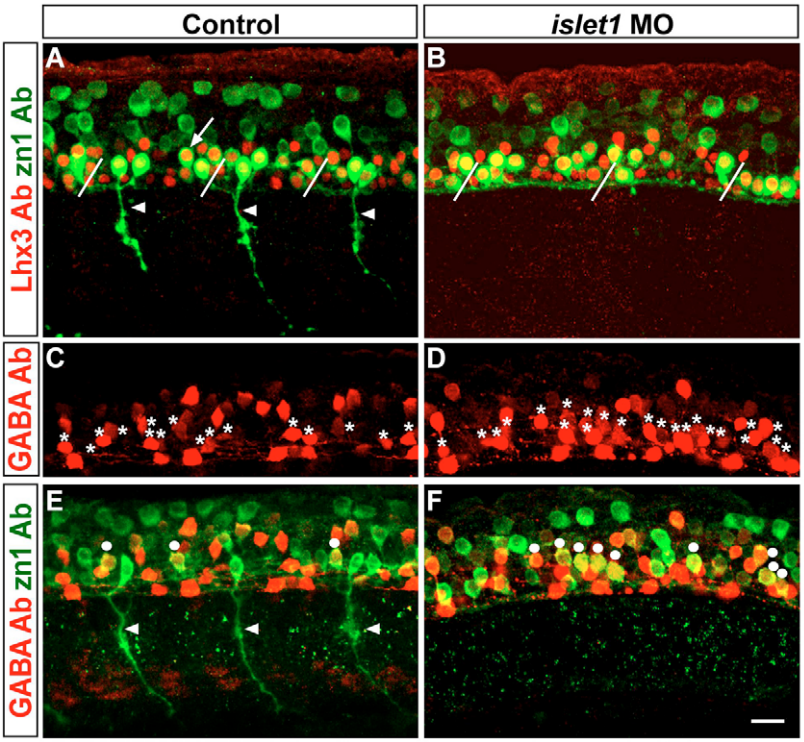


Fig. 4. Islet1 is required to inhibit interneuron formation. (**A**,**B**) Embryos co-stained with Lhx3 (red) and zn1 (green) Abs. (A) In control embryos, zn1 and Lhx3 were co-expressed in motoneurons that projected axons (arrowheads) out of the spinal cord and in VeLD interneurons (one is indicated by an arrow; slanted lines denote somite boundaries). (B) *islet1* MO-injected embryos had cells that co-expressed Lhx3 and zn1, but they did not project axons out of the spinal cord, and instead had axons that projected caudally within the spinal cord. (**C**,**D**) Embryos stained with GABA Ab. (C) In control embryos, GABA Ab reveals KA″, KA′, VeLD and other (unidentified) interneurons; cells in the V-K position are marked by asterisks. (D) Cells in the V-K position (asterisks) are more numerous in *islet1* MO-injected embryos. (**E**,**F**) The same embryos shown in C and D, but here showing co-labeling with GABA (red) and zn1 (green) Abs. Dots indicate cells co-expressing GABA and zn1. (E) In control embryos, only a few cells co-express these markers. Arrowheads in E indicate CaP axons. (F) In *islet1* MO-injected embryos there are many more cells that co-express zn1 and GABA. All embryos shown in this figure are at 28 hpf. Scale bar: 20 μm.

embryos, there were only an average of 11 extra GABA-positive cells in the V-K position (Table 3), suggesting that some PMNs did not develop into GABA-expressing interneurons. To determine whether this was the case, we co-labeled embryos with GABA and zn1 Abs. At 28 hpf these antigens co-localized in a few cells in the V-K position in control embryos (Fig. 4E). *islet1* MO-injected embryos had more cells in the V-K position that co-expressed GABA and zn1 (Fig. 4F). However, some of the zn1-positive interneuron-like cells did not co-express GABA (Fig. 4F), suggesting that some PMNs developed into a type of interneuron distinct from VeLDs or KA′ neurons. Alternatively, these cells may have adopted a hybrid identity in which they developed interneuron-like axonal projections, without expressing interneuron markers. Without interneuron type-specific markers, we are unable to distinguish between these possibilities. Together these data provide evidence that *islet1* MO-injected embryos have more pMN domain-derived interneurons than control embryos, and suggest that these supernumerary cells are PMNs that have become interneurons in the absence of Islet1.

islet1 RNA misexpression induces supernumerary motoneurons and inhibits interneuron formation

Our studies using *islet1* MO showed that Islet1 is required to promote PMN formation and to inhibit interneuron formation. To test whether *islet1* is sufficient to induce PMNs, we misexpressed *islet1* RNA at the one-cell stage and examined the number of PMNs at 28 hpf. Zn1 and znp1 Ab labeling showed that *islet1* RNA-injected embryos had supernumerary PMN cell bodies and thicker ventral motor nerves (Fig. 5A,B). Some *islet1* RNA-injected embryos had supernumerary *islet2*-positive cells in the ventral neural tube (Fig. 5C,D), consistent with the hypothesis that some of the supernumerary PMNs were CaPs whose axons contributed to the thicker ventral motor nerve. Interestingly, the supernumerary PMN cell bodies were not scattered throughout the spinal cord, but were localized in the normal dorsoventral position of PMNs, suggesting that the ability of Islet1 to specify PMN development is limited to cells derived from the pMN domain.

Table 3. Islet1 is required to inhibit interneuron formation

	V-K position		KA″ position	
	24 hpf	28 hpf	24 hpf	28 hpf
Control	15.1±4.7 (*n*=41)	20.7±3.6 (*n*=31)	9.2±2.4 (*n*=32)	13±1.7 (*n*=35)
islet1 MO	26.0±4.6* (*n*=22)	31.7±4.5* (*n*=20)	9.8±2.8‡ (*n*=20)	12.6±2.3‡ (*n*=16)
islet1 RNA	12.5±4.5† (*n*=27)	14.2±4.3* (*n*=22)	9.6±2.4‡ (*n*=18)	13.8±2.5‡ (*n*=21)

Number of GABA-positive interneurons at axial level 8-11.
Values given±s.d.
*P<0.00001; †P<0.05; ‡not significant; *n*, number of embryos.
Red cells in diagram represent the interneuron population counted in columns below picture.

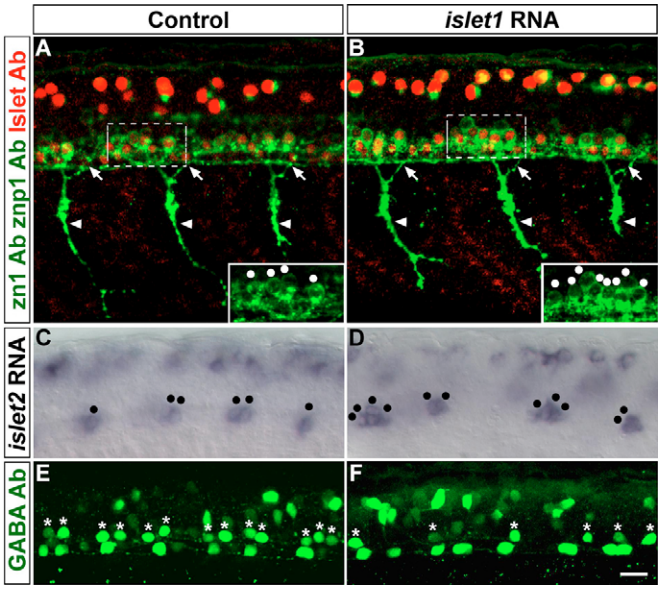


Fig. 5. Misexpression of *islet1* RNA induces PMN formation and inhibits interneuron formation. (**A**,**B**) 28 hpf embryos labeled with zn1 and znp1 (green) and Islet (red) Abs. Control embryos (A) have MiP axons (arrows), CaP axons (arrowheads) and several PMN cell bodies (inset; zn1 Ab; dots). Misexpression of *islet1* RNA (B) causes a thicker ventral motor nerve (arrowheads) and more zn1-positive cell bodies (inset, dots). (**C**,**D**) 20 hpf embryos labeled with *islet2* riboprobe. *islet2* is expressed in one or two PMNs per segment (black dots) in control embryos (C); the second cell is VaP, a duplicate CaP that is sometimes present and typically dies (Eisen et al., 1990). *islet2* is expressed in two to four PMNs per segment in embryos misexpressing *islet1* RNA (D). (**E**,**F**) 28 hpf embryos labeled with GABA Ab. The number of cells in the V-K position (asterisks) is decreased in embryos misexpressing *islet1* RNA (F) when compared with controls (E). Scale bar: 20 μm.

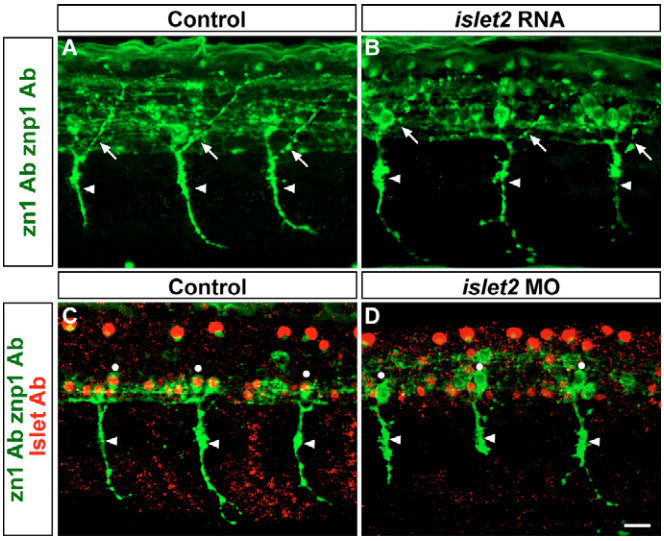


Fig. 6. Formation of CaP subtype identity is independent of Islet2. (**A**,**B**) Embryos labeled with zn1 and znp1 Abs (green). Arrows indicate MiP axons, arrowheads indicate CaP axons. At 28 hpf, control embryos (A) have both CaP and MiP axons. Embryos misexpressing *islet2* RNA (B) also have normal MiP and CaP axons. (**C**,**D**) Embryos labeled with zn1 and znp1 (green), and Islet (red) Abs. Arrowheads indicate CaP axons. CaP cell bodies (dots) co-label with Islet and zn1 Abs and project axons ventrally at 28 hpf in control embryos (C). *islet2* MO-injected embryos (D) lack Islet staining in CaP cell bodies and have abnormal CaP axons. Scale bar: 20 μm.

To learn whether the supernumerary PMNs in *islet1* RNA-injected embryos correlated with a loss of ventral interneurons, we stained *islet1* RNA-injected embryos with GABA Ab and counted the number of pMN domain-derived interneurons. There was a significant reduction of cells in the V-K position in *islet1* RNA-injected embryos, but the number of cells in the KA″ position was unchanged from controls (Fig. 5E,F; Table 3). Thus, *islet1* RNA misexpression inhibited interneuron formation and promoted PMN formation, but this effect appeared limited to a subset of interneurons derived from the pMN domain. These results support our hypothesis that Islet1 is required to promote PMN fate and inhibit interneuron fate. However, they contrast with results from mouse in which *islet1* misexpression did not induce motoneuron formation (Thaler et al., 2002).

CaP subtype specification is independent of Islet2

The specific expression of Islet2 in CaPs, but not in MiPs, led us to hypothesize that Islet2 is required for CaP subtype identity. CaPs transiently co-express *islet1* and *islet2*, but they downregulate expression of *islet1* prior to axogenesis (Appel et al., 1995). Recent characterization of narrow somite mutants revealed that PMNs that maintain co-expression of Islet1 and Islet2 develop a CaP axon trajectory (Lewis and Eisen, 2004), suggesting that Islet2 is sufficient to cause a PMN to become a CaP, even when Islet1 is not downregulated. Therefore, we asked whether misexpression of Islet2 could turn MiPs into CaPs. We found that misexpression of *islet2* RNA had no effect on formation of dorsally projecting MiP axons (Fig. 6A,B). Thus, MiPs maintained their subtype identity despite co-expressing Islet1 and Islet2, providing evidence that Islet2 is not sufficient to turn a MiP into a CaP.

To further test whether Islet2 is required for CaP subtype identity, we injected embryos with *islet2* MO to block Islet2 protein formation. As a control, we stained embryos with Islet Ab and looked for loss of Islet protein in CaPs. After 15 hpf, CaPs express Islet2, but not Islet1 (Appel et al., 1995); therefore, we were able to use Islet Ab staining after 15 hpf to assay loss of Islet2 in CaPs. Islet protein was absent from CaP cell bodies in *islet2* MO-injected embryos, indicating the MO was able to knock down Islet2 protein (Fig. 6C,D). *islet2* MO-injected embryos had some CaPs with truncated axons and some with abnormally branched axons; however, many CaPs were normal. These results suggest that Islet2 is not required for formation of the ventral axon that defines the CaP subtype identity, but that it is involved in later aspects of CaP axon pathfinding. Together with our finding that *islet1* RNA can rescue all PMNs in *islet1* MO-injected embryos even when *islet2* mRNA expression is not induced (Fig. 2A-C), this result suggests that Islet1 alone is sufficient for specification of CaP subtype identity.

Islet2 can promote motoneuron formation and substitute for Islet1 in MiP formation

Our observation that Islet1 alone was sufficient for specification of CaP subtype identity prompted us to ask whether Islet2 was similarly sufficient to specify CaP. We tested whether Islet2 could promote CaP formation in the absence of Islet1 by misexpressing *islet2* RNA in *islet1* MO-injected embryos and labeling them with zn1 and znp1 Abs. We found that CaP formation was restored in these embryos (Fig. 7A,B; Table1), revealing that, similar to Islet1, Islet2 is sufficient to specify CaP subtype identity. These results also reveal that Islet2 can substitute for Islet1 in motoneuron formation.

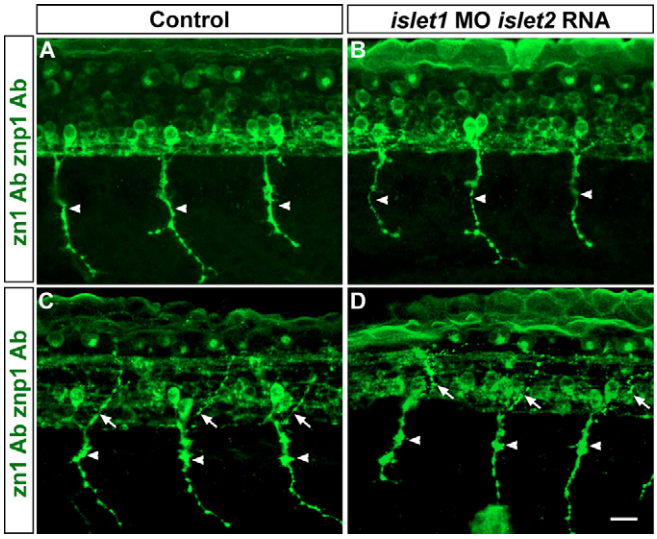


Fig. 7. Islet1 and Islet2 can function redundantly in PMN formation. (A-D) 28 hpf embryos stained with zn1 and znp1 (green) Abs. CaP axons are indicated by arrowheads; MiP axons are indicated by arrows. Control embryos (**A**) have normal CaP axons. Embryos co-injected with *islet2* RNA and *islet1* MO (**B**) have normal CaP axons. Control embryos (**C**) have dorsally projecting MiP axons. MiP axons are present in embryos co-injected with *islet1* MO and *islet2* RNA (**D**). Scale bar: 20 μm.

Our results suggest that the differences between the Islet1 and Islet2 proteins are not important for CaP formation. Therefore, we asked whether the differences between these proteins mattered for formation of other PMN subtypes. MiPs never express Islet2, thus we investigated whether Islet2 could substitute for Islet1 in MiP formation. We misexpressed *islet2* RNA in *islet1* MO-injected embryos and found that this restored normal MiP development (Fig. 7C,D; Table1). Our results reveal that Islet1 or Islet2 is sufficient to specify both CaP and MiP subtype identity. Thus, the differences between these proteins cannot be what controls PMN subtype specification. These results do not support our original hypothesis, that the differences between the Islet1 and Islet2 proteins are responsible for the differences between the MiP and CaP subtypes. Instead, they suggest that PMN subtype specification depends on upstream factors that regulate the differential expression of *islet1* and *islet2* in MiP and CaP, or on factors that act in parallel with Islet1 and/or Islet2.

DISCUSSION

We report three key findings. First zebrafish Islet1 protein is required not only to promote PMN formation, but also to inhibit interneuron formation. Second, despite distinct expression patterns, Islet2 can substitute for Islet1 to promote PMN formation. Finally, PMN subtype specification is independent of the differences between the Islet1 and Islet2 proteins.

Islet1 promotes motoneuron formation at the expense of interneuron formation

Zebrafish Islet1 is required for both SMN and PMN formation, and appears to mediate a switch between motoneuron and interneuron fates in the pMN domain. This apparently contrasts with the reported role of Islet1 in mouse, to promote motoneuron survival (Pfaff et al., 1996). However, several additional studies raise the possibility that in mouse and chick, Islet1 may also inhibit interneuron formation. For example, transplanting neural tubes from Islet1-deficient mice into chicks prevents the death of nascent motoneurons. These surviving cells express interneuron markers (Thaler et al., 2004), although it is unclear whether they project motoneuron-like axons out of the spinal cord or interneuron-like axons within the spinal cord. Similarly, mouse embryos with a targeted deletion of the Mnx family member Hb9 initially express Islet1 in nascent motoneurons, allowing these cells to develop as motoneurons and extend axons out of the spinal cord. However, Islet1 expression is very quickly extinguished in these mice, and motoneurons express interneuron markers (Arber et al., 1999; Thaler et al., 1999), suggesting that both Hb9 and Islet1 may participate in inhibiting interneuron formation. Together, these results support the idea that in mouse and chick, as in zebrafish, Islet1 may play a role in inhibiting interneuron formation.

Whether Islet1 normally mediates a decision between motoneuron and interneuron fates may depend whether these cells are derived from the same progenitor population. Although lineage studies in chick using recombinant retroviruses provided evidence that an individual spinal cord progenitor cell can generate both motoneurons and interneurons (Leber et al., 1990), more recent studies in both chick and mouse have advocated the idea that motoneurons arise from the pMN domain, whereas interneurons are generated from adjacent p3 and p2 domains, as well as from other domains that are more distal from the pMN domain (Briscoe and Ericson, 2001; Briscoe et al., 2000). In mouse and chick, pMN domain-derived motoneurons co-express Lhx3 and Islet1, whereas V2 interneurons, which are derived from the p2 progenitor domain situated just dorsal to the pMN domain, express Lhx3 but not Islet1 (Ericson et al., 1992; Sharma et al., 1998; Tanabe et al., 1998). Studies in the chick spinal cord show that misexpression of Islet1 alone has no effect on motoneuron formation, whereas misexpression of Lhx3 alone promotes V2 interneuron formation (Tanabe et al., 1998; Thaler et al., 2002). Misexpression of both Lhx3 and Islet1 causes cells to become motoneurons, even when they do not originate from the pMN domain (Thaler et al., 2002). As in mouse and chick, zebrafish PMNs co-express Islet1 and Lhx3, whereas VeLD interneurons express Lhx3 but not Islet1 (Appel et al., 1995). However, in contrast to mouse and chick, PMNs and VeLD interneurons are both derived from the pMN domain (Park et al., 2004). Clonal analysis in zebrafish reveals that a single ventral neural tube progenitor in the pMN domain can generate PMNs, interneurons, or both PMNs and interneurons; however, there is no consistent lineage relationship among these cell types (Kimmel et al., 1994; Park et al., 2004). Zebrafish lacking Islet1 lack PMNs, but have a normal number of $Lhx3^+$ pMN domain cells, consistent with the idea that loss of Islet1 results in pMN domain-derived cells that express only Lhx3 and therefore develop as interneurons. In contrast to chick, misexpression of zebrafish Islet1 alone leads to formation of supernumerary PMNs. However, these cells only form in the normal PMN position, suggesting that $Lhx3^+$ cells within the pMN domain become PMNs when they co-express Islet1. Thus, we suspect that in zebrafish the fate decision between PMNs and interneurons is determined by the interaction of transcription factors, such as Islet1, that are motoneuron-specific within the pMN domain and Lhx3, which is expressed by both motoneurons and interneurons. This is similar to what Thaler and colleagues proposed happens in chick (Thaler et al., 2002), except that in zebrafish the fate decision appears to occur between cell types generated within the same progenitor domain, whereas in chick it appears to occur between cell types generated in adjacent progenitor domains. If our interpretation is correct, then

Islet1 may only normally mediate a switch between motoneuron and interneuron fates in cells that co-express Lhx3 and are derived from the same progenitor population.

An outstanding question that remains to be addressed is the identity of the supernumerary interneurons that form in zebrafish in the absence of Islet1. The pMN domain generates at least four types of interneurons: VeLD, KA′, KA″ and CiD (Park et al., 2004). Unfortunately, we currently have few markers other than cell morphology to distinguish these cells (Lewis and Eisen, 2003). Using GABA as a marker for VeLD, KA′ and KA″ interneurons, we found that the number of cells in the V-K position increased in *islet1* MO-injected embryos, whereas the number of cells in the KA″ position was unchanged. Interestingly, the number of GABA-positive, supernumerary interneurons in *islet1* MO-injected embryos was about half the number of PMNs that were lost, raising the possibility that some PMNs were only partially transformed into interneurons, and changed their axon trajectory without expressing GABA. Alternatively, there might be an increase in another type of pMN domain-derived interneuron that was not detectable with our markers, or there could have been an increase in several types of interneurons, only some of which express GABA. We are unable to distinguish among these possibilities with the available interneuron markers. Thus, it is crucial to identify cell-type specific markers for pMN domain derivatives to further assess the fates of these cells under different conditions.

Islet1 and Islet2 are functionally redundant

Previous studies have suggested that Islet1 and Islet2 may have redundant functions during motoneuron formation; however, this has not previously been tested. Thaler and colleagues (Thaler et al., 2004) proposed that the level of Islet protein, not the specific type of Islet protein, determines whether a cell becomes a visceral motoneuron. We have tested directly whether Islet1 and Islet2 have redundant functions by co-injecting embryos with *islet1* MO and *islet2* RNA to learn whether Islet2 can substitute for Islet1 during motoneuron formation in zebrafish. We found that Islet2, like Islet1, could promote motoneuron formation, consistent with the hypothesis that the differences between Islet1 and Islet2 proteins are unimportant for motoneuron formation.

CaP and MiP subtype specification is independent of the differences between the Islet1 and Islet2 proteins

What is most surprising is that our results provide evidence that, despite the exquisite and dynamic regulation of expression of *islet1* and *islet2* in zebrafish PMNs (Appel et al., 1995; Inoue et al., 1994; Korzh et al., 1993; Tokumoto et al., 1995), the differences between these proteins are not important in establishing the differences between the different PMN subtypes. Islet2 is expressed only in CaPs, yet our data suggest that either Islet1 or Islet2 is sufficient for specification of CaP subtype identity. Previous studies from our laboratory suggested that Islet2 expression might force PMNs to develop as CaPs, because in some mutants, PMNs expressing both Islet1 and Islet2 formed CaP axon projections and not MiP axon projections (Lewis and Eisen, 2001). Thus, it was surprising that misexpression of *islet2* RNA did not prevent formation of MiP dorsal projections, indicating that in the context of wild-type embryos, Islet2 is insufficient to inhibit MiP development. We also found that knockdown of Islet2 protein resulted in only minor defects in CaP axon outgrowth. These results contrast with a previous study showing that expression of a dominant negative Islet2 LIM domain caused severe defects in CaP projections and in some cases caused CaPs to develop into interneurons (Segawa et al., 2001). However, the same study found that Islet2 knockdown using MOs resulted in a much less severe effect on CaPs that appears to be very similar to what we have described. One possible way to reconcile these results is to imagine that the dominant-negative Islet2 LIM domain interfered with some, but not all Islet1 functions, consistent with the finding of Thaler and colleagues (Thaler et al., 2002) that LIM domains of different LIM-HD proteins can have overlapping and non-overlapping functions. If this were the case, it could significantly lower the efficacy of both Islet2 and Islet1 proteins, resulting in insufficient Islet function to repress interneuron formation. This would then be similar to the result we got from knocking down Islet1 alone, and fits well with the model that the overall levels of Islet protein are important in motoneuron formation (Thaler et al., 2004). Together, these results lead to the surprising conclusion that Islet2 is not required for CaP subtype identity, despite its specific expression in CaP motoneurons.

Islet1 expression is maintained in MiPs but not in CaPs; therefore, we hypothesized that this late expression of Islet1 is required for MiP subtype identity. However, when we substituted Islet2 for Islet1 in embryos co-injected with *islet2* RNA and *islet1* MOs, MiPs formed normal, dorsally projecting axons. These results do not support our original hypothesis, but instead indicate that Islet1 protein is not required for MiP subtype specification if another Islet protein is available.

There have been previous reports that highly related proteins can substitute for one another, despite their distinct expression patterns (Geng et al., 1999; Hanks et al., 1995; Hirth et al., 2001; Wang and Jaenisch, 1997; Wang et al., 1996). Sequence analysis of zebrafish Islet1 and Islet2 proteins indicate they are highly related [98% identity in the DNA-binding homeodomain and 92% or 70% identity in the first and second LIM domains, respectively; Tokumoto et al. (Tokumoto et al., 1995)]. Our data show that Islet1 and Islet2 are also able to substitute for one another functionally during motoneuron formation, suggesting that the regulation of *islet1* and *islet2* transcript expression, rather than the distinct sequences of the proteins they encode, establishes their specific functions. Therefore, transcription factors expressed very early in motoneuron development are likely determinants of PMN subtype identity. *islet1* is the earliest reported gene expressed in PMNs following expression of so-called patterning genes, such as *olig2* (Park et al., 2002) and *nkx6.1* (Cheesman et al., 2004), that are expressed in pMN domain progenitor cells as well as in post-mitotic PMNs (Cheesman et al., 2004; Park et al., 2002). Thus, it will be important to determine whether any of the known patterning genes, or patterning genes yet to be discovered, plays a role in PMN subtype specification by regulating *islet* expression.

Islet proteins function in motoneuron development in other taxa

Islet1 appears to be expressed in all vertebrate motoneurons and in every instance in which it has been examined, it seems to be necessary for their formation. By contrast, the single *islet* gene of the fruit fly, *Drosophila melanogaster*, is expressed only in a subset of motoneurons that project their axons ventrally (Thor and Thomas, 1997) and thus cannot be required to confer ‘motoneuron-ness’ (Thor and Thomas, 2002). Similar to zebrafish, in the absence of Islet function Islet-expressing fruit fly motoneurons are present, but their axonal projections are aberrant. In most cases, the cells still send axons into the periphery, but they fail to make appropriate neuromuscular connections. Consistent with this, overexpression of

Islet also causes some motoneurons to project to inappropriate muscles. Interestingly, however, two Islet-positive fruit fly motoneurons apparently do not project axons into the periphery in the absence of Islet function, but instead project axons within the CNS, in essence acting as though they have become interneurons, similar to what we have reported for zebrafish PMNs in the absence of Islet1. No *islet* homolog has been reported in the nematode worm, *Caenorhabditis elegans*. However, three related LIM-HD genes, *lin-11* (Hobert and Ruvkun, 1998), *lim-6* (Hobert et al., 1999) and *lim-4* (Tsalik et al., 2003), function in aspects of development of specific *C. elegans* motoneurons: axon pathfinding in the case of *lin-11*, neurotransmitter receptor expression in the case of *lim-4* and axon pathfinding and neurotransmitter synthesis in the case of *lim-6*. Thus far, mouse is the only species in which a LIM-HD protein, in this case Islet1, appears required for motoneuron survival (Pfaff et al., 1996). Other LIM-HD proteins are expressed in tetrapod vertebrate motoneurons (Sharma et al., 1998; Tsuchida et al., 1994), but like the LIM-HD proteins of flies, worms and zebrafish, these all seem to function in later aspects of motoneuron development, especially axon pathfinding and neurotransmitter choice. Thus, it will be important to study motoneuron development and LIM-HD protein function in other species to fully understand how Islet1 function has changed over time.

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