

Prdm14 acts upstream of *islet2* transcription to regulate axon growth of primary motoneurons in zebrafish

Chao Liu^{1,2}, Weirui Ma^{1,2}, Wenjing Su^{1,2} and Jian Zhang^{1,*}

SUMMARY

The precise formation of three-dimensional motor circuits is essential for movement control. Within these circuits, motoneurons (MNs) are specified from spinal progenitors by dorsoventral signals and distinct transcriptional programs. Different MN subpopulations have stereotypic cell body positions and show specific spatial axon trajectories. Our knowledge of MN axon outgrowth remains incomplete. Here, we report a zebrafish gene-trap mutant, *short lightning* (*slg*), in which *prdm14* expression is disrupted. *slg* mutant embryos show shortened axons in caudal primary (CaP) MNs resulting in defective embryonic movement. Both the CaP neuronal defects and behavior abnormality of the mutants can be phenocopied by injection of a *prdm14* morpholino into wild-type embryos. By removing a copy of the inserted transposon from homozygous mutants, *prdm14* expression and normal embryonic movement were restored, confirming that loss of *prdm14* expression accounts for the observed defects. Mechanistically, Prdm14 protein binds to the promoter region of *islet2*, a known transcription factor required for CaP development. Notably, disruption of *islet2* function caused similar CaP axon outgrowth defects as observed in *slg* mutant embryos. Furthermore, overexpression of *islet2* in *slg* mutant embryos rescued the shortened CaP axon phenotypes. Together, these data reveal that *prdm14* regulates CaP axon outgrowth through activation of *islet2* expression.

KEY WORDS: Prdm14, Zebrafish, Motoneuron, Axon outgrowth, Islet2

INTRODUCTION

The formation of interconnected neural circuit requires the proper positioning of different populations of neurons, the correct projection of axons and dendrites, and the remodeling of complicated neural connections. Extrinsic cues, such as neurotrophic factors, have long been thought to play major roles in setting up neural circuits, especially in promoting axon outgrowth (Markus et al., 2002). In addition to extrinsic cues, accumulating evidence also indicates that cell-intrinsic programs are required for axon growth and target projection. For example, the upregulation of transcription factors such as SnoN, Id2, NFAT and CREB has been reported to induce axon elongation (Kanning et al., 2010; Lee and Pfaff, 2001; Mizuguchi et al., 2001; Pattyn et al., 2003). Interestingly, activation of some of the transcription factors is dependent on extrinsic cues, suggesting that, at least in some cases, extrinsic cues and intrinsic cues are interconnected (Lee and Pfaff, 2001; Mizuguchi et al., 2001; Pattyn et al., 2003). Thus, combinational functions of intrinsic and extrinsic cues might be essential for properly setting up neural circuits.

Among neural networks, motor circuits are responsible for the execution and coordination of animal movement. Within motor circuits, motoneurons (MNs) provide the action connections between the nervous system and motor output. A number of transcription factors have been identified to contribute to the initial MN diversification and in maintaining the identities of MN subtypes (Kanning et al., 2010; Lee and Pfaff, 2001; Lewis and Eisen, 2003). MNs come from the ventrolateral MN domain

region, in which the transcription factors Nkx6.1 and Pax6 are expressed. Combinational activities of these two factors allow expression of Olig2, a bHLH transcription factor, in the domain (Kanning et al., 2010; Pattyn et al., 2003). Olig2, combined with another bHLH factor, Neurogenin 2, activates MN-specific transcription factors such as Mnr2, Hb9, Lim3 and Islet1/2 to specify MN progenitors (Mizuguchi et al., 2001). Two classes of transcription factors encoded by the LIM-HD and Hox genes, in combination of extrinsic factors, also control MN axon outgrowth (Bonanomi and Pfaff, 2010).

In zebrafish, the primary MNs (PMNs) are derived from the ventral spinal cord regions on both sides of the floor plate. PMNs are classified into caudal primary (CaP), middle primary (MiP) and rostral primary (RoP) MNs. Separately, variable primary (VaP) MNs initially develop equivalently to CaP but later degenerate (Eisen et al., 1986; Eisen et al., 1990; Myers et al., 1986; Westerfield et al., 1986). The three major PMNs can be recognized by their stereotypical cell body positions and axon projection patterns (Westerfield et al., 1986). The projection patterns are due to their initial developmental axon selections, rather than being the result of the elimination of wrongly formed projections (Eisen et al., 1986). CaP, MiP and RoP axons leave the spinal cord sequentially at the same ventral root and extend ventrally until they reach the horizontal myoseptum (HMS). After a brief pause at the HMS, the three axon subtypes diverge in their growth pathways within the myotomes. CaP axons further extend ventrally, whereas MiP axons branch and extend dorsally, and RoP axons extend laterally in the HMS (Eisen et al., 1986; Myers et al., 1986; Westerfield et al., 1986).

It is conceivable that different PMNs require unique molecular programs for their specification and morphogenesis (Kanning et al., 2010; Lewis and Eisen, 2003). Among key factors controlling zebrafish PMN development, two LIM-HD transcription factors, Islet1 and Islet2, are essential for PMN development. *islet1* expression is initially detected in all postmitotic PMNs, but its

¹State Key Laboratory of Molecular Developmental Biology, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing 100101, China.

²Graduate School of Chinese Academy of Sciences, Beijing 100039, China.

*Author for correspondence (jianzhang84@genetics.ac.cn)

expression is only maintained in MiP and RoP at later stages [15 hours postfertilization (hpf)], whereas *islet2* expression enriches specifically in CaP and VaP (Appel et al., 1995; Tokumoto et al., 1995). In the absence of *Islet1*, zebrafish PMN precursors switch fates and form interneurons (INs) (Hutchinson and Eisen, 2006). By contrast, inhibition of zebrafish *Islet2* function by a dominant-negative mutant LIM^{islet2} results in failure of CaP neurons to project their axons beyond the HMS (Segawa et al., 2001), indicating that *Islet2* is essential for CaP axon outgrowth. Despite the highly regulated expression patterns of *Islet1* and *Islet2*, their functions may be redundant for PMN specification in terms of axon projection trajectories (Hutchinson and Eisen, 2006). Thus, PMN subtype establishment and axon outgrowth differences might be controlled by different upstream transcription factors that differentially regulate *islet1* and *islet2*. For example, late-phase *islet1* expression in MiP is activated by *Nkx6*. Disruption of *Nkx6* causes MiP to develop a more IN-like morphology (Hutchinson et al., 2007). However, the immediate upstream regulator of *islet2* remains unknown.

In addition to the transcription factors mentioned above, a number of Prdm family (PRDI-BF1 and RIZ homologous region) proteins play diverse roles at different stages of neural development. For example, mouse Prdm16 is expressed in neural stem cells and has been shown to promote cell identity by modulating oxidative stress (Chuikov et al., 2010). In *Drosophila*, Hamlet, the homolog of Prdm16, controls odorant gene choice and axon targeting specificity through modifying cellular responses to Notch signals in a context-dependent manner (Endo et al., 2011). In zebrafish, Prdm1a is expressed at the edge of the neural plate and is required for the specification of neural crest and Rohon-Beard (RB) sensory neurons (Hernandez-Lagunas et al., 2005; Olesnicki et al., 2010; Rossi et al., 2009). Prdm6, 8, 12, 13 and 16 were shown to be expressed in the developing mouse CNS (Kinameri et al., 2008), but their exact functions remain to be defined. Another important member of the family is Prdm14, a role for which has not been reported in neural development. Instead, it is indispensable for the establishment of primordial germ cells (PGCs), with roles in the reacquisition of pluripotency and epigenetic reprogramming (Ohinata et al., 2009; Yamaji et al., 2008). Recent studies have revealed that PRDM14 is also a key component of the core transcriptional regulatory network for maintaining embryonic stem cell identity by directly regulating the pluripotency gene *POU5F1*, and may prevent embryonic stem cells from differentiating into extra-embryonic endoderm (Chia et al., 2010; Ma et al., 2011; Tsuneyoshi et al., 2008).

Here, we report a zebrafish gene-trap mutant, *slg*, in which *prdm14* gene expression is disrupted, resulting in axon growth defects in CaP. We show that Prdm14 is required for *islet2* activation and that *Islet2* mediates major functions of Prdm14 in CaP. These results strongly argue that, in addition to extrinsic factors, intrinsic factors such as Prdm14 specifically control CaP axon outgrowth in zebrafish.

MATERIALS AND METHODS

Zebrafish and gene trapping

Zebrafish husbandry and embryo manipulations were performed as described (Westerfield, 2000). The T2ASAd gene-trap vector was modified from T2AL200R150 (Urasaki et al., 2006) by replacing the *EF1a* promoter and second intron of rabbit β -globin with the splicing acceptor of the first intron of zebrafish *bcl2* and adding transcription termination signals (see supplementary material Table S1 for sequences). T2ASAd plasmid (25 ng/ μ l) and *Tol2* transposase mRNA (50 ng/ μ l) were injected (1 nl each) into 1-cell embryos. Over 300 injected embryos were raised and outcrossed

with wild-type fish. GFP expression was examined at different developmental stages until 72 hpf. Ten gene-trap fish lines with obvious EGFP expression were identified, four of which showed neuronal EGFP expression patterns, including the *slg* mutant.

Tail-PCR and cloning of zebrafish *prdm14*

To identify the gene trapped in the *slg* mutant, we used Tail-PCR (Liu and Chen, 2007). *prdm14* sequence (GenBank NM_001163831.1) was used to design primers *prdm14-5* (5'-AAAGGATCCATGGCTATGTCGGTT-TCTCTCTCCAG-3') and *prdm14-3'* (5'-AAACTCGAGTTAGTTC-CAGGGTCTGTACTC-3'). The resulting cDNA was cloned into the pCS2+ plasmid (from Dr David Turner, University of Michigan, USA) to generate CS2-Prdm14.

RNA in situ hybridization and immunohistochemistry

Digoxigenin-labeled or fluorescein-labeled probes were synthesized using an in vitro transcription system (Roche) and probes longer than 2 kb were hydrolyzed using limited alkaline hydrolysis buffer. Whole-mount in situ hybridization and double-labeling in situ hybridization were performed as described (Jowett, 2001). Whole-mount immunohistochemistry was performed as described (Macdonald, 1999). Embryos were photographed with a Leica MZ16F or Nikon Elips50i microscope using a Nikon DS-Ri1 digital camera or imaged with an Olympus FV1000 confocal microscope. Region of interest (ROI) scores of *Islet* signals were calculated and analyzed using FV10-ASW 2.1 software (Olympus).

Morpholinos

Morpholino (MO) (Gene Tools) sequences (5'-3') are: *prdm14* splicing MO (*prdm14* SplMO), TGATTCTTGACCAACCTGTGCTGG; *islet2* ATG MO (*islet2* MO), GAATATCCACCATACAGGAGGGTTA (Segawa et al., 2001); *prdm1a* splicing MO (*prdm1a* MO), TGGTGTCATA-CCTCTTTGGAGTCTG (Roy and Ng, 2004). MOs were injected into embryos at the 1- to 2-cell stage. Approximately 1 nl of each MO was injected at 1.5 μ g/ μ l for *prdm14* SplMO, 6 μ g/ μ l for *islet2* MO and 0.5 μ g/ μ l for *prdm1a* MO. For more information on MOs, see supplementary material Table S2, Fig. S1.

Behavior assays

For spontaneous coiling, embryos at 22-24 hpf were kept without stimulus and recorded for 1 minute using a Nikon DS-Qi1MC camera. For touch response, embryos at 28-30 hpf were dechorionated and kept in the resting state and then stimulated with a pin. The stimulus was repeated at ~1- to 2-second intervals and recorded using a Nikon DS-Qi1MC camera at 50 frames per second for 1 minute. Movies were analyzed with NIS-Element software (Nikon) or QuickTime (Apple). Typical twist movement frames in each group were extracted and assembled with Adobe Photoshop.

Transgene expression in PMNs

A promoter including three copies of the 125 bp *mxn1* enhancer (*mxn1*) was used to drive transgene expression in PMNs (Zelenchuk and Brusés, 2011). *mxn1-islet2* or *mxn1-prdm14* and *mxn1-mRFP* were injected together with transposase mRNA into 1-cell *slg* mutant embryos; 1 nl of each plasmid (25 ng/ μ l) and transposase mRNA (50 ng/ μ l) was injected.

Chromatin immunoprecipitation (ChIP)-PCR

ChIP-PCR was performed essentially as described previously (Lindeman et al., 2009) with minor modifications. Briefly, nuclei pellets from embryos were incubated in crosslink buffer NIM (0.25 mM sucrose, 25 mM KCl, 5 mM MgCl₂, 10 mM Tris-HCl, pH 7.4) plus 1% formaldehyde for 8 minutes at room temperature. The reaction was quenched by adding glycine. The nuclei were then washed with NIM buffer and resuspended in lysis buffer. The chromatin lysate was sonicated and precleared with protein A beads (Millipore). The precleared supernatant was incubated with 2 μ l rabbit polyclonal Prdm14 antibodies (see supplementary material Fig. S2). Protein A beads were then added and incubated at 4°C. The cross-linking of the bound complexes was reversed by adding proteinase K and NaCl. The recovered DNA was used as template for PCR. For ChIP-PCR in cell culture, human HCT116 cells were cotransfected with equal amounts of CS2-Prdm14 and GL3-*Pislet2* [~3 kb *islet2* promoter inserted

into GL3-Basic (Promega)] and ChIP-PCR performed similarly to above. Primers are described in supplementary material Table S2.

RESULTS

Identification of the *short lightning* mutant with GFP expression in neurons

In a *Tol2* transposon-mediated gene-trap screen intended to study neural development, we identified a trapped fish line showing restricted GFP expression patterns in a subset of neurons (Fig. 1A). Because CaP neurons of the homozygous mutant embryos show shortened axons, we named the mutant *short lightning* (*slg*). In the trunk region, GFP marks RB, CaP, VaP and their axons (Fig. 1B,C). However, RoP and MiP are notably GFP negative. Additionally, another subpopulation of spinal neurons is GFP positive (Fig. 1D). These cells might be INs, but further characterization is required to define their identity.

To better identify neuronal types among the GFP-positive cells, we stained the neurons with a *znp1* antibody (a PMN axon marker). Colocalization of GFP with *znp1* staining in ventral projected axons (Fig. 1E-E'') indicates that the GFP-positive cells

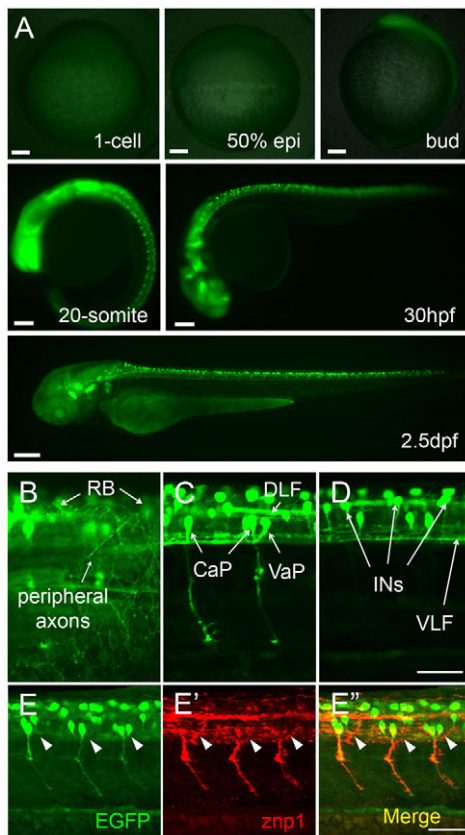


Fig. 1. Neuron-specific expression of GFP transgene in *slg*^{+/-} zebrafish embryos. (A) GFP expression starts from the bud stage and is restricted to a subset of embryonic neurons. (B-D) GFP expression in RB, CaP, VaP and INs at 32 hpf. (E-E'') CaP axons labeled by *znp1* antibody staining are GFP positive in 26-hpf embryos, whereas MiP axons (arrowheads) are GFP negative. All images are lateral views. Animal pole is to the top for 1-cell and 50% epiboly; dorsal to the right for bud and 20-somite; dorsal to the top and anterior to the left for 30 hpf and 2.5 dpf in A and for B-E''. CaP, caudal primary motoneurons; DLF, dorsal longitudinal fasciculus; INs, interneuron-like cells; RB, Rohon-Beard neurons; VaP, variable primary motoneurons; VLF, ventral longitudinal fasciculus. Scale bars: 100 μ m in A; 50 μ m in B-E''.

are CaP/VaP neurons. GFP also labels statoacoustic ganglion (SAG) neurons, trigeminal neurons (TGNs), olfactory sensory neurons (OSNs), midbrain and forebrain neurons (MBNs and FBNs), Mauthner cells (MCs) and reticulospinal neurons (RSNs), but not other types of cells (supplementary material Fig. S3). Together, these results indicate that GFP expression in this fish line is mostly in neurons.

The *prdm14* gene is trapped in the *slg* mutant

We used Tail-PCR to identify the gene trapped in the *slg* mutant (Liu and Chen, 2007). Sequencing indicated that all DNA fragments flanking the inserted transposon were derived from the *prdm14* locus (Fig. 2A). The conceptually translated zebrafish Prdm14 protein contains a PR/SET (PR) domain and a six zinc-finger (ZF) domain, which are highly conserved from fish to human (Fig. 2B,C; supplementary material Fig. S4). *prdm14* regulatory elements are likely to drive the observed GFP expression, as heterozygous embryos consistently showed weaker GFP fluorescence than homozygotes (Fig. 2D).

prdm14 is initially expressed at the dorsal region at 30% epiboly (Fig. 2E) and later in the anterior neural plate region and a part of the posterior neural plate region around the 75% epiboly and bud stages (Fig. 2F,G). Consistent with the GFP expression patterns, *prdm14* RNA in the anterior neural plate region is expressed in MBNs and the preplacodal ectoderm region, then in OSNs, SAG neurons and TGNs (Fig. 2H-J). In the posterior neural plate region, *prdm14* is restricted to spinal cord neuron precursors, including ventral neuron precursors and dorsal neuron precursors at the 1-somite stage. Around the 22-somite stage, the RNA can be detected in spinal cord neurons (Fig. 2K-M). *prdm14* RNA also partially colocalizes with *ngn1* (*neurog1*; a pan-neuronal marker) in the anterior region of the trunk and in brain at the 3-somite stage (Fig. 2N-O''). At the 26-somite stage, *prdm14* shares expression patterns with *islet2* (an RB and CaP marker) (Fig. 2P-P''), which is consistent with the GFP expression patterns in these neurons. In summary, GFP expression profiles of heterozygotes are highly similar to those of *prdm14* RNA, indicating that the trapped gene in the *slg* mutant is *prdm14*.

prdm14 expression is reduced in the *slg* mutant

To study functions of *prdm14* in neuron development, we investigated whether *prdm14* expression is disrupted in *slg* mutants. Sequencing data from Tail-PCR indicate that the transposon is inserted in the first intron of *prdm14* and is likely to disrupt *prdm14* expression (Fig. 3A). Indeed, *prdm14* expression was greatly reduced in the homozygotes (Fig. 3B,C). Injection of a *prdm14* morpholino (SplMO), which spans the boundary of exon 2 and intron 2, disrupted *prdm14* RNA splicing (Fig. 3A,D). *prdm14* expression in *slg* mutants was reduced to ~20% of that in wild type (Fig. 3E). The Prdm14 protein level was also greatly reduced in *slg* mutants and *prdm14* SplMO (*splmo*) morphants (Fig. 3F).

A previous study showed that the *Tol2* transposon could be mobilized efficiently by injection of *Tol2* transposase mRNA (Urasaki et al., 2008). To further establish that the transposon insertion in the *prdm14* gene is responsible for its reduced expression, we excised the *Tol2* transposon by injecting transposase mRNA into 1- to 2-cell homozygous embryos obtained from F0 *slg* mutants. The injected F1 embryos were bred and outcrossed with wild-type fish to obtain F2 embryos. If the insertion was excised in the germ lineage of F1, some F2 progeny would carry a wild-type allele and a *Tol2* excised allele at the *prdm14* locus and should be GFP negative. Indeed, up to 22.69% of F2 embryos were GFP

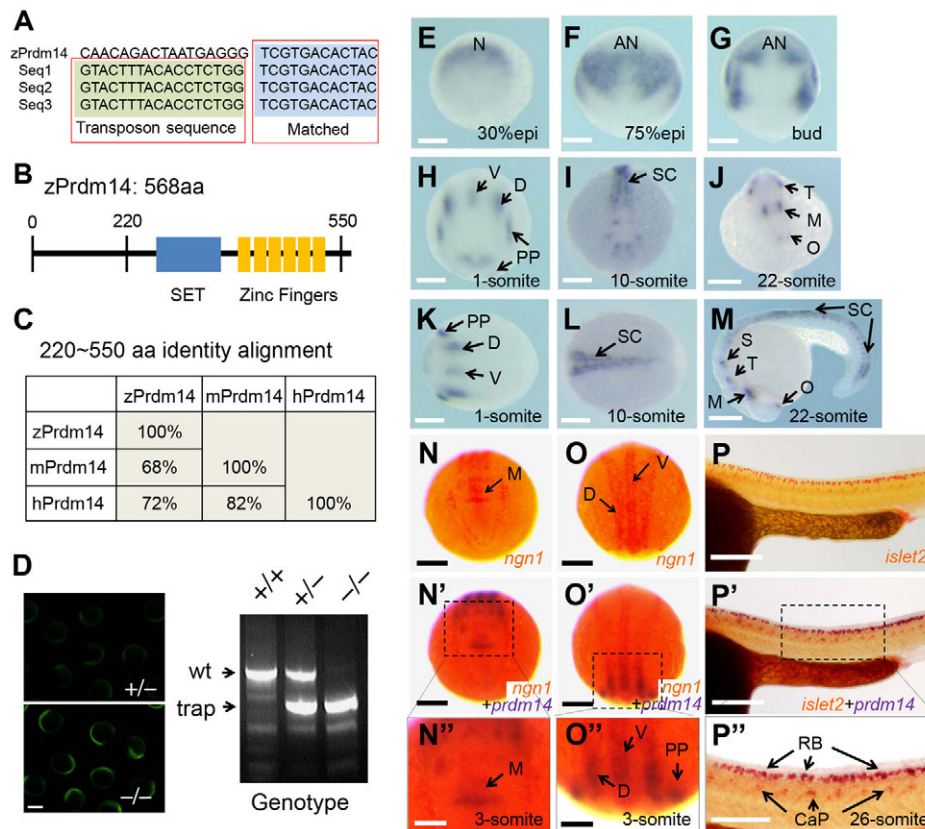


Fig. 2. *prdm14* is trapped in the *slg* mutant. (A) Boundary sequences of the inserted transposon and *prdm14* gene. (B,C) Zebrafish Prdm14 protein contains a SET domain and a six zinc-finger domain, which are highly conserved. z, zebrafish; m, mouse; h, human. (D) PCR genotyping confirms that the *prdm14* gene is disrupted in *slg* mutants (right). Heterozygotes and homozygotes show different GFP intensities (left). (E-M) *prdm14* RNA expression patterns detected by in situ hybridization. *prdm14* is expressed in neural plate at 30% epiboly (E) and in the anterior neural plate at 75% epiboly and bud stages (F,G). At the 1-somite stage, *prdm14* RNA is restricted to dorsal and ventral neuron precursors and precordal plate (H,K) and at the 10-somite stage *prdm14* is detected in neuron precursors of the spinal cord (I,L). At the 22-somite stage *prdm14* is further restricted to the ventral midbrain, olfactory sensory neurons, statoacoustic neurons, trigeminal neurons and spinal cord (J,M). (N-O'') *prdm14* RNA is co-expressed with *ngn1* at the 3-somite stage as detected by double in situ hybridization. (P-P'') *prdm14* is co-expressed with *islet2* in RB and CaP at the 26-somite stage. Animal view, dorsal to the top in E-G; anterior view, dorsal to the top in H-J,N-N''; dorsal view, anterior to the left in K,L; lateral view, anterior to the left in M,P-P''; dorsal view, anterior to the bottom in O-O''. N, neural plate; AN, anterior neural plate; V and D, ventral and dorsal neuron precursors; PP, precordal plate; SC, spinal cord; M, ventral midbrain; O, olfactory sensory neurons; S, statoacoustic ganglion neurons; T, trigeminal neurons. Scale bars: 500 μ m in D; 200 μ m in E-P'; 100 μ m in N''-P''.

negative in several independent experiments, suggesting that *Tol2* excision occurred in these F1 germ cells. The F1 fish were designated as mosaic F1 revertant and the *prdm14* allele excised in the mosaic F1 revertant germ line was defined as the *prdm14* revertant allele (*r*). We crossed the mosaic F1 revertant fish with *prdm14*^{-/-} and obtained *prdm14*^{r/-} progeny and their *prdm14*^{-/-} siblings. The *prdm14*^{r/-} and *prdm14*^{-/-} embryos can be distinguished by their different GFP intensity. We found that *prdm14* expression is restored in *prdm14*^{r/-} embryos (Fig. 4E,F), suggesting that the insertion is likely to be the sole cause of the reduced expression. In summary, *prdm14* expression is substantially disrupted in *slg* mutants and *splmo* morphants.

Prdm14 is required for full growth of CaP axons

Expression of *prdm14* in neural tissues in early zebrafish embryos prompted us to examine whether *prdm14* is involved in early neural development. We monitored the expression of *olig2* (an MN progenitor marker), *foxd3* (a neural crest progenitor marker), *cxcr4b* (an OSN progenitor marker), *ngn1* and *huC* (*elavl3* – Zebrafish Information Network) (pan-neural markers).

All these markers showed similar expression patterns and expression levels in *slg* mutant and wild-type embryos (supplementary material Fig. S5A). In addition, cell body positions and cell numbers of examined neurons were unaffected in the mutant embryos at 2.5 days postfertilization (dpf) (supplementary material Fig. S5B,C). These results suggest that reduction of *prdm14* expression does not affect early neuronal specification.

In this report, we focus on Prdm14 functions in neuronal development in the trunk, specifically CaP development. One CaP neuron is centrally positioned in each myotome and extends its axon to the distal part of the ventral muscle, which can be easily recognized in 1-day-old embryos (Fig. 4A). CaP cell numbers and cell body positions are normal in *slg* mutant and *splmo* morphant embryos (supplementary material Fig. S6A). Expression of GABA, an IN transmitter that is often misexpressed in fate-changed PMNs, is not detected in CaP in *slg* mutants (supplementary material Fig. S6B), suggesting that the CaP neurons might not switch to an IN-like fate. These results indicate that the early specification of CaP neurons in *slg* mutants is largely normal.

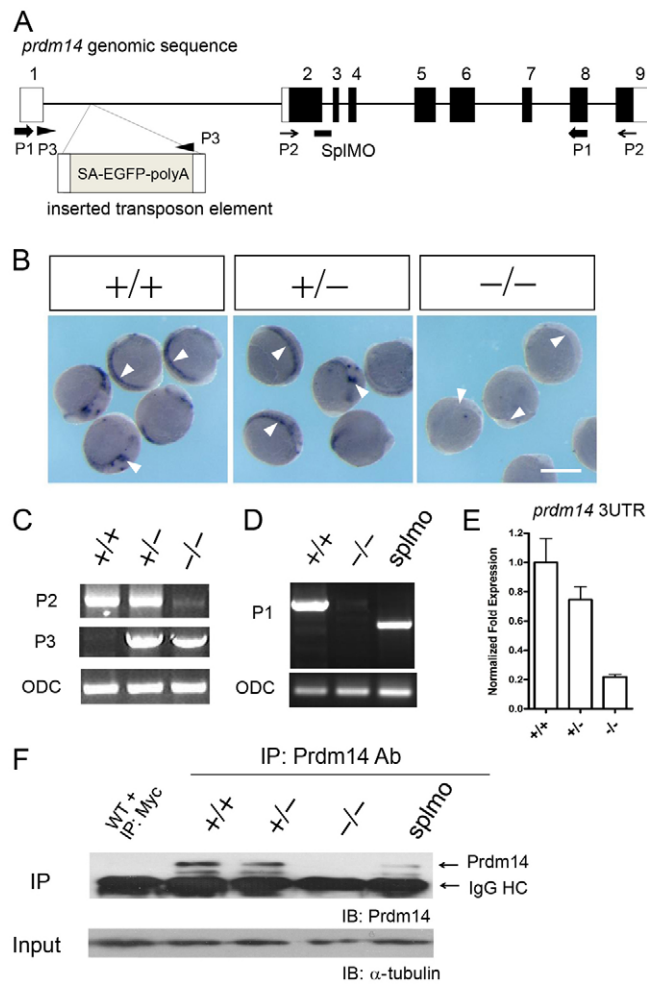


Fig. 3. *prdm14* expression is reduced in *slg* mutant and *splmo* morphant embryos. (A) The zebrafish *prdm14* genomic locus. The transposon is inserted in the first intron. Primers used in C and D are indicated. (B,C) RNA in situ hybridization (B, arrowheads) and RT-PCR (C) indicate that *prdm14* expression is greatly reduced in *slg* mutant embryos. ODC, *ornithine decarboxylase 1* internal standard. (D) *prdm14* splicing is disrupted in *splmo* morphants. (E) Real-time RT-PCR results indicate that *prdm14* expression is reduced to ~75% in *slg* heterozygotes and to ~20% in homozygotes. Error bars indicate s.e.m. of triplicate experiments. (F) Immunoprecipitation (IP) showing that Prdm14 protein levels are greatly decreased in *slg* homozygotes and *splmo* morphants. Scale bar: 500 μ m.

In wild-type embryos, CaP growth cones leave the spinal cord at ~17 hpf and extend ventrally until they arrive at the first intermediate target – the muscle pioneers located at the HMS. The growth cones pause at the HMS for 1-2 hours and then continue to extend along the ventral myotome next to the notochord and finally reach the ventral-most region of ventral myotome. Four regions along the axon pathway can be defined: HMS; ventral edge of the notochord (VNC); proximal portion of the ventral muscle (PVM); and distal portion of the ventral muscle (DVM) (Hilario et al., 2009; Rodino-Klapac and Beattie, 2004) (Fig. 4A). One of the most prominent cellular phenotypes in *slg* mutant embryos is their significantly shorter CaP axons, as compared with the heterozygotes at 26 hpf (Fig. 4B-C'). Similarly, CaP axons in *splmo* morphants are also shortened (Fig. 4D,D'). Moreover, abnormal axon branching was

evident in some CaP neurons in *slg* mutant and *splmo* morphant embryos (Fig. 4C',D'). Staining by *znp1* and SV2 antibodies (PMN axon markers) confirmed the axon outgrowth defects in the mutant and morphant embryos (Fig. 4E; supplementary material Fig. S6C). Statistical analysis indicated that the majority of the CaP axons in *slg* mutant embryos only reached the PVM, with some growth cones even stalled in the HMS and VNC (Fig. 4F). In the *prdm14*^{-/-} revertant embryos, CaP axon morphology was restored to that observed in the heterozygous embryos (Fig. 4E,F). Together, these results indicate that Prdm14 is required for CaP axon outgrowth to the ventral-most regions in the trunk at 26 hpf.

Prdm14 is required for embryonic movement

The CaP axon defects in *slg* mutants clearly suggest that their early embryonic movements might be compromised. In zebrafish, there are two major types of embryonic movement at early stages (17-30 hpf): spontaneous tail coiling and the touch response. The embryos begin to exhibit spontaneous tail coiling shortly after PMN axons first extend out of the spinal cord (17 hpf). At later stages (21 hpf), they can respond to touch with an over-the-head fast coiling of the trunk, known as the touch response. Both movements require a functional early spinal circuit, including PMNs (Brustein et al., 2003).

We compared spontaneous tail coiling in wild-type, *slg* heterozygous, homozygous and *splmo* morphant embryos at 22-24 hpf. The coiling frequency of homozygous or morphant embryos was reduced to less than once per minute compared with two to three times per minute in wild-type and heterozygous embryos (Fig. 5A). Moreover, coiling amplitude was also greatly decreased in the mutants and morphants (data not shown). We also checked the touch response of embryos at 28-30 hpf, at which time embryos are touch responsive but no longer coil spontaneously (Tallafuss and Eisen, 2008). Wild-type embryos responded to tail touch with a stereotyped bend of the trunk. In *slg* homozygous and *splmo* morphant embryos, the trunk bending amplitude was much smaller and the movement was uncoordinated (Fig. 5B,C; supplementary material Movies 1-4). As expected, the *prdm14*^{-/-} revertant embryos showed normal spontaneous tail coiling and touch response, as in wild-type or heterozygous embryos (Fig. 5B,C; supplementary material Movie 5). Thus, the disruption of the *prdm14* locus is likely to be the sole cause of the movement defects.

In addition to the neuron defects, abnormal muscle development can also cause defective movement (Granato et al., 1996). To exclude this possibility, we examined the development of muscle pioneers, fast muscle and slow muscle. Immunohistochemistry results indicated that muscle development appeared normal in *slg* mutants (supplementary material Fig. S7A-C). Thus, it is axon shortening caused by *prdm14* disruption that contributes to the embryonic movement defects in *slg* mutant and *splmo* morphant embryos.

islet2 acts as a downstream target of Prdm14 in CaP

Prdm14 belongs to the PR domain-containing protein family (Fig. 2B), some members of which show protein methyltransferase activity (Hayashi et al., 2005; Kim et al., 2003). We could not detect any methylation activity of zebrafish Prdm14, however, using various substrates (supplementary material Fig. S8; data not shown). Previously, mouse and human PRDM14 were shown to directly bind to regulatory elements of pluripotency-related genes (Chia et al., 2010; Ma et al., 2011), suggesting that they might function as transcription factors. Zebrafish Prdm14 is a nuclear protein and its

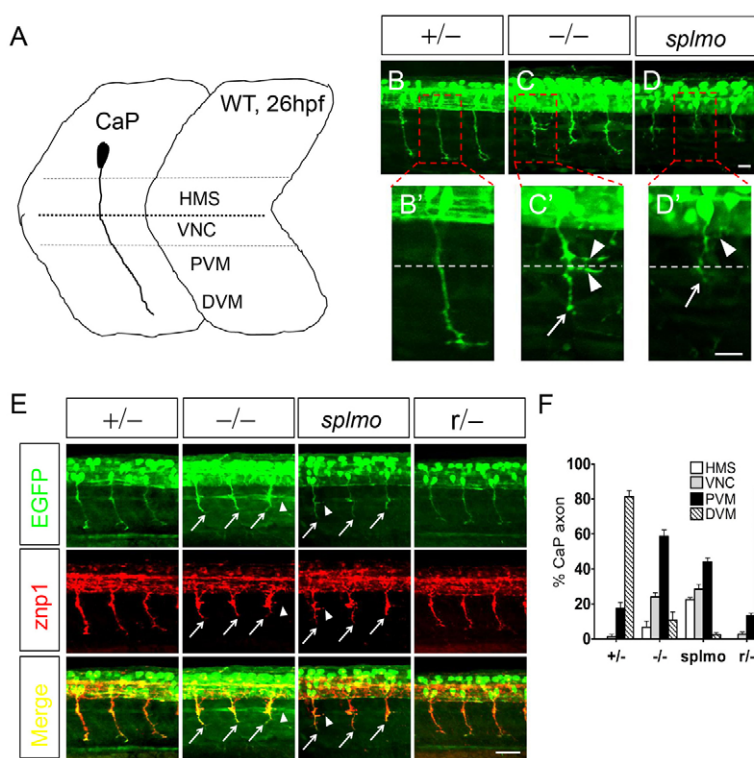


Fig. 4. CaP axons are shortened in *slg* mutant and *splmo* morphant zebrafish embryos. (A) CaP axon projection in 26-hpf wild type. Four regions are defined to describe CaP axon outgrowth: HMS, horizontal myoseptum; VNC, myotome adjacent to the ventral edge of the notochord; PVM and DVM, proximal and distal portion of the ventral myotome. (B–D') *slg* mutant and *splmo* morphant embryos show greatly shortened CaP axons at 26 hpf. The boxed regions are magnified in B'–D'. Dashed line indicates horizontal septum. (E) *znp1* immunostaining shows that the shortened axons are CaP in *slg* mutant and *splmo* morphant embryos. (F) Summary of CaP axon outgrowth (hemisegments 8–12) in *slg* heterozygous, *slg* homozygous, *splmo* morphant and *slg* revertant embryos. For each group, a total of 25 axons from five embryos were scored as HMS, VNC, PVM or DVM. Error bars indicate s.e.m. of triplicate experiments. Lateral views, dorsal to the top and anterior to the left. Arrows indicate shortened CaP axons; arrowheads indicate abnormal branched axons. Scale bars: 20 μ m in B–D'; 50 μ m in E.

localization depends on its intact ZF domain (supplementary material Fig. S9A,B); thus, it might also function as a transcription factor in MNs. To identify potential Prdm14 targets, we analyzed a number of genes involved in PMN differentiation/maturation. Among them, *islet2* is specifically expressed in zebrafish CaP, but not in RoP or MiP. Consistent with its expression pattern, *Islet2* plays an important role in the late-stage differentiation/maturation of CaP (Segawa et al., 2001). Further, *islet2* morphants also show shortened CaP axons (Fig. 6A) (Segawa et al., 2001). Importantly, the *islet2* RNA level is greatly decreased in CaP in *slg* mutant and *splmo* morphant embryos (Fig. 6B). By contrast, *islet1* expression in RoP and MiP is unaffected (Fig. 6B). In addition, the expression of *mnx1* (a ventral PMN marker), *olig2* (a ventral neuron marker), *huC* (a pan-neuronal marker) and *pax2a* (an IN and midbrain-hindbrain boundary marker) is not altered in *slg* mutants (Fig. 6B,D; supplementary material Fig. S10A).

We also examined *Islet2* protein expression. Because a zebrafish *Islet2*-specific antibody is not available, we relied on a general *Islet* antibody that recognizes both *Islet1* and *Islet2*. Although it was difficult to distinguish the *Islet1* signal from that of *Islet2*, we showed that *Islet*-positive cells (one or two cells in each somite segment) with GFP expression are CaP or VaP (Fig. 1C; Fig. 6C), suggesting that *Islet2* and Prdm14 overlap in expression in CaP/VaP. Indeed, previous studies have shown that *Islet2* is expressed in these cells (Segawa et al., 2001; Tokumoto et al., 1995). Thus, we refer to *Islet* staining in GFP-positive cells as indicating *Islet2* and *Islet* staining in GFP-negative cells in ventral spinal cord as indicating *Islet1*. *Islet2* staining in CaP (GFP positive) greatly decreased in *slg* mutant and *splmo* morphant embryos [Fig. 6C; ROI values (mean \pm s.e.m.): *slg* heterozygote, 3147.2 \pm 162.6; *slg* homozygote, 1567.8 \pm 147.1; *splmo*, 1829.3 \pm 90.8]. By contrast, the *Islet1* signal in GFP-negative cells (presumably RoP and MiP) remained unchanged, consistent with the fact that *prdm14* is not expressed in RoP and MiP

[Fig. 6C; ROI values (mean \pm s.e.m.): *slg* heterozygote, 3161.6 \pm 79.8; *slg* homozygote, 3284.1 \pm 52.1; *splmo*, 3436.6 \pm 150.2].

Although *prdm14* is also expressed in RB (Fig. 1B; Fig. 2P–P''), *islet2* expression is not downregulated in RB in *slg* mutant embryos (Fig. 6B,D). Previous studies suggested that another PR domain protein, Prdm1a, regulates *islet2* expression in RB but not in CaP (Olesnick et al., 2010). Thus, it is likely that Prdm1a and Prdm14 regulate *islet2* expression in RB and CaP, respectively. To test this hypothesis, we reduced Prdm14 and Prdm1a expression separately or in combination. Downregulation of *prdm14* led to decreased *islet2* expression in CaP but not in RB (Fig. 6B,Da'). Conversely, *prdm1a* MO reduced *islet2* expression in RB but not in CaP (Fig. 6Da''). Injection of *prdm1a* MO into *slg* mutant embryos resulted in loss of *islet2* expression in both RB and CaP (Fig. 6Da'''). As a control, *islet2* expression at the distal end of the yolk extension (cloaca) was not affected (Fig. 6Da-a''). Expression of *mnx1* and *islet1* was largely intact for all manipulations (Fig. 6Db–c''). These data indicate that *islet2* expression is mainly regulated by Prdm1a in RB and by Prdm14 in CaP.

prdm14 expression precedes that of *islet2* (supplementary material Fig. S9C,D). We examined whether zebrafish Prdm14 can directly bind to the *islet2* promoter. Using a conserved Prdm14 binding sequence identified in mammals to scan the zebrafish *islet2* promoter region, we identified a putative binding site (pBS) for Prdm14 near the transcription start site (Fig. 6E). In ChIP-PCR, Prdm14 antibody specifically pulled down an *islet2* PCR fragment containing the pBS (Fig. 6F). However, the antibody failed to precipitate the fragment from *slg* mutant embryos (Fig. 6G). In addition, Prdm14 antibody can precipitate an *islet2* DNA fragment containing the pBS from the cell extract of mammalian cells expressing Prdm14 (Fig. 6H). When Prdm14 was not expressed or the pBS was mutated, the antibody failed to pull down the fragments (Fig. 6H). In addition, the Prdm14 pBS in the *islet2* promoter may be required for *islet2* expression in CaP (supplementary material Fig. S11). Taken

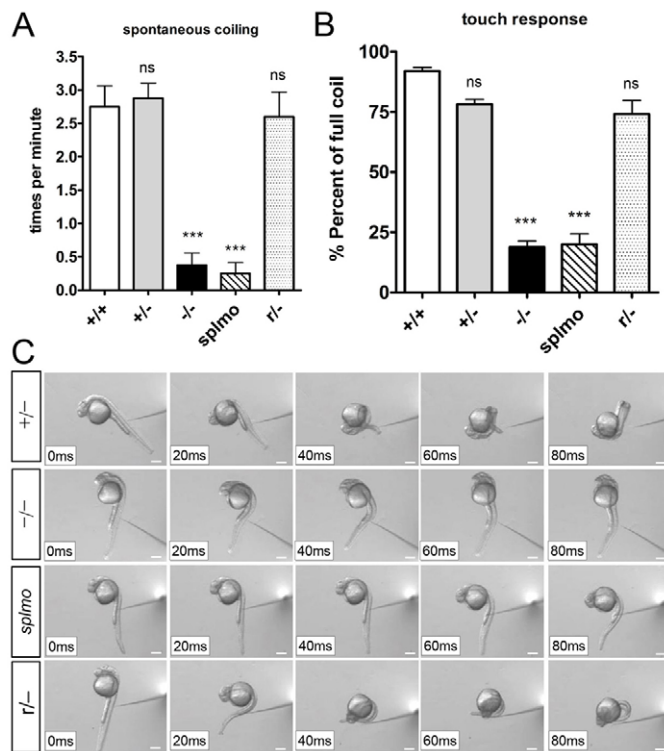


Fig. 5. Reduction of Prdm14 expression causes embryonic movement defects. (A) Frequencies of spontaneous tail coiling in *slg* mutant and *splmo* morphant embryos are significantly reduced compared with wild-type and heterozygous embryos. The normal tail coiling is restored in revertant embryos. Ten embryos were examined in each group. Error bars indicate s.e.m. (B) The percentage of full tail coil in *slg* mutant and *splmo* morphant embryos after touch stimulus is significantly decreased compared with wild-type and heterozygous embryos. The normal movements were largely restored in revertant embryos. A total of 30–45 touch responses were monitored in each embryo and the percentage of full tail coil calculated. In each group, at least three embryos were recorded. Error bars indicate s.e.m. of triplicate experiments. *** $P < 0.001$; ns, not significant; compared with wild type (two-tailed unpaired *t*-test). (C) Frame shots of typical twist movement in the touch response. See supplementary material Movies 1–5. Scale bars: 200 μm .

together, Prdm14 can bind to the conserved pBS in the *islet2* promoter and may therefore play a role in activating *islet2* transcription.

***islet2* mediates major *prdm14* functions in CaP**

Because Prdm14 activates *islet2* expression, it is possible that *islet2* might mediate the major output of *prdm14* functions in CaP. To test this, we used an *islet2* transgene to rescue the CaP axon defects in *slg* mutant embryos. Previously, *mnx1* was shown to be strictly expressed in PMNs, and an *mnx1*-3 \times 125bp enhancer sequence (*mnx1*) was used successfully to drive expression of a GFP reporter in PMNs (Zelenchuk and Brusés, 2011). We used the *mnx1* sequence to drive *islet2* or *prdm14* transgene expression in PMNs and used the *mnx1*-*mRFP* transgene to label PMNs with *islet2* or *prdm14* transgene expression (Fig. 7A). Expression of *prdm14* or *islet2* transgenes in CaP rescued the axon outgrowth defects of *slg* mutants (Fig. 7B,C). These results suggest that *islet2* is a major downstream target of *prdm14* and is likely to mediate major functions of Prdm14 in CaP (Fig. 7D).

DISCUSSION

Here, we show that *prdm14* is expressed in selected neurons, including CaP, in early zebrafish embryos. Prdm14 is required for CaP axon outgrowth and early embryonic movement. We further identify *islet2* as a major downstream target of Prdm14 in CaP (Fig. 7D). Our results provide an example of an intrinsic factor that is crucial for the development of a specific MN axon.

Zebrafish Prdm14 is expressed in the nervous system

We showed that *prdm14* expression is mainly restricted to neural lineages in early zebrafish embryos (Fig. 1; supplementary material Fig. S3). Despite the early expression of *prdm14* in the neural system, we did not observe neural specification defects in *slg* mutant embryos, suggesting that Prdm14 might be dispensable for this early process (supplementary material Fig. S5A). It remains possible that redundant factors mask the impact of Prdm14 loss in early neural specification. Thus, we cannot rule out the possibility that *prdm14* might play a role in early neural development.

At 26 hpf, *prdm14* is prominently expressed in RB, INs and PMNs. In PMNs, *prdm14* is only expressed in CaP/VaP, but not RoP and MiP. Similarly, only a proportion of INs are marked by *prdm14*. These observations suggest that expression of *prdm14* itself is highly regulated. *prdm14* is also expressed in the nervous system of mouse and human (Kinameri et al., 2008) (Allen Brain Atlas, <http://www.brain-map.org/>). In human embryonic stem cells, PRDM14 may regulate genes involved in neurogenesis (Chia et al., 2010). Thus, it would be intriguing to investigate whether PRDM14 also plays roles in mammalian neuron development, especially PMN axon outgrowth.

Roles of Prdm14 in CaP axon outgrowth and early embryonic movement

CaP development is a well-characterized system used to study mechanisms that regulate branching and projection of MN axons (Lewis and Eisen, 2003). Using *slg* mutants and *prdm14* morphants, we found that disruption of Prdm14 expression does not appear to affect CaP specification and initial axon extension, but leads to premature branching and shortened axon outgrowth (Fig. 4). Muscle development was largely normal in the mutant (supplementary material Fig. S7). Thus, the defects are likely to be due to a cell-autonomous function of Prdm14 in the neurons. Previous studies showed that Plexin:Nrp1a/Semaphorin, the extracellular matrix (ECM) component Tenascin-C, the glycosyltransferase Lh3 and secreted glycoprotein PGRN are all essential for CaP axon development (Chitramuthu et al., 2010; Feldner et al., 2007; Sato-Maeda et al., 2006; Schneider and Granato, 2006; Schweitzer et al., 2005; Tanaka et al., 2007). It remains to be investigated whether Prdm14 directly or indirectly regulates these factors. Previously, the Prdm family protein Hamlet was implicated in the control of axon targeting in *Drosophila* (Endo et al., 2011). Thus, some members of the Prdm family might be widely used to regulate axon morphogenesis.

It is not surprising that *slg* mutants display defective embryonic movements in spontaneous tail coiling and touch response (Fig. 5) given that CaP axons in the mutants failed to reach the DVM region (Fig. 4). However, swimming behaviors are generally unaffected in *slg* mutant embryos at 2.5 dpf (data not shown), suggesting that the underlying neuron circuits function normally at later stages. In agreement with this observation, the majority of CaP axons reach the ventral-most myotome and form connections with muscles at this stage (supplementary material Fig. S5C).

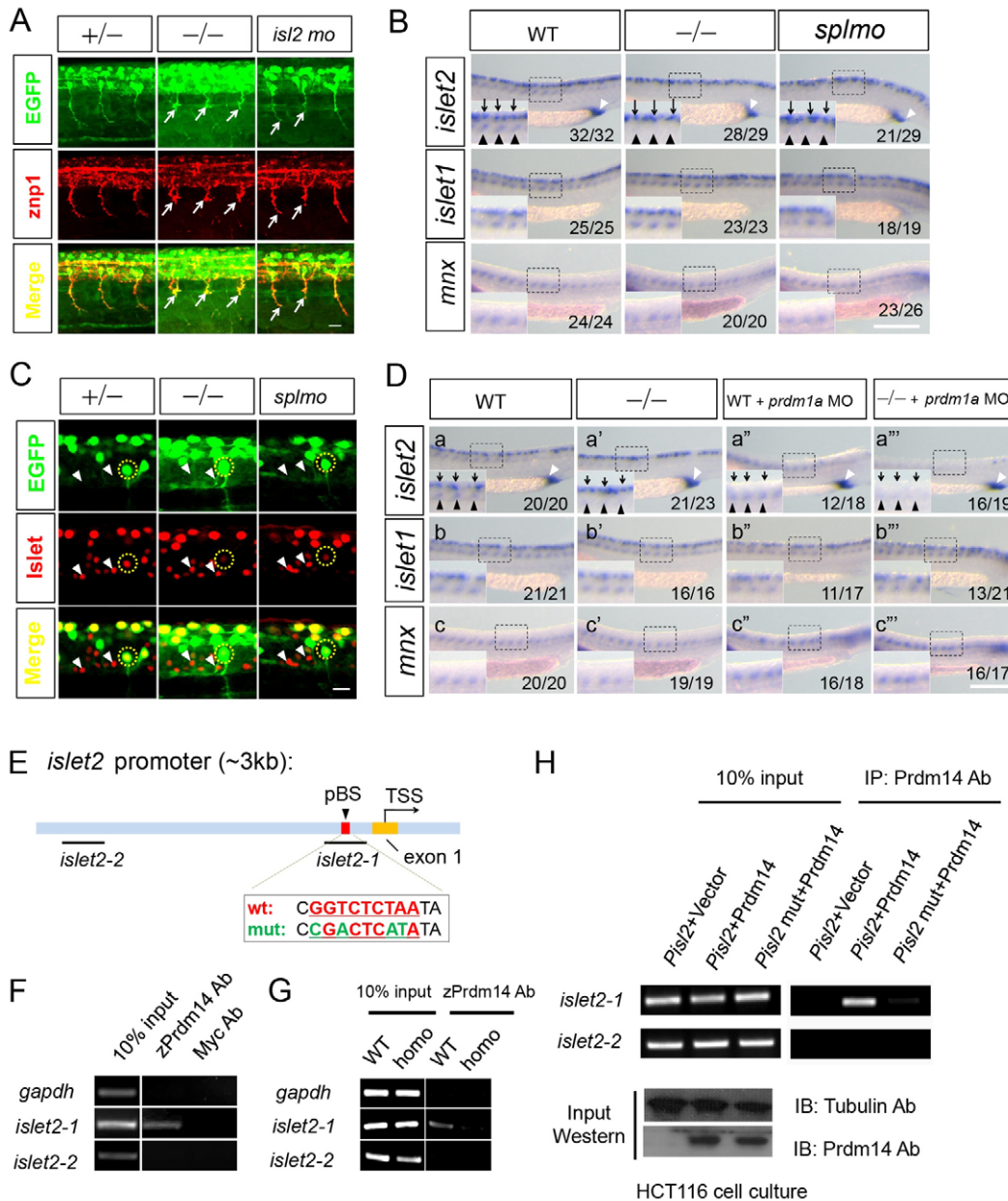


Fig. 6. *islet2* is a downstream target of Prdm14. (A) CaP axons are shortened in *islet2* morphants and *slg* mutants. The embryos are stained with znp1 antibody. Arrows indicate shortened CaP axons. (B) *islet2* RNA expression is only downregulated in CaP (black arrowheads) in *slg* mutant and *splmo* morphant embryos, whereas *islet2* expression in RB (black arrows) and cloaca (white arrowhead) is unaffected. *islet1* and *mnx1* expression are unaffected by Prdm14 reduction. (C) Islet antibody staining indicates that the signal intensity of Islet2 (circled) in the CaP neurons is substantially reduced in *slg* mutants and *splmo* morphants compared with heterozygotes. Islet1 signal (arrowheads) in GFP-negative neurons (non-CaP) showed comparable staining intensity in embryos of all genotypes. (D) (a-a'') *islet2* RNA expression is decreased specifically in CaP (black arrowheads) in *slg* mutants (a'). *islet2* is specifically reduced in RB (black arrows) in *prdm1a* morphants (a''). *prdm1a* MO injection into *slg* mutants results in greatly decreased *islet2* expression in both RB and CaP (a'''). *islet2* in cloaca remains unchanged (white arrowheads). (b-c'') *islet1* and *mnx1* expression in RB and CaP remains largely unchanged when *prdm14* and *prdm1a* are downregulated. (B,D) The number of embryos showing this phenotype out of the total examined is indicated bottom right. (E) The *islet2* promoter contains a putative Prdm14 binding site (pBS). The conserved sequence among zebrafish, mouse and human is indicated in red; mutated nucleotides are in green. TSS, transcription start site. (F) ChIP-PCR shows that Prdm14 antibodies specifically precipitate an *islet2* promoter fragment containing the pBS in wild-type embryos. (G) The ChIP signal from the Prdm14 antibodies is greatly decreased in *slg* mutants. (H) Zebrafish Prdm14 expressed in HCT116 cells efficiently pulls down the pBS-containing fragment when an *islet2* promoter (*PisI2*) plasmid is cotransfected. The PCR signal is greatly reduced when a pBS mutant (*PisI2* mut) plasmid is transfected or Prdm14 protein is not expressed in the cells. Scale bars: 20 μ m in A,C; 200 μ m in B,D.

These data suggest that Prdm14 could be required for the timely connection of the axons to their targets. Alternatively, the residual Prdm14 expression in *slg* mutant embryos might be enough for full axon extension, albeit at a slow rate. We favor the second

possibility because *prdm14* SpIMO injection, which is more efficient in blocking *prdm14* expression (supplementary material Fig. S6D), causes more severe axon outgrowth defects and slows the full growth of the axons to their final targets (data not shown).

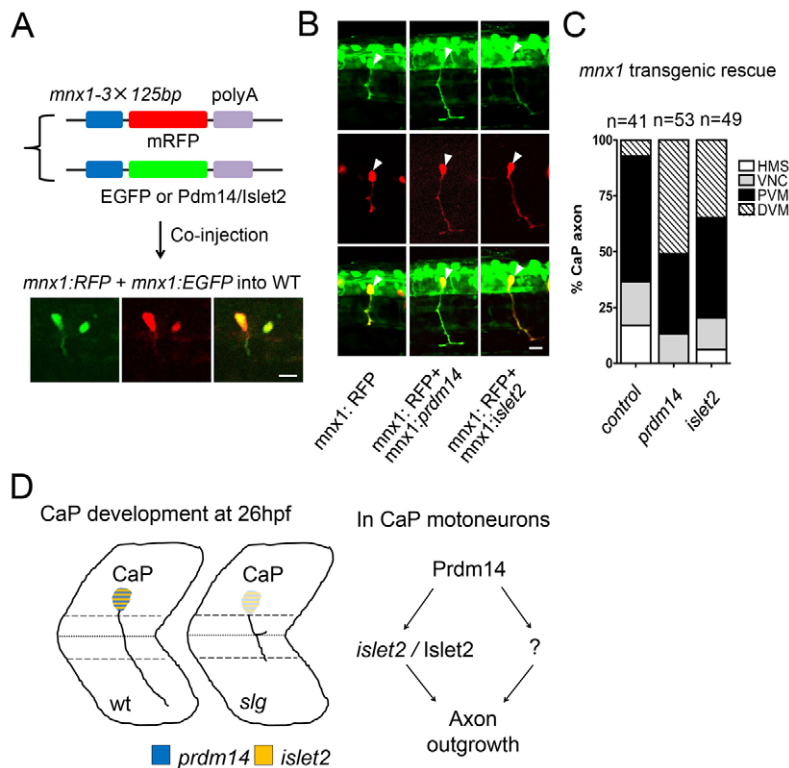


Fig. 7. Transgenic *islet2* expression in *slg* mutants rescues the axon outgrowth defect. (A) An *mnx1*-3x125bp enhancer sequence (*mnx1*) was used to direct protein co-expression in the same primary motoneurons (PMNs) from two injected plasmids (*mRFP* and *GFP*). (B) Expression of Prdm14 or Islet2 in CaP rescues the axon outgrowth defect of *slg* mutant zebrafish embryos. Arrowheads indicate CaP cell bodies. (C) Summary of results in B. *n*, number of RFP-labeled CaP neurons. *n*=41 from 20 injected embryos in the *mnx1:RFP* group (control); *n*=53 from 33 injected embryos in the *mnx1:RFP* + *mnx1:prdm14* group (*prdm14*); *n*=49 from 28 injected embryos in the *mnx1:RFP* + *mnx1:islet2* group (*islet2*). (D) Proposed regulation of CaP axon outgrowth. Prdm14 regulates CaP axon outgrowth through *islet2* and as yet unidentified factors (question mark). Scale bars: 20 μ m.

Generation and analysis of a *prdm14* null mutant might provide further insight into the requirements of Prdm14 activity in CaP axon development.

Islet2 mediates Prdm14 functions in CaP

Although *islet1* and *islet2* show specific expression patterns in later PMNs, either is sufficient to specify MiP and CaP subtypes (Hutchinson and Eisen, 2006). It is hypothesized that factors upstream of the *Islet* genes regulate the specification of different PMNs. Indeed, Nkx6 proteins expressed in MiP act upstream of *islet1* and are required to maintain MiP identity (Cheesman et al., 2004; Hutchinson et al., 2007). An upstream regulator of *islet2* in CaP had not been identified prior to this study. We show here that Prdm14 directly binds to the promoter region of the *islet2* gene and is required for *islet2* expression in CaP (Fig. 6). *Islet2* plays a major role in neuronal subtype specification and MN subclass differentiation (Lee and Pfaff, 2001; Shirasaki and Pfaff, 2002). In addition, *islet2* is an essential factor for Slit2 to induce axonal branching and elongation of TGNs, suggesting that downstream factors of *Islet2* are crucial for mediating axonal development (Yeo et al., 2004). We show here that *Islet2* is a major mediator of Prdm14 function in CaP axon outgrowth.

To further investigate axon development in CaP, it would be helpful to identify downstream targets of *Islet2*. Several proteins have been implicated in CaP axon morphogenesis. For example, Plexin A3 and Nrp1a expressed in CaP are required for axon outgrowth (Feldner et al., 2005; Feldner et al., 2007; Sato-Maeda et al., 2006). However, the expression patterns of *plexin A3* and *nrp1a* are not altered in *slg* mutants (supplementary material Fig. S10B). Similarly, expression of *agrin* and *c-met*, which encode two further factors involved in CaP axon growth (Kim et al., 2007; Tallafuss and Eisen, 2008), is largely unaffected in *slg* mutants (supplementary material Fig. S10B). However, other changes, such as post-translational modifications to these molecules in CaP of *slg*

mutants, cannot be ruled out. In addition, components of the ECM, such as Tenascin-C and Collagen XVIII, are also important in CaP axon outgrowth (Schneider and Granato, 2006; Schweitzer et al., 2005). Possible changes to signal transducers of the ECM in CaP could result in *slg* mutant phenotypes. It would be interesting to investigate connections between the ECM and Prdm14 or *Islet2* activities. *prdm14* is also expressed in RB (Fig. 1). However, *islet2* expression is only downregulated in CaP but not in RB in *slg* mutant embryos. Our data suggest that Prdm14 and Prdm1a regulate *islet2* expression in CaP and RB, respectively (Fig. 6D) (Olesnicki et al., 2010). The functions of Prdm14 in RB remain to be investigated.

Acknowledgements

We thank members of the J.Z. laboratory for discussions; Dr Shanshan Zhu for bioinformatics assistance; Drs Catherina G. Becker, Kristin Bruk Artinger, Yoshihiro Yoshihara and Juan L. Bruses for plasmids; and Dr Koichi Kawakami for the original *To2* system.

Funding

This work was supported by grants from the Ministry of Science and Technology of China (MOST) [2011CB943800, 2005CB522502]; the Chinese Academy of Sciences (CAS) [XDA01010108]; and the National Natural Science Foundation of China (NSFC) [90408001, 30425013].

Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material available online at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.083055/-/DC1>

References

- 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.
- Bonanomi, D. and Pfaff, S. L. (2010). Motor axon pathfinding. *Cold Spring Harb. Perspect. Biol.* **2**, a001735.

- Brustein, E., Saint-Amant, L., Buss, R. R., Chong, M., McDermid, J. R. and Drapeau, P. (2003). Steps during the development of the zebrafish locomotor network. *J. Physiol. Paris* **97**, 77-86.
- 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.
- Chia, N. Y., Chan, Y. S., Feng, B., Lu, X., Orlov, Y. L., Moreau, D., Kumar, P., Yang, L., Jiang, J., Lau, M. S. et al. (2010). A genome-wide RNAi screen reveals determinants of human embryonic stem cell identity. *Nature* **468**, 316-320.
- Chitramuthu, B. P., Baranowski, D. C., Kay, D. G., Bateman, A. and Bennett, H. P. (2010). Progranulin modulates zebrafish motoneuron development in vivo and rescues truncation defects associated with knockdown of Survival motor neuron 1. *Mol. Neurodegener.* **5**, 41.
- Chuikov, S., Levi, B. P., Smith, M. L. and Morrison, S. J. (2010). Prdm16 promotes stem cell maintenance in multiple tissues, partly by regulating oxidative stress. *Nat. Cell Biol.* **12**, 999-1006.
- Eisen, J. S., Myers, P. Z. and Westerfield, M. (1986). Pathway selection by growth cones of identified motoneurons in live zebra fish embryos. *Nature* **320**, 269-271.
- Eisen, J. S., Pike, S. H. and Romancier, B. (1990). An identified motoneuron with variable fates in embryonic zebrafish. *J. Neurosci.* **10**, 34-43.
- Endo, K., Karim, M. R., Taniguchi, H., Krejci, A., Kinameri, E., Siebert, M., Ito, K., Bray, S. J. and Moore, A. W. (2011). Chromatin modification of Notch targets in olfactory receptor neuron diversification. *Nat. Neurosci.* **15**, 224-233.
- Feldner, J., Becker, T., Goishi, K., Schweitzer, J., Lee, P., Schachner, M., Klagsbrun, M. and Becker, C. G. (2005). Neupilin-1a is involved in trunk motor axon outgrowth in embryonic zebrafish. *Dev. Dyn.* **234**, 535-549.
- Feldner, J., Reimer, M. M., Schweitzer, J., Wendik, B., Meyer, D., Becker, T. and Becker, C. G. (2007). PlexinA3 restricts spinal exit points and branching of trunk motor nerves in embryonic zebrafish. *J. Neurosci.* **27**, 4978-4983.
- Granato, M., van Eeden, F. J., Schach, U., Trowe, T., Brand, M., Furutani-Seiki, M., Haffter, P., Hammerschmidt, M., Heisenberg, C. P., Jiang, Y. J. et al. (1996). Genes controlling and mediating locomotion behavior of the zebrafish embryo and larva. *Development* **123**, 399-413.
- Hayashi, K., Yoshida, K. and Matsui, Y. (2005). A histone H3 methyltransferase controls epigenetic events required for meiotic prophase. *Nature* **438**, 374-378.
- Hernandez-Lagunas, L., Choi, I. F., Kaji, T., Simpson, P., Hershey, C., Zhou, Y., Zon, L., Mercola, M. and Artinger, K. B. (2005). Zebrafish narrowminded disrupts the transcription factor prdm1 and is required for neural crest and sensory neuron specification. *Dev. Biol.* **278**, 347-357.
- Hilario, J. D., Rodino-Klapac, L. R., Wang, C. and Beattie, C. E. (2009). Semaphorin 5A is a bifunctional axon guidance cue for axial motoneurons in vivo. *Dev. Biol.* **326**, 190-200.
- 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.
- Hutchinson, S. A., Cheesman, S. E., Hale, L. A., Boone, J. Q. and Eisen, J. S. (2007). Nkx6 proteins specify one zebrafish primary motoneuron subtype by regulating late islet1 expression. *Development* **134**, 1671-1677.
- Jowett, T. (2001). Double in situ hybridization techniques in zebrafish. *Methods* **23**, 345-358.
- Kanning, K. C., Kaplan, A. and Henderson, C. E. (2010). Motor neuron diversity in development and disease. *Annu. Rev. Neurosci.* **33**, 409-440.
- Kim, K. C., Geng, L. and Huang, S. (2003). Inactivation of a histone methyltransferase by mutations in human cancers. *Cancer Res.* **63**, 7619-7623.
- Kim, M. J., Liu, I. H., Song, Y., Lee, J. A., Halfter, W., Balice-Gordon, R. J., Linney, E. and Cole, G. J. (2007). Agrin is required for posterior development and motor axon outgrowth and branching in embryonic zebrafish. *Glycobiology* **17**, 231-247.
- Kinameri, E., Inoue, T., Aruga, J., Imayoshi, I., Kageyama, R., Shimogori, T. and Moore, A. W. (2008). Prdm proto-oncogene transcription factor family expression and interaction with the Notch-Hes pathway in mouse neurogenesis. *PLoS ONE* **3**, e3859.
- Lee, S. K. and Pfaff, S. L. (2001). Transcriptional networks regulating neuronal identity in the developing spinal cord. *Nat. Neurosci.* **4**, 1183-1191.
- Lewis, K. E. and Eisen, J. S. (2003). From cells to circuits: development of the zebrafish spinal cord. *Prog. Neurobiol.* **69**, 419-449.
- Lindeman, L. C., Vogt-Kielland, L. T., Aleström, P. and Collas, P. (2009). Fish'n ChIPs: chromatin immunoprecipitation in the zebrafish embryo. *Methods Mol. Biol.* **567**, 75-86.
- Liu, Y. G. and Chen, Y. (2007). High-efficiency thermal asymmetric interlaced PCR for amplification of unknown flanking sequences. *Biotechniques* **43**, 649-656.
- Ma, Z., Swigut, T., Valouev, A., Rada-Iglesias, A. and Wysocka, J. (2011). Sequence-specific regulator Prdm14 safeguards mouse ESCs from entering extraembryonic endoderm fates. *Nat. Struct. Mol. Biol.* **18**, 120-127.
- Macdonald, R. (1999). Zebrafish immunohistochemistry. *Methods Mol. Biol.* **127**, 77-88.
- Markus, A., Patel, T. D. and Snider, W. D. (2002). Neurotrophic factors and axonal growth. *Curr. Opin. Neurobiol.* **12**, 523-531.
- Mizuguchi, R., Sugimori, M., Takebayashi, H., Kosako, H., Nagao, M., Yoshida, S., Nabeshima, Y., Shimamura, K. and Nakafuku, M. (2001). Combinatorial roles of olig2 and neurogenin2 in the coordinated induction of pan-neuronal and subtype-specific properties of motoneurons. *Neuron* **31**, 757-771.
- Myers, P. Z., Eisen, J. S. and Westerfield, M. (1986). Development and axonal outgrowth of identified motoneurons in the zebrafish. *J. Neurosci.* **6**, 2278-2289.
- Ohinata, Y., Ohta, H., Shigeta, M., Yamanaka, K., Wakayama, T. and Saitou, M. (2009). A signaling principle for the specification of the germ cell lineage in mice. *Cell* **137**, 571-584.
- Olesnicki, E., Hernandez-Lagunas, L. and Artinger, K. B. (2010). prdm1a Regulates sox10 and islet1 in the development of neural crest and Rohon-Beard sensory neurons. *Genesis* **48**, 656-666.
- Pattyn, A., Vallstedt, A., Dias, J. M., Sander, M. and Ericson, J. (2003). Complementary roles for Nkx6 and Nkx2 class proteins in the establishment of motoneuron identity in the hindbrain. *Development* **130**, 4149-4159.
- Rodino-Klapac, L. R. and Beattie, C. E. (2004). Zebrafish topped is required for ventral motor axon guidance. *Dev. Biol.* **273**, 308-320.
- Rossi, C. C., Kaji, T. and Artinger, K. B. (2009). Transcriptional control of Rohon-Beard sensory neuron development at the neural plate border. *Dev. Dyn.* **238**, 931-943.
- Roy, S. and Ng, T. (2004). Blimp-1 specifies neural crest and sensory neuron progenitors in the zebrafish embryo. *Curr. Biol.* **14**, 1772-1777.
- Sato-Maeda, M., Tawarayama, H., Obinata, M., Kuwada, J. Y. and Shoji, W. (2006). *Sema3a1* guides spinal motor axons in a cell- and stage-specific manner in zebrafish. *Development* **133**, 937-947.
- Schneider, V. A. and Granato, M. (2006). The myotomal diwanka (lh3) glycosyltransferase and type XVIII collagen are critical for motor growth cone migration. *Neuron* **50**, 683-695.
- Schweitzer, J., Becker, T., Lefebvre, J., Granato, M., Schachner, M. and Becker, C. G. (2005). Tenascin-C is involved in motor axon outgrowth in the trunk of developing zebrafish. *Dev. Dyn.* **234**, 550-566.
- Segawa, H., Miyashita, T., Hirate, Y., Higashijima, S., Chino, N., Uyemura, K., Kikuchi, Y. and Okamoto, H. (2001). Functional repression of Islet-2 by disruption of complex with Ldb impairs peripheral axonal outgrowth in embryonic zebrafish. *Neuron* **30**, 423-436.
- Shirasaki, R. and Pfaff, S. L. (2002). Transcriptional codes and the control of neuronal identity. *Annu. Rev. Neurosci.* **25**, 251-281.
- Tallafuss, A. and Eisen, J. S. (2008). The Met receptor tyrosine kinase prevents zebrafish primary motoneurons from expressing an incorrect neurotransmitter. *Neural Dev.* **3**, 18.
- Tanaka, H., Maeda, R., Shoji, W., Wada, H., Masai, I., Shiraki, T., Kobayashi, M., Nakayama, R. and Okamoto, H. (2007). Novel mutations affecting axon guidance in zebrafish and a role for plexin signalling in the guidance of trigeminal and facial nerve axons. *Development* **134**, 3259-3269.
- 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.
- Tsuneoyoshi, N., Sumi, T., Onda, H., Nojima, H., Nakatsuji, N. and Suemori, H. (2008). PRDM14 suppresses expression of differentiation marker genes in human embryonic stem cells. *Biochem. Biophys. Res. Commun.* **367**, 899-905.
- Urasaki, A., Morvan, G. and Kawakami, K. (2006). Functional dissection of the Tol2 transposable element identified the minimal cis-sequence and a highly repetitive sequence in the subterminal region essential for transposition. *Genetics* **174**, 639-649.
- Urasaki, A., Asakawa, K. and Kawakami, K. (2008). Efficient transposition of the Tol2 transposable element from a single-copy donor in zebrafish. *Proc. Natl. Acad. Sci. USA* **105**, 19827-19832.
- Westerfield, M. (2000). *The Zebrafish Book: a Guide for the Laboratory Use of Zebrafish (Danio rerio)*. Eugene, OR: University of Oregon Press.
- Westerfield, M., McMurray, J. V. and Eisen, J. S. (1986). Identified motoneurons and their innervation of axial muscles in the zebrafish. *J. Neurosci.* **6**, 2267-2277.
- Yamaji, M., Seki, Y., Kurimoto, K., Yabuta, Y., Yuasa, M., Shigeta, M., Yamanaka, K., Ohinata, Y. and Saitou, M. (2008). Critical function of Prdm14 for the establishment of the germ cell lineage in mice. *Nat. Genet.* **40**, 1016-1022.
- Yeo, S. Y., Miyashita, T., Fricke, C., Little, M. H., Yamada, T., Kuwada, J. Y., Huh, T. L., Chien, C. B. and Okamoto, H. (2004). Involvement of Islet-2 in the Slit signaling for axonal branching and defasciculation of the sensory neurons in embryonic zebrafish. *Mech. Dev.* **121**, 315-324.
- Zelenchuk, T. A. and Brusés, J. L. (2011). In vivo labeling of zebrafish motor neurons using an *mxn1* enhancer and Gal4/UAS. *Genesis* **49**, 546-554.

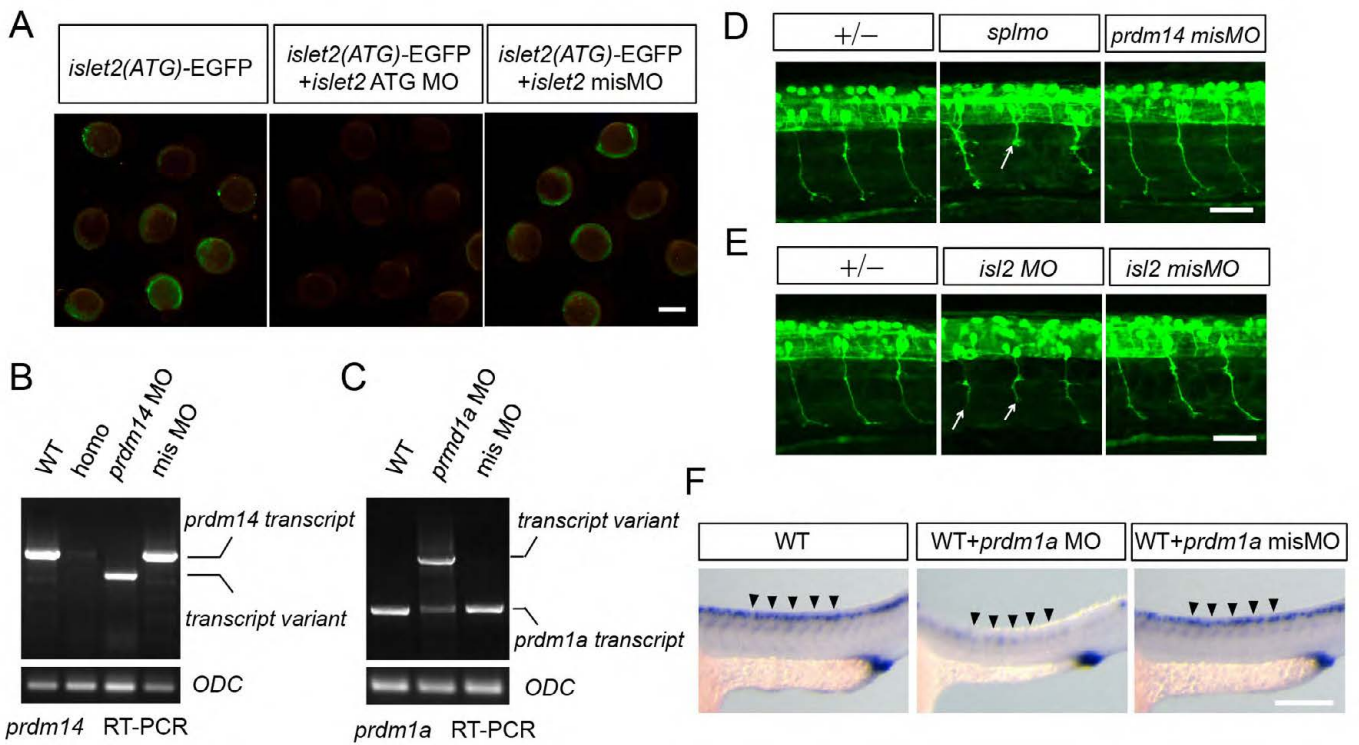


Fig. S1. Morpholinos used in this study are efficient and specific. (A) *islet2* MO is sufficient to suppress translation of *EGFP* mRNA containing the MO binding site (*islet2* ATG). *islet2* misMO shows no effect on the *EGFP* reporter translation. (B,C) *prdm14* and *prdm1a* splicing MO injections result in reduction of their respective transcripts and appearance of aberrant transcript variants. The control mismatched MOs have no effect. (D,E) *prdm14* and *islet2* misMO injections have no effects on CaP axon outgrowth. (F) *prdm1a* misMO injection affects *islet2* expression in RB. Arrows (D,E) indicate shortened CaP axons; arrowheads (F) indicate RB neurons. Scale bars: 500 μ m in A; 50 μ m in D,E; 200 μ m in F.

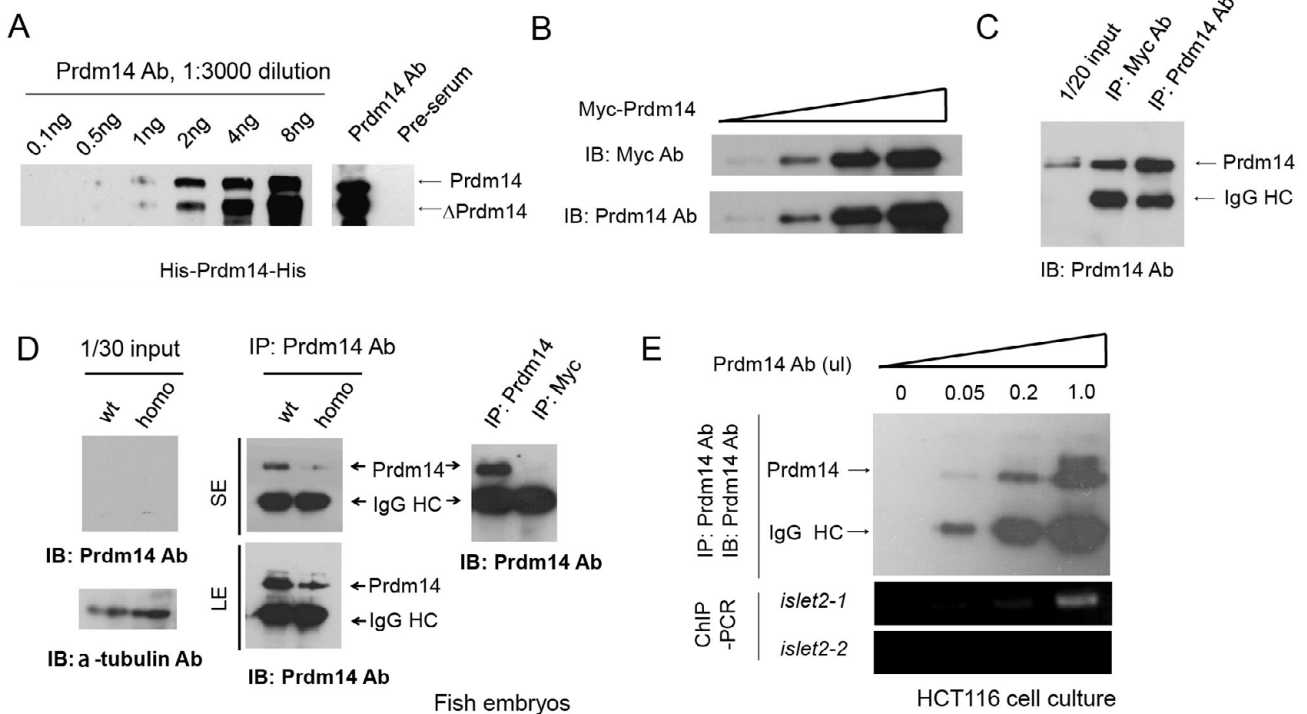


Fig. S2. Purified Prdm14 antibodies can pull down endogenous Prdm14. (A) Prdm14 antibodies (Ab) (diluted 1:3000) recognize His-Prdm14-His expressed from BL21 bacteria. (B) Prdm14 Ab recognize Myc-Prdm14 expressed from HEK293 cells. The detection efficiency is comparable to a commercialized Myc Ab (MBL, cat. 562). (C) Prdm14 Ab pull down Myc-Prdm14 with a comparable or even higher efficiency compared with Myc Ab. (D) Prdm14 Ab enrich and pull down endogenous Prdm14 protein from zebrafish embryo lysate (~200 embryos at 24 hpf). SE, short-time exposure; LE, long-time exposure. (E) Prdm14 Ab dose-dependently precipitate fragments containing pBS in the ChIP-PCR assay from HCT116 lysate.

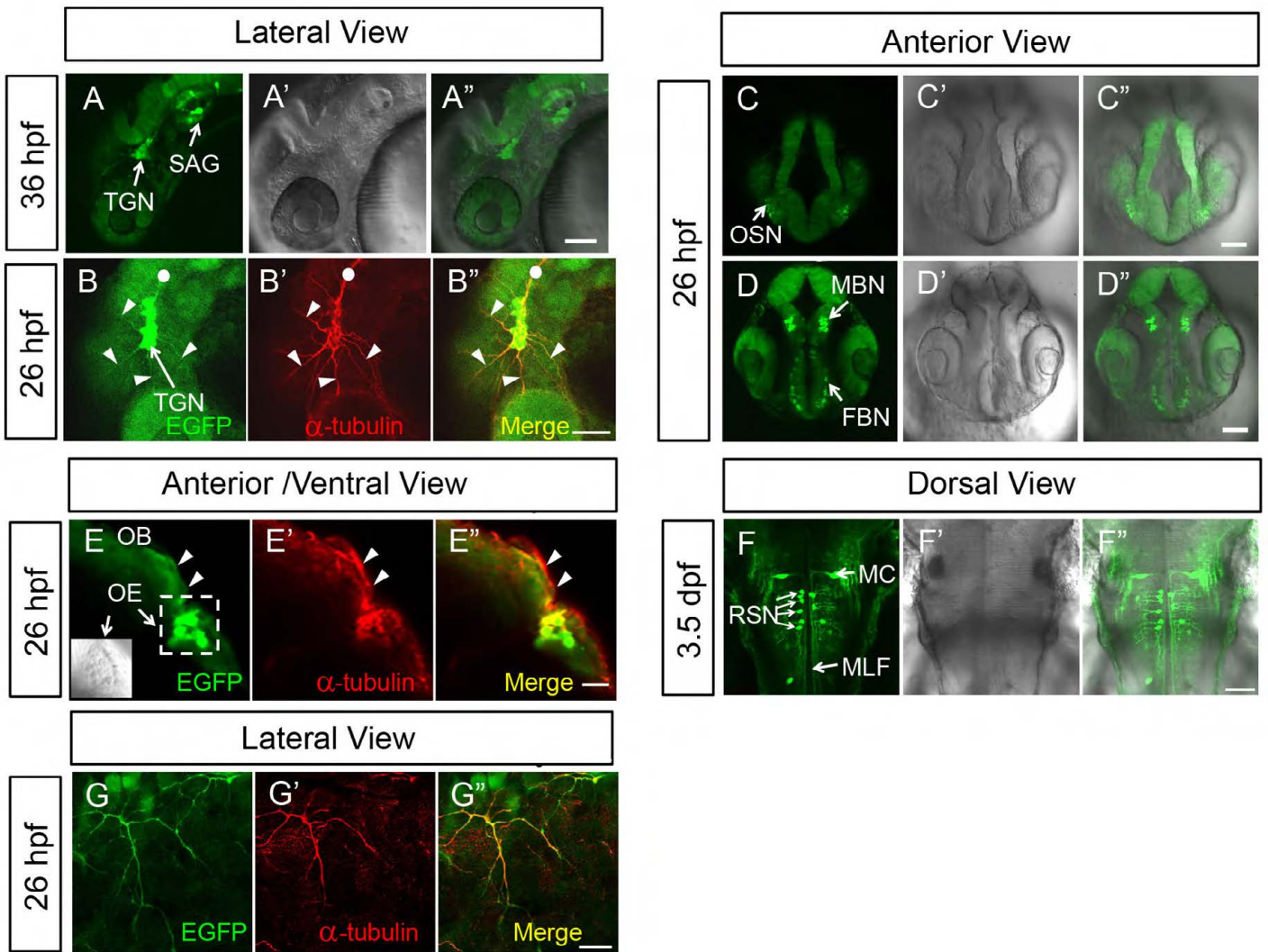


Fig. S3. Expression patterns of the trapped gene in other neurons. (A-A'') GFP expression in TGN and SAG in 36-hpf embryos. (B-B'') Acetylated α -tubulin expression in TGN in 26-hpf embryos (circle, central axon; arrowheads, peripheral axons). (C-D'') GFP expression in OSN, FBN and MBN in 26-hpf embryos. (E-E'') Acetylated α -tubulin in OSN in 26-hpf embryos (square, OE; arrowheads, axons projected from OSN to OE). (F-F'') GFP expression in MC, RSN and their afferents (MLF) in 3.5-dpf embryos. (G-G'') Acetylated α -tubulin in RB in 26-hpf embryos. FBN, forebrain neurons; MBN, midbrain neurons; MC, Mauthner cells; MLF, medial longitudinal fasciculus; OB, olfactory bulb; OE, olfactory epithelium; OSN, olfactory sensory neurons; RSN, reticulospinal neurons; SAG, statoacoustic ganglion neurons; TGN, trigeminal neurons. Scale bars: 50 μ m in A-D'', F-F''; 20 μ m in E-E'', G-G''.

Human	QTLDKDSLQLPEGLCLMQTVFGEVPHFGVFCSSFIAKGVRFGPF	GKVVNASEVRYTYGDNSVMWEIFEDGHLSHFIDGKG	319
Mouse	KTLDKDSLQLPEGLCLMQTISFGDVPHF'GVFCSDFIKGVRF'GPF	GRVNVNASEVKAHRDNSRMWEIFEDGHLSHFIDGKG	309
Zebrafish	GKLVNTSEIKTYDDNTLMWEIFENGRLSHFVDGRG	217
Consensus		g vn se k dn mweife g lshf dg g	
Human	GTGNWMSYVNCARFPKEQNLVAVQCOGHIEYESCKEIHQNQELLVWYGDCYEKFLDIFVSLQVTEPG.KQPSGPS	EESAE	398
Mouse	.SGNWMSYVNCARFPKEQNLAVQHGGQIEYESCRDIQRNQELLVWYNGYKFTIGVPMNLRVTEQGGQQLSESS	EESAE	388
Zebrafish	APGNWMSLVKCARFPKEQNLVAVQCDGQIYYEACKETIRAGQELLVWYDCYVQFI GIPILTLEKFIDD..SEALP	EDSGE	295
Consensus	gnwms v carfp eqnl avq g i ye c i qellvwyg y fl p l	e s e	
Human	GYRCERCGRVETTKYKYRDKHLKYTPCVDKQDRKFPCSLCKRSFEKRDRLRIHILHVHEKHRPHKCS	TCGKCFSQSSSLNK	478
Mouse	CYRCERCGRVETTKYKYRDKHLKYTPCVDKQDRKFPCSLCKRSFEKRDRLRIHILHVHERHPYLCST	TCGKSFQSSSLNK	468
Zebrafish	GFKCDRCGRVETTKYKYRDKHLKYTRCVDQDRKFPCFLCNRSFEKRDRLRIHILHVHEKHRPHKCS	VCGKSFQSSSLNK	375
Consensus	g c rcgkvf ykyyrdkhlkylt cvd qdrkfpcl c rsfekrdrlrihilhvhe hrp cs cgk fsqssslnk		
Human	HMRVHSGDRPYQCVYCTKRFETASSILRTHIRQHSGERPFKCKYCGKSFASHAAHDSHVRRSH	KEDDGCSCSICGKIFSDQ	558
Mouse	HMRVHSGDRPYQCVYCTKRFETASSILRTHIRQHSGERPFKCKHCGKAFASHAAHDSHVRRSH	KDNGRSSCDICCKGFLDQ	548
Zebrafish	HMRVHSGDRPYKCVYCNKRFETASSILRTHIRQHSGERPFKCKHCGKAFASHAAHDSHVRRTH	AKDKQLSCDVCGATFQEA	455
Consensus	hmrvhsg rpy cvyc k ftassilrthirqhsge pfkck cgk fashaahdshvrr h	sc cg f	
Human	ETFYSHMKFHEDY.....		571
Mouse	EAFYAHMRLHKT.....		561
Zebrafish	QELKYHMKAHKKRPLLESSVVPADENALFSTKESLHAQTQLADTFSPFGMTSISSEYRPW		516
Consensus	hm h		

Fig. S4. Prdm14 protein contains a highly conserved PR domain and a ZF domain. Alignment of zebrafish, mouse and human Prdm14 proteins. The blue box indicates the PR domain sequences and the red box indicates the ZF domain.

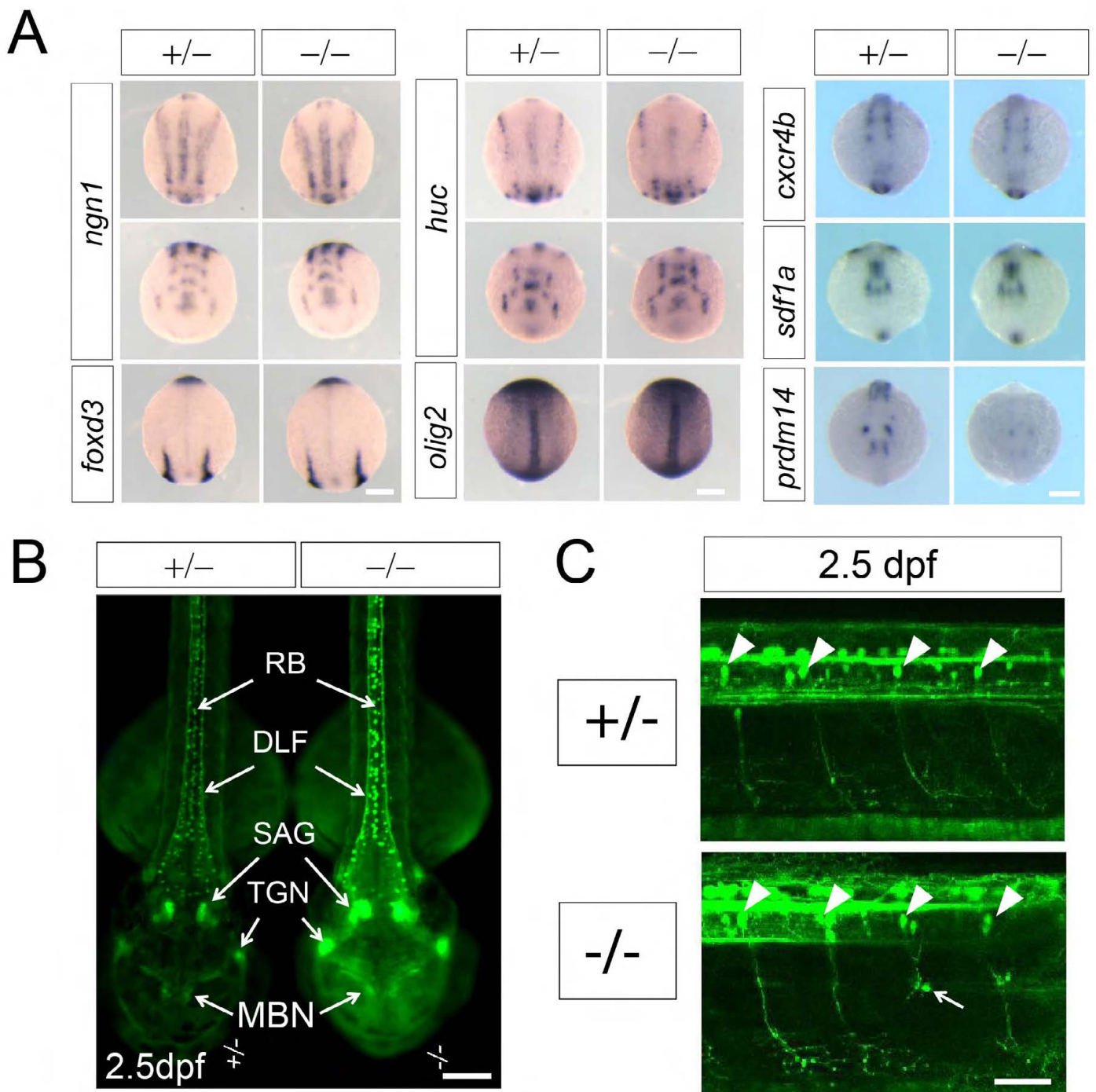


Fig. S5. Reduction of Prdm14 expression may not affect neuron specification. (A) Expression of the early neuron markers *ngn1*, *foxd3*, *huc*, *olig2* and *cxcr4b* is not substantially changed in *slg* mutant embryos at the 1- to 3-somite stage. *sdf1a* is used as a control. (B) At 2.5 dpf, neuron positions and cell numbers of MBN, TGN, SAG and RB are not greatly affected in *slg* mutant embryos. (C) At 2.5 dpf, CaP neurons (arrowheads) are present and the majority of the CaP neurons extend their axons to the ventral targets and form interactions in *slg* mutant embryos; a few shortened CaP axons are indicated by an arrow. Scale bars: 200 μ m in A; 50 μ m in B,C.

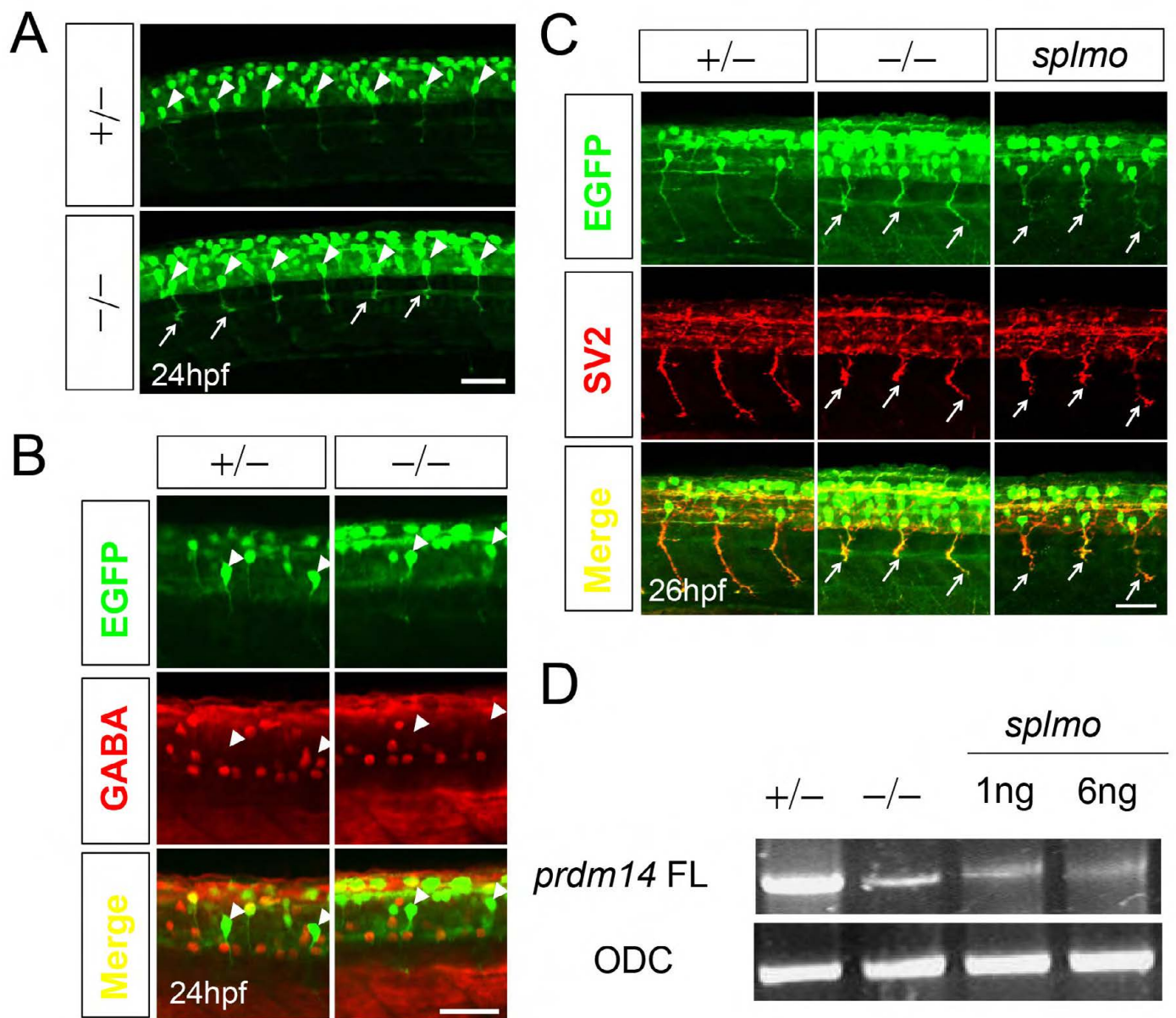


Fig. S6. Reduction of Prdm14 expression may not affect CaP specification. (A) Cell body position and cell number of CaP are not significantly changed in homozygous *slg* mutant embryos at 24 hpf, as compared with heterozygous embryos. Arrowheads indicate CaP neurons, arrows indicate shortened CaP axons. (B) No ectopic GABA expression in GFP-positive CaP neurons (arrowheads) in *slg* mutant embryos at 24 hpf. (C) At 26 hpf, CaP axons stained with the axon-specific marker SV2 show a shortened axon outgrowth phenotype in *slg* mutant and *splmo* morphant embryos. (D) In *slg* mutant, full-length *prdm14* is greatly reduced. In *splmo* morphant, *prdm14* reduction is dose dependent and is even more severe than that seen in the *slg* mutant. Scale bars: 50 μ m.

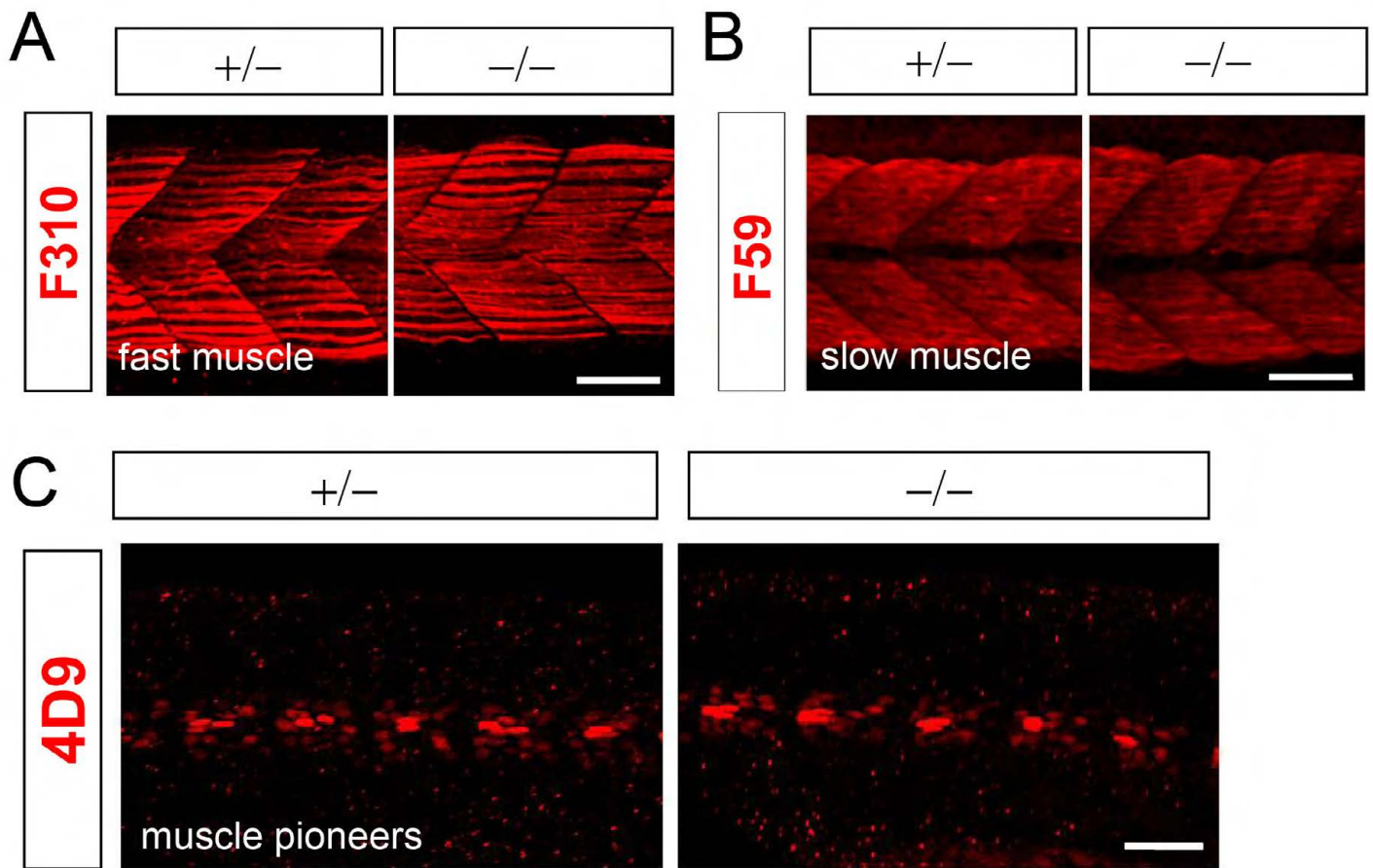


Fig. S7. Reduction of Prdm14 may not affect muscle development. Fast muscle (A), slow muscle (B) and muscle pioneer (C) cells are stained with F310, F59 and 4D9 antibodies, respectively. No obvious difference in muscle development is observed between wild-type and *slg* mutant embryos. Scale bars: 50 μ m.

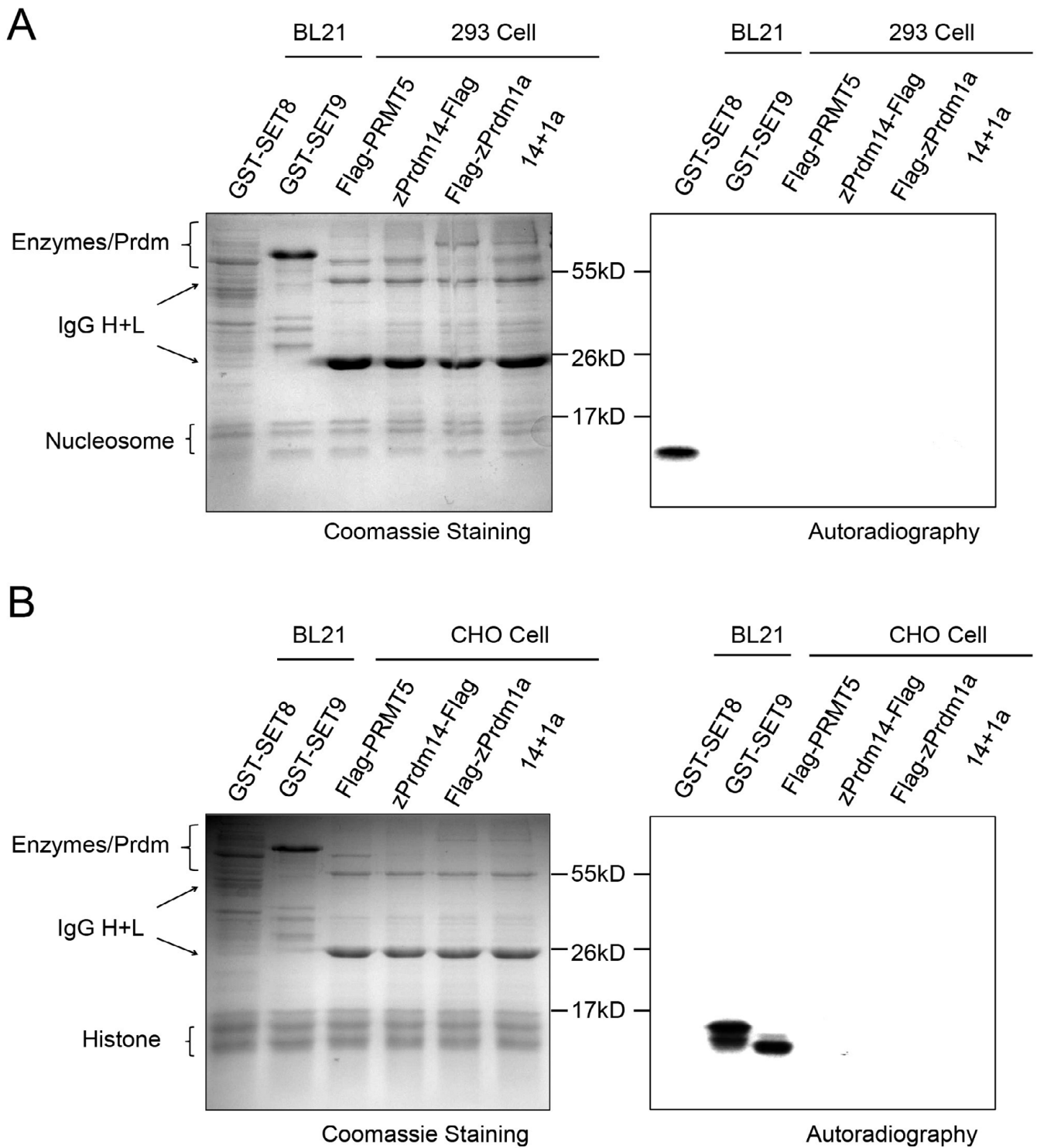


Fig. S8. Prdm14 protein may not possess methyltransferase activity. (A) Prdm14 expressed in HEK293T cells shows no methyltransferase activity with recombinant nucleosomes as substrate. (B) Prdm14 expressed in CHO cells shows no methyltransferase activity with histone octamers as substrate. As controls, SET8 methylates H3 in nucleosomes, SET9 methylates H3 and PRMT5 methylates H4 in the histone octamer.

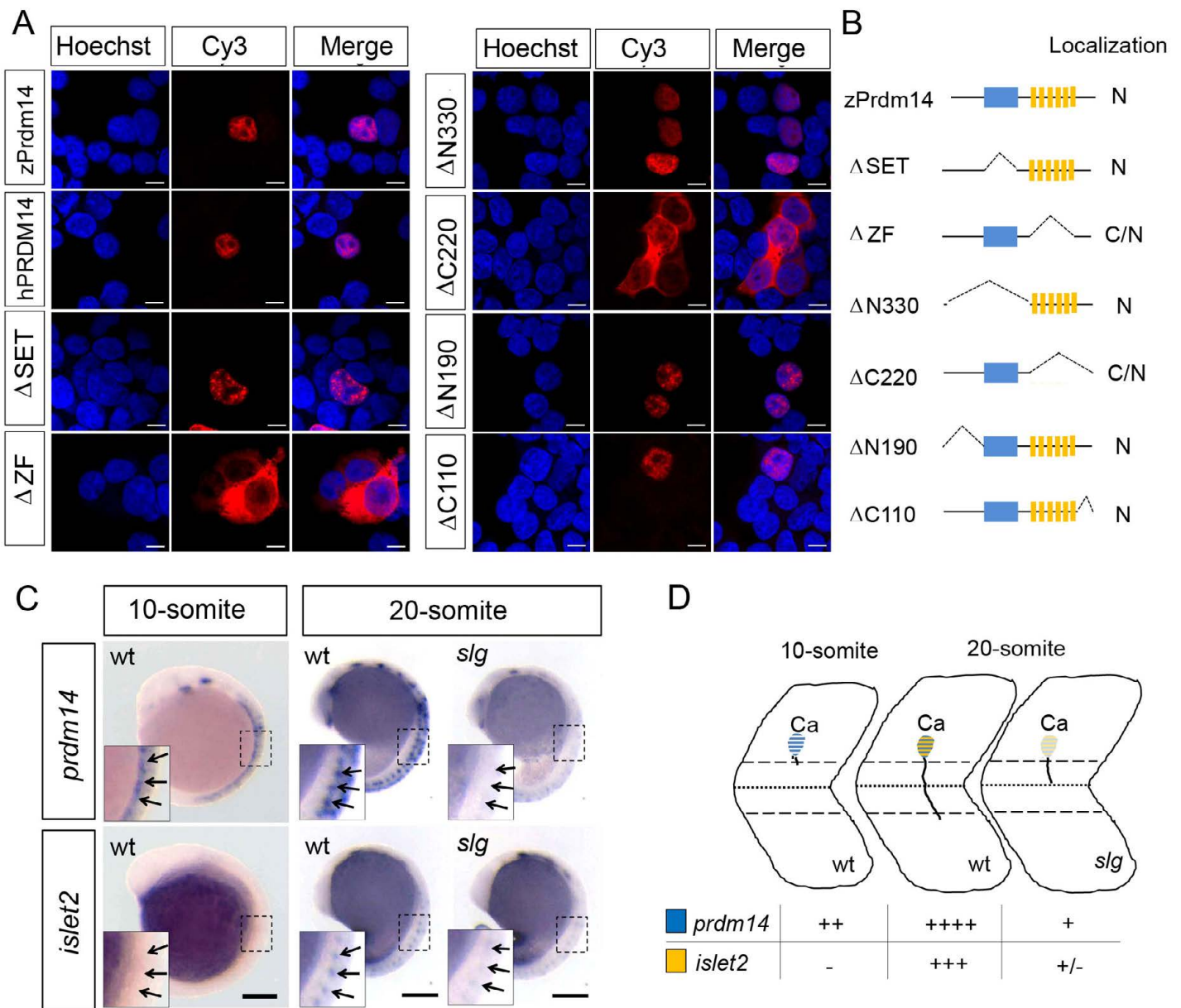


Fig. S9. Prdm14 is a nuclear protein and *prdm14* is expressed before *islet2* in zebrafish embryos. (A) Zebrafish Prdm14 is localized to the nucleus, similar to human PRDM14. The localization is ZF domain dependent. (B) The localization of different zebrafish Prdm14 isoforms is summarized. N, nucleus localized; C, cytoplasm localized. (C) *prdm14* is expressed earlier than *islet2* in CaP (arrows) at the 10-somite stage. Prdm14 downregulation causes decreased *islet2* expression at the 20-somite stage. (D) Diagram illustrating the results in C. Scale bars: 10 μ m in A; 200 μ m in C.

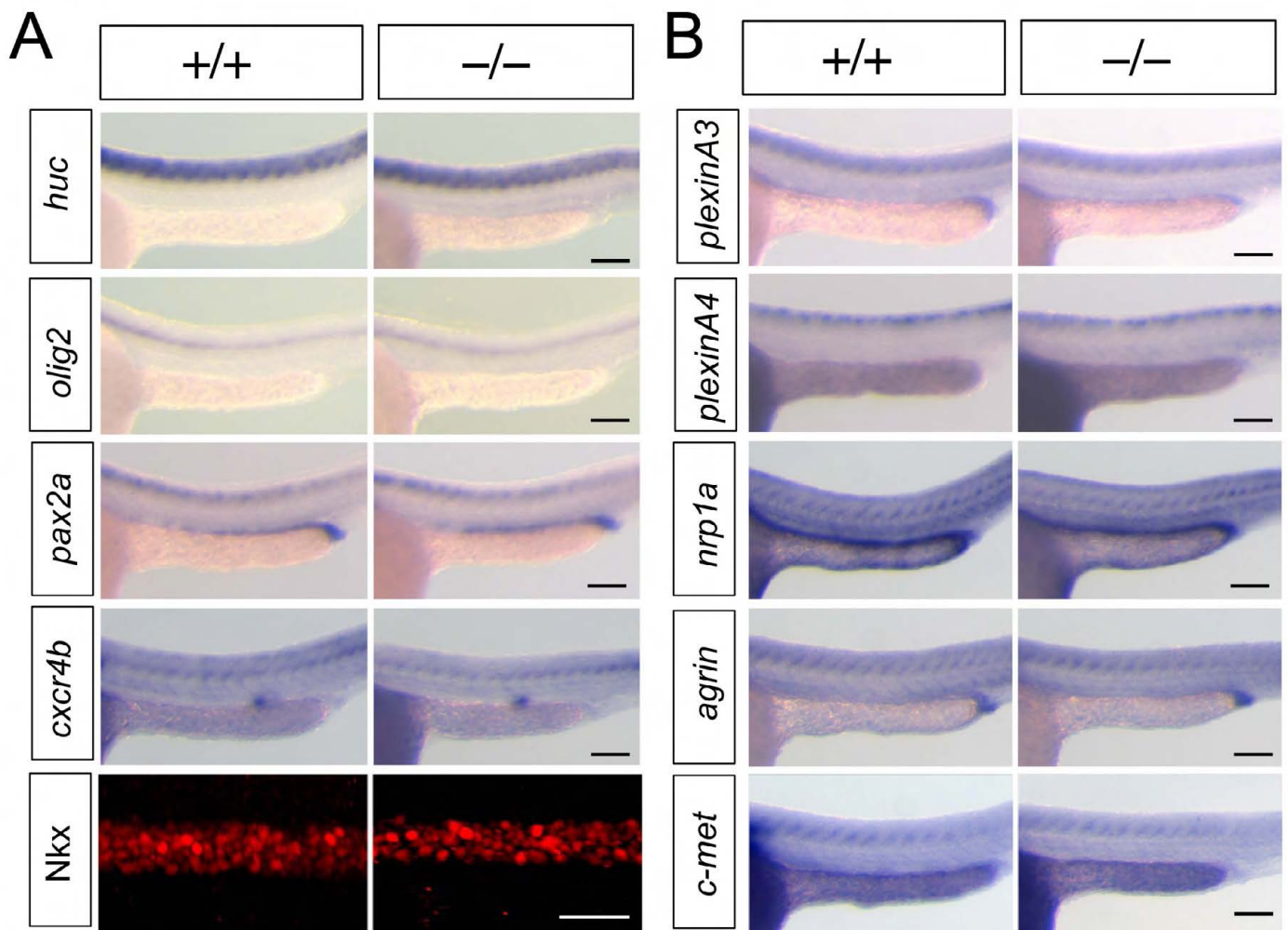
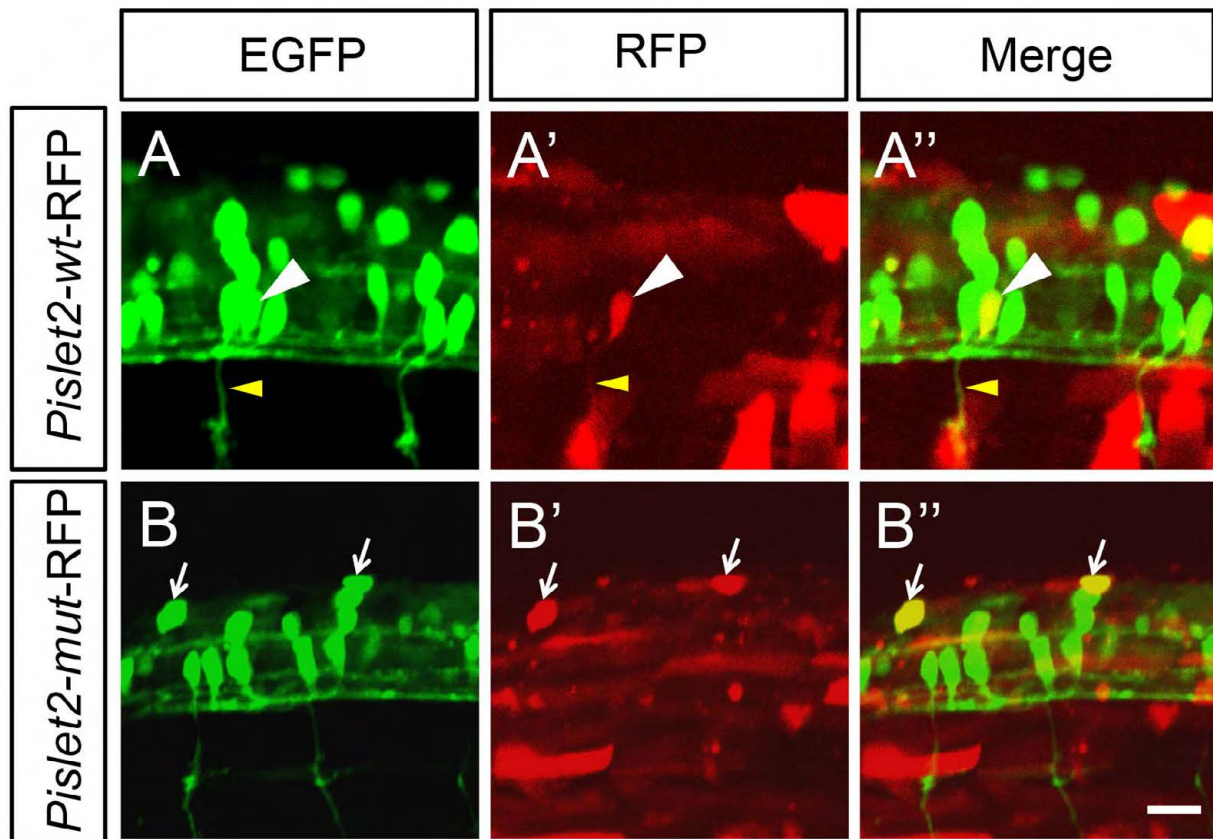


Fig. S10. Neuronal transcription factors and effectors are not substantially affected in the *slg* mutant. (A) Transcription factors involved in neuron development, including *huc*, *olig2*, *pax2a* and Nkx6.1, are not greatly changed in *slg* mutant embryos. (B) Effectors that might function downstream of Prdm14 in CaP axon outgrowth, including *cxcr4b*, *nrp1a*, *plexin 3A*, *plexin 4*, *agrin* and *c-met*, are not greatly changed in *slg* mutant embryos. However, *c-met* may be slightly downregulated in the mutant. Scale bars: 100 μ m.



C	Transient RFP Expression Pattern	
	RB positive	CaP positive
<i>Pislet2-wt-RFP</i> (N=27)	n=31, N=12	n=4, N=4
<i>Pislet2-mut-RFP</i> (N=36)	n=50, N=18	N=0

Fig. S11. The Prdm14 binding site (BS) may be required for *islet2* expression in CaP. (A-A'') The *islet2* promoter with wild-type BS drives RFP expression in CaP (white arrowheads, cell body; yellow arrowheads, axon). (B-B'') A promoter with a mutated BS does not affect RFP transgene expression in RB (arrow), but no longer drives RFP expression in CaP. (C) Summary of A and B. N, number of embryos; n, RFP-positive RB or CaP neurons. Scale bar: 20 μ m.

Table S1. Sequences of *bcl2* splicing acceptor and bovine growth hormone poly(A) and pause site

Name	Sequence (5'-3')	Other information
<i>bcl2</i> splicing acceptor (<i>bcl2</i> SA)	GGGCCCTGACCCTTATCCGCTCAATCTGTA ACAATGCAGATAAAAAGAAAATGAGTAACT GTATAACTAAATACTGTATAATTAGATGCT GTCAGTACACCAGTATTTTACATTTGAGTG TCTATGCAAACATAAATAGGCTAAATCAT AGAACCATACTTTATTTAAAAAAATACTTT ATTTATATATAATTATTTAAATATTTGTGA GAATTATCACAGTAATGTTGAGGCAAATC AAATCTAATCAAAGATGCTGTAAGACTGT AATAACAACCCATTTTACCTTAATTCATTT AGCAGTTTCATGCACCATAGACCGCAGGG GCAAGCAAAGGGTATAAAAATAGATACATA CATAGGAAATTTGCTGCAAGTTTGGTGGTC ATTCCACAAACAAACAAACATTTAATTTG ATTTATTTGGCATTATTTATCCATGCTTTGC TATTTTCACTAGTGCAATAATGTGATTTCT AATTGTCTGCTCCTAATACCCTTCTGTTTCT CTTTCAGGATGCCTTCGTGGAGATGTACGG TCAGCAGAGAGACTCTGTGTTCCACCCGTT TTCCATGG	Zebrafish <i>bcl2</i> splicing acceptor was cloned into T2AL200R150 with <i>ApaI/NcoI</i> to replace <i>EF1a</i> promoter and second intron of rabbit β -globin
Bovine growth hormone poly(A) and pause site (bGHpA.PS)	ATCGATCGACTGTGCCTTCTAGTTGCCAGC CATCTGTTGTTTGCCCCCTCCCCCGTGCCTT CCTTGACCCTGGAAGGTGCCACTCCCCTG TCCTTTCCTAATAAAAATGAGGAAATTGCAT CGCATTGTCTGAGTAGGTGTCATTCTATTC TGGGGGGTGGGGTGGGGCAGGACAGCAA GGGGGAGGATTGGGAAGACAATAGCAGG CATGCTGGGGATGCGGTGGGCTCTATGGC TTCTGAGAATTCAACATACGCTCTCCATCA AAACAAAACGAAACAAAACAAACTAGCA AAATAGGCTGTCCCCAGTGCAAGTGCAGG TGCCAGAACATTTCTCTATCGAT	Bovine growth hormone poly(A) and pause site were cloned upstream of original SV40 poly(A) with <i>ClaI/ClaI</i>

Table S2. Mismatched MOs and primers

Mismatched MO	Sequence (mutations underlined)
<i>prdm14</i> misMO	TCATTGTTGGAGCAAC <u>CGTGTGGTGG</u>
<i>islet2</i> misMO	GAT <u>TATGCACGATAGAGGACGGT</u> TAA
<i>prdm1a</i> misMO	TGCTGTGATACGTCTTT <u>CGAGTGTG</u>

Primers	Sequence (5'-3')
Tail-PCR	
LAD1-1	ACGATGGACTCCAGAGGC(G/C/A)N(G/C/A)NNNGGAA
LAD1-2	ACGATGGACTCCAGAGGC(G/C/T)N(G/C/T)NNNGGTT
LAD1-3	ACGATGGACTCCAGAGGC(G/C/A)(G/C/A)N(G/C/A)NNNCCAA
LAD1-4	ACGATGGACTCCAGAGGC(G/C/T)(G/A/T)N(G/C/T)NNNCGGT
AC1	ACGATGGACTCCAGAG
T3-1	CTCTAGATCAGATCTAATACTCAAGTACAA
T3-2	ACGATGGACTCCAGTCCGGCCACTCAAGTAAGATTCTAGCCAGATACTT
T3-3	CCTAAGTACTTGTACTTTCACTTGAGTAA
T5-1	GACTGTAAATAAAAATTGTAAGGAGTAAAAAGTACT
T5-2	ACGATGGACTCCAGTCCGGCCGTACTCAAGTAAAGTAAAATCCCCAAAAA
T5-3	CAAGTAAAATTACTCAAGTACTTTACACCT
<i>prdm14</i> genotyping	
<i>prdm14</i> G T up	GAGGCTTCATTAATGGTGACC
<i>prdm14</i> G T down	CAACAATATGCAGGTCAGACAC
<i>mutantG</i> T down	CGGTCTATGGTGCATGAACT
P1, P2, P3 (see Fig. 3)	
P1-up	ACACCAGACCTCTTTTCATC

P1-down	ATGTGTGTGCGAAGGATGCT
P2-up	ATGTCGGTTTCTCTCTCCAG
P2-down	TTAGTTCCAGGGTCTGTACTC
P3-up	ACACCAGACCTCTTTTCATC
P3-down	GCCTGCTATTGTCTTCCCAA
Cloning mRFP, EGFP, <i>islet2</i>, <i>prdm14</i> into DEST394 destination vector (containing <i>mnx1-3_125bp</i> promoter) by GATEWAY system	
mRFP attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCTGGATCCATG GCCTCCTCC
mRFP attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTTTAGGCGCCG GTGGAGTG
EGFP attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCTGCCACCATG GTGAGCAAG
EGFP attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTTTATCTAGAT CCGGTGGGA
Prdm14 attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCTGCAGGATCC ACCATGGCT
Prdm14 attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTTTAGTTCCAG GGTCTGTACTC
Iset2 attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCTGCAGGATCC ACCATGGCT
Iset2 attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTTTACGTCTCC ACGGGACTG
Antisense probes (SP6 promoter sequence is in bold)	
<i>olig2</i> up	GAGTGAAGTGGATAGCCTTA
<i>olig2</i> down	GATTTAGGTGACACTATAGTGGTGGCTTCTCAAAGTTCT
<i>pax2a</i> up	CGACCTCAGTCGATTATCTT
<i>pax2a</i> down	GATTTAGGTGACACTATAGAATCCCTCTGACCATTCAGA
<i>huc</i> up	ATGGAAACTCAGGTGTCCAA
<i>huc</i> down	GATTTAGGTGACACTATAGGATGACCTTGACGTTTGTGA
<i>c-met</i> up	GCTTCCATCCTAATCATCCT
<i>c-met</i> down	GATTTAGGTGACACTATAGTCGCTCAGAGTAAATGCACT

<i>mnx</i> up	CGAGGCGTTAATCTGTTTGT
<i>mnx</i> down	GATTTAGGTGACACTATAGTCGCTCAGAGTAAATGCACT
<i>agrin</i> up	TACCTGAAAGGCAAGACCAT
<i>agrin</i> down	GATTTAGGTGACACTATAGTGGCGTTGAACTTACAACCA
<i>plexinA3</i> up	AAGGTCTGGAATCATGAGGT
<i>plexinA3</i> down	GATTTAGGTGACACTATAGTCAGTGTCATTGGAGCACAT
<i>plexinA4</i> up	AAGGTCTGGAATCATGAGGT
<i>plexinA4</i> down	GATTTAGGTGACACTATAGTACTCATCTCCATCCCATCA
Testing splicing MO	
<i>prdm14</i> mo-up	ACACCAGACCTCTTTTCATC
<i>prdm14</i> mo-down	ATGTGTGTGCGAAGGATGCT
<i>prdm1a</i> mo-up	GTCACTTACCATCTGGACTA
<i>prdm1a</i> mo-down	GGTTCTTGCAGCACATCTTT
Cloning <i>islet2</i> ATG to upstream of EGFP	
<i>islet2</i> ATG up	GATCTCTGTAGCCTTCTGCCTTAACCCTCCTGTATGGTGG ATATTCTACC
<i>islet2</i> ATG	CATGGGTAGAATATCCACCATACAGGAGGGTTAAGGCAG AAGGCTACAGA