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prdm12b specifies the p1 progenitor domain and reveals a role for V1 interneurons in swim movements

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ABSTRACT

Proper functioning of the vertebrate central nervous system requires the precise positioning of many neuronal cell types. This positioning is established during early embryogenesis when gene regulatory networks pattern the neural tube along its anteroposterior and dorsoventral axes. Dorsoventral patterning of the embryonic neural tube gives rise to multiple progenitor cell domains that go on to differentiate unique classes of neurons and glia. While the genetic program is reasonably well understood for some lineages, such as ventrally derived motor neurons and glia, other lineages are much less characterized. Here we show that *prdm12b*, a member of the *PR domain containing*-family of transcriptional regulators, is expressed in the p1 progenitor domain of the zebrafish neural tube in response to Sonic Hedgehog signaling. We find that disruption of *prdm12b* function leads to dorsal expansion of *nkx6.1* expression and loss of p1-derived *eng1b*-expressing V1 interneurons, while the adjacent p0 and p2 domains are unaffected. We also demonstrate that *prdm12b*-deficient fish exhibit an abnormal touch-evoked escape response with excessive body contractions and a prolonged response time, as well as an inability to coordinate swimming movements, thereby revealing a functional role for V1 interneurons in locomotor circuits. We conclude that *prdm12b* is required for V1 interneuron specification and that these neurons control swimming movements in zebrafish.

Introduction

Formation of the central nervous system during vertebrate embryogenesis requires the initial establishment of multipotent progenitor cells that subsequently give rise to all mature neuronal and glial cell populations. The identity of such progenitor cells is defined, at least in part, by their position along the dorsoventral and anteroposterior axes of the developing neural tube. In particular, the neural tube is divided into multiple domains along its dorsoventral axis (reviewed in (Goulding, 2009)), such that neurons forming in the ventral domains (pd6, p0, p1, p2, pMN and p3) primarily contribute to motor circuits – with pMN-derived cells becoming motor neurons and glial cells, whereas cells from pd6 and p0–p3 develop into interneurons that modulate motor activity. In contrast, neurons arising from dorsal domains (pd1–pd5) are primarily involved in sensory pathways.

Progenitor domains along the dorsoventral axis are specified in response to opposing gradients of Sonic hedgehog (Shh; produced in the notochord and floor plate; reviewed in (Jessell and Sanes, 2000)) and bone morphogenetic proteins (BMPs; produced in roof plate and overlying epidermis; reviewed in (Lee and Jessell, 1999)). For instance, ventral progenitor cells respond to Shh in a graded fashion such that cells closest to the Shh source activate expression of *nkx2.2* in the p3 domain, while cells at a slightly greater distance activate *olig2* expression in the pMN domain (Ericson et al., 1997a; Lu et al., 2000). Other genes are induced more broadly by Shh – such as *nkx6.1* that is expressed in the p3, pMN and p2 domains (Qiu et al., 1998). In addition, Shh negatively regulates genes such as *pax6* and *irx3*, thereby restricting their expression to more dorsal domains (Ericson et al., 1997b; Briscoe et al., 2000). Domain-restricted expression of genes is reinforced by cross-repressive interactions. One such example is the pMN/p2 boundary where *olig2* represses *irx3* to establish its ventral expression limit and *irx3* reciprocally represses *olig2* to define its dorsal expression boundary (Novitch et al., 2001; Zhou and Anderson, 2002). The end result of these regulatory interactions is a combinatorial code of gene expression along the dorsoventral axis that leads to the specification of all motor neurons, interneurons and glial cells in the neural tube. Perturbation of this process leads to aberrant dorsoventral patterning and cell type misspecification. For example, loss of *olig2* function causes the pMN domain to give rise to V2 interneurons and astrocytes instead of motor neurons and oligodendrocytes (Zhou and Anderson, 2002).

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We recently identified *prdm12b* in a screen for genes expressed in the zebrafish nervous system (Choe et al., 2011). Prdm proteins are defined by an N-terminal PR domain and a variable number of zinc fingers (Fog et al., 2012). In general, Prdm proteins act as transcription factors with the ability to activate or repress transcription depending on the context and several *Prdm* genes function in the nervous system. For instance, *Prdm14* acts upstream of *isl2* to regulate outgrowth of motor axons (Liu et al., 2012) and Prdm8 forms a complex with Bhkhb5 (an Olig-related transcription factor) to regulate axon targeting during neural circuit assembly (Ross et al., 2012). Previous work demonstrated expression of *Prdm12b* in the mouse nervous system (Kinameri et al., 2008), but it is unclear if *prdm12b* functions in neural development. Here we report that *prdm12b* is expressed in the ventral neural tube, at least partially in response to Shh. We map *prdm12b* expression to the p1 domain and demonstrate that loss of *prdm12b* function leads to dorsal expansion of *nkx6.1* expression into the p1 domain, as well as to loss of *eng1b* expression in the p1 domain. The p1 domain normally gives rise to inhibitory interneurons, known as circumferential ipsilateral ascending (CiA) neurons in zebrafish (Bernhardt et al., 1990; Higashijima et al., 2004). CiA neurons are rhythmically active during zebrafish swimming and have been shown to make contacts with motor neurons (Higashijima et al., 2004), but their functional role in locomotion has not been explored. We find that zebrafish lacking *prdm12b* function are depleted of CiA interneurons, allowing us to examine the function of this class of neurons. Strikingly, *prdm12b*-depleted fish exhibit an exaggerated touch-evoked escape response and an inability to organize productive swimming movements. We conclude that *prdm12b* is required for specification of the p1 domain and that zebrafish V1 interneurons provide functionally important inhibitory signals to zebrafish locomotor circuits.

Material and methods

Wild type and transgenic zebrafish

Wild type and transgenic zebrafish were raised in the University of Massachusetts Medical School Zebrafish Facility. Embryos were staged according to morphological criteria (Kimmel et al., 1995) and hours post-fertilization (hpf). The *Tg(olig2:egfp)*^{vu12} (Shin et al., 2003), *Tg(isl1:egfp)*^{rw0} (Higashijima et al., 2000) and *Tg(hoxb1a:egfp)* (Choe et al., 2012) transgenic lines were used.

In situ RNA hybridization

Embryos were fixed in 4% paraformaldehyde (pfa) and stored in 100% methanol at −20 °C. In situ RNA hybridization was performed as described (Hauptmann and Gerster, 2000) followed by a color reaction using NBT/BCIP or INT/BCIP in 10% polyvinyl alcohol. Embryos were dissected from the yolk and flat mounted in 70% glycerol for imaging on bridged coverslips or sectioned as described (Zannino and Appel, 2009). Images were captured using a Nikon Eclipse E600 microscope equipped with Spot RT Color camera (model 2.2.1). Images were imported into Adobe Photoshop and adjustments were limited to contrast, levels, color matching settings, and cropping where all adjustments were applied to the entire image.

Immunocytochemistry

Primary antibodies: mouse anti-Isl (39.4D5, 1:100; Developmental Studies Hybridoma Bank [DSHB] (Ericson et al., 1992)), mouse anti-Zn8 (1:1000; DSHB (Trevarrow et al., 1990)), mouse anti-BrdU (G3G4, 1:200; DSHB (George-Weinstein et al., 1993)), mouse 3A10 (1:100; DSHB (Hatta, 1992)), and rabbit anti-MBP (1:1000; kind gift from B. Appel (Kucenas et al., 2009)). Alexa Fluro secondary antibodies: 488, 568 and 647 goat anti-mouse and 568 goat anti-rabbit (all at 1:200; Molecular Probes). Staining with secondary antibodies alone did not produce specific signals. Embryos were fixed in 4% AB fix (4% paraformaldehyde, 8% sucrose, 1 × PBS) for 2 h at room temperature (RT) or overnight at 4 °C. Whole-mount fluorescent labeling was performed as described (Zannino et al., 2012). All imaging was conducted on an upright Zeiss Imager.M2 equipped with either a 20 × water immersion objective [numerical aperture (NA)=1.0] or a 40 × water immersion objective (NA=1.2), mounted on a Nano-Drive, and a Perkin-Elmer Ultraview system. Embryos for sectioning were treated as described (Zannino and Appel, 2009). Fluorescently labeled sections were covered with Vectashield (Vector Laboratories) and imaged and processed as described above.

Fluoromyelin treatment

Embryos were fixed for 10 min at room temp in 2% pfa/1% TCA (trichloroacetic acid) followed by 3 × 15 min washes in PBS. Embryos were then incubated in Fluoromyelin (1:300 in PBS; Molecular Probes) for four hours at room temperature, followed by 2 × 15 min washes in PBS. Finally the embryos were de-yolked and imaged on the confocal microscope as described above.

In vitro protein synthesis

The TnT Reticulocyte Lysate System with the Transcend Non-Radioactive Translation Detection System (Promega) was used to make Prdm12b protein from a pCS2 vector containing the *prdm12b* gene according to the manufacturer's instructions.

Cyclopamine treatments

Wild type embryos were incubated in 100 μM cyclopamine (Santa Cruz Biotechnology) in egg water at 28 °C. Cyclopamine was diluted from a 10 mM stock in ethanol and an equivalent dilution of ethanol was used as vehicle control. Treatment began at 6 hpf, as previously described (Park et al., 2004).

Bromodeoxyuridine (BrdU) labeling

Embryos were dechorionated and incubated with 10 mM BrdU in 10% DMSO in embryo medium for 20 min on ice followed by incubation with 10 mM BrdU solution in embryo medium at 28.5 °C for 40 min. The embryos were then fixed using 4% pfa in PBS and sectioned as described above. Prior to anti-BrdU immunocytochemistry the sections were treated for 30 min with 2 M HCl. All sections were imaged using the confocal microscope described above.

Antisense morpholino oligonucleotide injections

An antisense morpholino (MO) oligonucleotide with the sequence 5′-GCAGGCAACACTGAACCCATGATGA-3′ (Gene Tools, LLC) was designed to the *prdm12b* translation start site, a corresponding 5 bp mismatch MO was used as control 5′-GCAcGCAAtACTtAACtCATGATtA-3′ (Gene Tools, LLC) and a splice blocking MO, 5′-AGGTCCTGGCGCAGTCATTCTCTGGAG-3′ was designed to exon1/intron1. All morpholinos were re-suspended in distilled water for a stock solution of 3 mM. The stock solution was further diluted with water and phenyl red and 1–2 ng was injected into the yolk of one- to two-cell stage embryos.

Behavioral analysis

Escape responses were elicited by a light tap to the head of an embryo with a 3.22/0.16 g of force Von Frey filament. A high-speed

digital camera (Fastec Imaging, San Diego, CA) mounted to a 35 mm lens (Nikon, Melville, NY), recorded each response at 1000 frames/s. Computer software developed in Gerald Downes' laboratory measured the head-to-tail angle for each frame, which was then plotted in Microsoft Excel (McKeown et al., 2012). The calculated escape response began in the frame preceding the first movement until movement was no longer observed. The number of body bends exceeding 110°, the length of time each fish swam and the distance each fish traveled was also calculated. Statistical significance was calculated using Student's *t*-test.

Results

prdm12b is expressed in the p1 progenitor domain of the ventral neural tube

At 10 hours post-fertilization (hpf), *prdm12b* is expressed in two anteroposterior stripes in the presumptive zebrafish hindbrain (HB; Fig. 1A), as well as in more anterior domains that appear to correspond to midbrain (MB) and cranial placodes (arrows; (Thisse and Thisse, 2004)). Hindbrain expression expands both anteriorly and posteriorly by 14hpf and *prdm12b* becomes expressed in several rhombomeres, as indicated by double labeling with *krox20* in rhombomere (r) 3 and r5 (Fig. 1B). This expression pattern is maintained at 18 hpf (Fig. 1C and D). By 24 hpf, *prdm12b* expression is observed medially throughout the hindbrain and extending into the spinal cord (Fig. 1E–H). Additionally, at this time point there is expression in midbrain and forebrain structures (Fig. 1E and F; Dien, Teg, and Tel). To better define the dorsoventral position occupied by the *prdm12b* expression domain, we carried out double in situ hybridizations between *prdm12b* and *olig2* (expressed in the pMN domain; (Lu et al., 2000; Zhou et al., 2000; Mizuguchi et al., 2001; Novitch et al., 2001)), followed by transverse sectioning of hindbrain and spinal cord. The sections demonstrate that *prdm12b* is expressed in a chevron-shaped domain with its apex dorsally at the midline and its two ends projecting ventrolaterally at 24 hpf (Fig. 1G–K). The *prdm12b* domain is dorsal to *olig2* expressing cells in both hindbrain and spinal cord. Additionally, segmental stripes of *prdm12b* expression

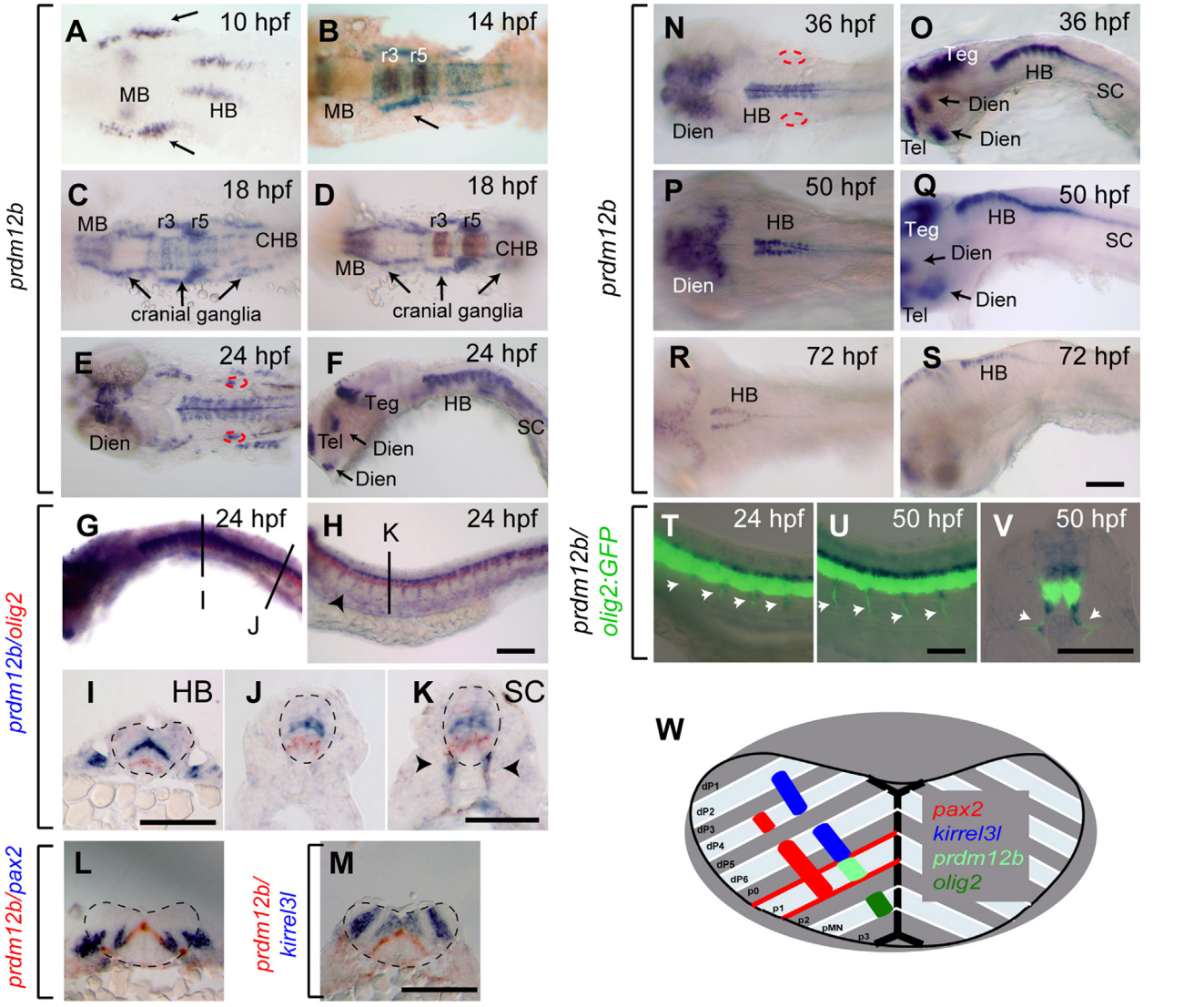

Fig. 1. *prdm12b* is expressed in the p1 domain. (A–V). Expression of *prdm12b* by itself (A, C, E, F, N–S) and together with *krox20* (B, D), *olig2* (G–K), *pax2* (*L*), *kirrel3l* (*M*) or *olig2:egfp* (T–V) in wild type embryos. Embryos are shown in dorsal (A–E, N, P, R) or lateral (F–H, O, Q, S–U) view with anterior to the left, or in cross section (I–M) with dorsal to the top. (W) Diagram of hindbrain gene expression. Scale bars are 100 μm, except (J–K, V) that are 50 μm. The otic vesicles and the neural tube are outline by red and black dashed lines, respectively. Arrows indicate cranial ganglia (A–D), or forebrain/midbrain structures (F, O, Q). Arrowheads mark motor axons (H, K, T–V). MB – midbrain, HB – hindbrain, CHB – caudal hindbrain, Teg – tegmentum, Dien – diencephalon, Tel – telencephalon, and *r* – rhombomere.

are found ventral to the neural tube and adjacent to the notochord (arrowheads in Fig. 1H and K). These stripes coincide with sites where segmentally arranged motor axons exit the neural tube, as evidenced by co-localization of *prdm12b* transcript with GFP fluorescence at the motor roots in *olig2*:*egfp* transgenic fish (Fig. 1T–V). We also note that a region of unlabeled tissue separates the *prdm12b* and *olig2* expression domains along the dorsoventral axis (Fig. 1I–K). To examine this in greater detail, we simultaneously detected *prdm12b* and *pax2* (expressed in p1-dP6 and in dP4; (Nornes et al., 1990; Burrill et al., 1997); Fig. 1L), as well as *prdm12b* and *kirrel3l* (expressed in p0-dP6 and in dP2–dP4; Fig. 1M). We find that *prdm12b* expression overlaps with ventral *pax2* expression, which corresponds to p1 (Fig. 1L) and is immediately ventral to *kirrel3l* expression in p0 (Fig. 1M), indicating that *prdm12b* is expressed in p1 (expression patterns are summarized in Fig. 1W). Lastly, *prdm12b* expression persists in spinal cord and hindbrain at 36 hpf and 50 hpf (Fig 1N–Q), but decreases by 72 hpf (Fig. 1R and S).

It is well established that Shh produced by the notochord and floor plate acts to induce ventral fates in the neural tube (reviewed in (Dessaud et al., 2008)). In order to determine whether Shh is required for expression of *prdm12b*, we examined embryos treated with cyclopamine (a small molecule inhibitor of Shh signaling; (Cooper et al., 1998)). Cyclopamine treatment eliminates expression of the ventral neural tube marker *nkx6.1*, (Fig. 2K–N), as previously reported (Guner and Karlstrom, 2007). In contrast, we find that expression of *prdm12b* is variably affected in cyclopamine treated embryos (Fig. 2F–J). Specifically, *prdm12b* expression is completely lost at the motor roots (compare Fig. 2B, E–G, and J), but is only partially lost in hindbrain and spinal cord (Fig. 2A–J) and appears unaffected at the MHB (Fig. 2A, and F; asterisk). Hence, *prdm12b* expression appears variably dependent on Shh as a result of distance, such that *prdm12b* expression at the motor roots (immediately adjacent to the notochord) is more highly dependent on Shh than domains located further from a Shh source. We conclude that *prdm12b* is expressed in the ventral p1 domain of the neural tube, as well as in a region near the ventral motor roots, in a partially Shh-dependent manner.

prdm12b is required for specification of engrailed1b-expressing V1 interneurons

Progenitor cell domains along the dorsoventral axis of the neural tube give rise to the full complement of motor neurons, interneurons and glial cells of the hindbrain and spinal cord (reviewed by (Melton et al., 2004; Wilson and Maden, 2005)). Formation of each progenitor population requires a unique combination of genes that also serve as selective markers for that particular progenitor type. In particular, V1 interneurons that arise from the p1 domain are uniquely defined by their expression of *Engrailed1* (*En1*) in mouse and *engrailed1b* (*eng1b*) in zebrafish (Higashijima et al., 2004; Alvarez et al., 2005; Gosgnach et al., 2006). Since *prdm12b* expression is restricted to the p1 domain, we examined if *prdm12b* is required for *eng1b* expression and V1 interneuron specification.

In 24hpf wild type embryos, *eng1b* is expressed in two longitudinal stripes of V1 interneurons in the hindbrain and spinal cord (arrows in Fig. 3A and B), as well as in a weakly staining transverse band in the hindbrain (bracket in Fig. 3A) that corresponds to r4 (based on double labeling with *krox20*; Fig. S1A). *eng1b* is also expressed at the midbrain–hindbrain boundary (MHB) and in muscle pioneer cells at the somites (arrowhead in Fig. 3B). To test the role of *prdm12b*, we made use of an antisense morpholino oligonucleotide (PTB MO) that targets the *prdm12b* translation start site and that disrupts translation of the *prdm12b* mRNA (Fig. S1B). At 24 hpf, *eng1b* expression in hindbrain and spinal cord is almost completely lost in embryos injected with PTB MO (Fig. 3H–L), except that some *eng1b* expression may persist in the r4 domain of the hindbrain (bracket in Fig. 3H; asterisk in Fig. 3J). Specifically, 72% of PTB MO-injected embryos (23/32) completely lack V1 interneurons and 28% (9/32) show a reduction (Fig. 3Z). In contrast, *eng1b* expression is unaffected at the MHB (Fig. 3H) and in muscle pioneer cells – where *prdm12b* is not expressed – (Fig. 3I and L) of PTB MO-injected embryos, demonstrating that *prdm12b* regulates *eng1b* expression only in the p1 domain. The loss of *eng1b* expression in the neural tube of PTB

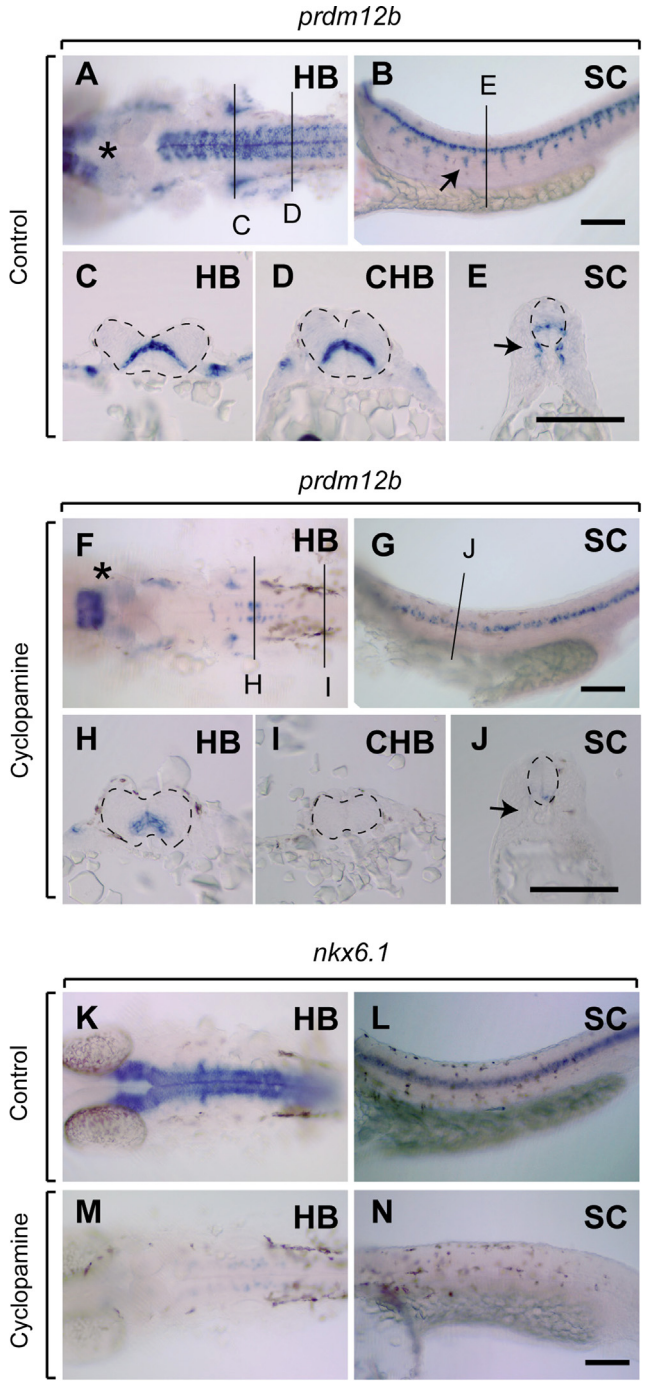

Fig. 2. *prdm12b* is partially regulated by Shh. (A–N) *prdm12b* (A–J) or *nkx6.1* (K–N) expression in control (A–E, K, L) or cyclopamine treated (F–J, M, N) embryos. Embryos are shown in dorsal (A, F, K, M) or lateral (B, G, L, N) view with anterior to the left, or in cross section (C–E, H–J) with dorsal to the top. Scale bars are 100 μm. Arrows indicate motor axons (B, E, J). Asterisk marks unaffected anterior *prdm12b* expression (A, F). HB – hindbrain, CHB – caudal hindbrain, and SC – spinal cord.

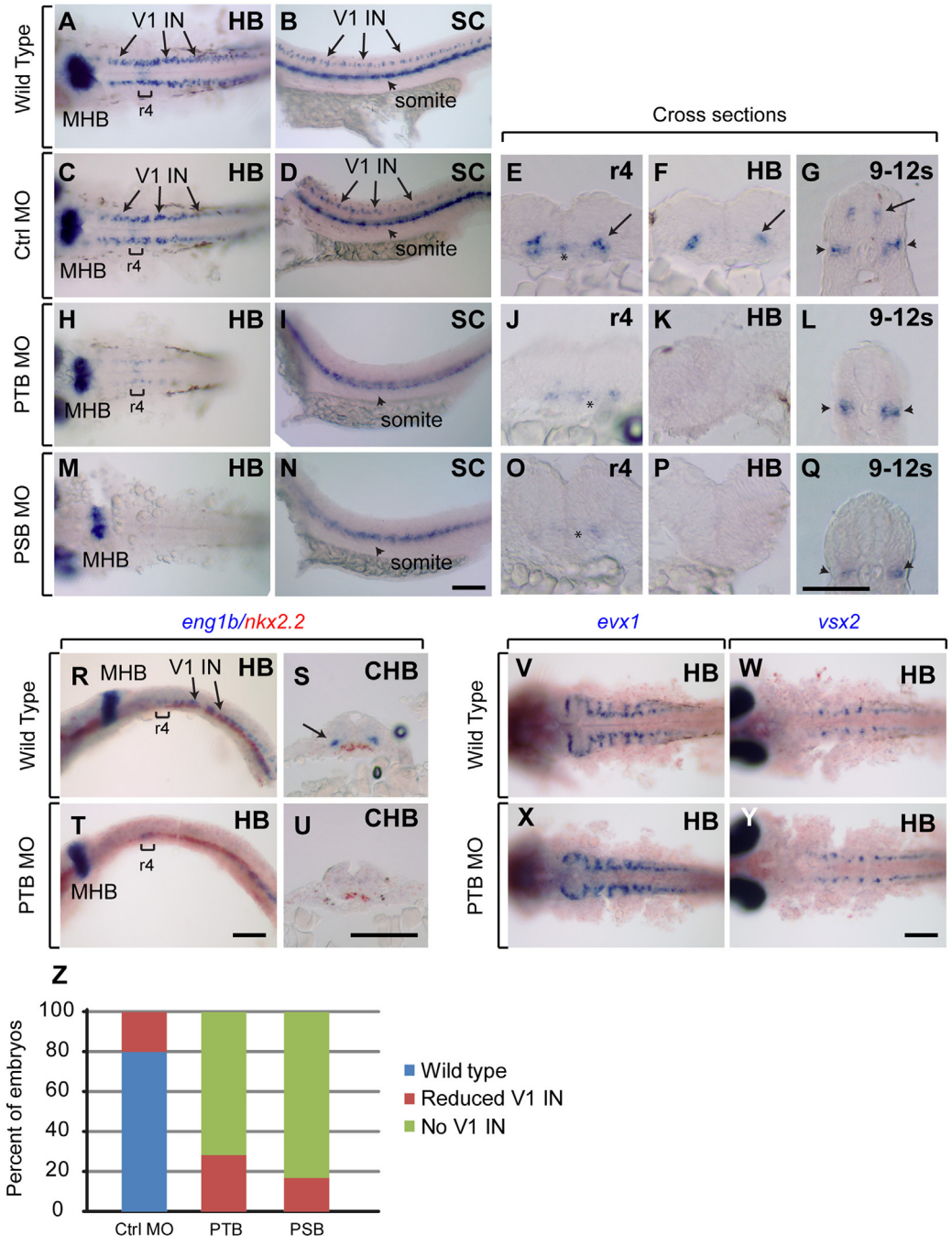

Fig. 3. *prdm12b* is required for *eng1b* expression in the p1 domain. (A–Q) *eng1b* Expression in 24hpf wild type (A, B), control MO-injected (C–G), PTB MO-injected (H–L), PSB MO-injected (M–Q) embryos. (R–U) *eng1b* and *nkx2.2* expression in 24 hpf wild type (R, S) and PTB MO-injected (T, U) embryos. (V, X) *evx1* Expression in wild-type (V) and PTB MO-injected (X) embryos. (W, Y) *vsx2* Expression in wild-type (W) and PTB MO-injected (Y) embryos. Embryos are shown in dorsal (A, C, H, M, V–Y) or lateral (B, D, I, N, R, T) view with anterior to the left, or in cross section (E–G, J–L, O–Q, S, U) with dorsal to the top. Sections were taken through r4, the hindbrain, the caudal hindbrain or the level of somites 9–12, as indicated. (Z) Quantification of *eng1b* phenotype. Scale bars are 100 μm. Brackets and asterisks indicate r4, arrows mark V1 interneurons and arrowheads mark somites. MHB – midbrain–hindbrain boundary, HB – hindbrain, CHB – caudal hindbrain, and SC – spinal cord.

MO-injected embryos persists at least to 50 hpf (Fig. S1C and D). Embryos injected with a 5 bp mismatch control MO show normal *eng1b* expression in all tissues (Fig. 3C–G, Z; $n=20$) and a second MO designed to disrupt splicing of the *prdm12b* transcript (PSB MO) produces the same phenotype as the PTB MO (Fig. 3M–Q and Z), demonstrating the specificity of the observed phenotype (20/24 embryos with no V1 INs and 4/24 with a reduction). To further establish the specificity of the MO effect, we used *prdm12b* mRNA to rescue the PSB MO phenotype at 24 hpf. 20% of embryos (3/15) show a reduction and 80% (12/15) completely lack V1 interneurons following injection of PSB MO alone (Fig. 4C, D, and K). Upon co-injection of *prdm12b* mRNA together with the PSB MO, 27% of embryos (4/15) display a wild type complement, 47% (7/15) display an intermediate phenotype and 27% (4/15) lack V1 interneurons (Fig. 4G–K), indicating that *prdm12b* mRNA can rescue PSB MO-mediated loss of *eng1b*-positive V1 interneurons.

We next examined gene expression in progenitor domains adjacent to the *prdm12b*-expressing p1 domain. In particular, *nkx2.2* is expressed in the p3 domain (Ericson et al., 1997b), as seen in lateral views (Fig. 3R) and transverse sections (Fig. 3S). As expected, injection of PTB MO blocks *eng1b* expression in the p1 domain, but not in the MHB or the somites (Fig. 3T and U).

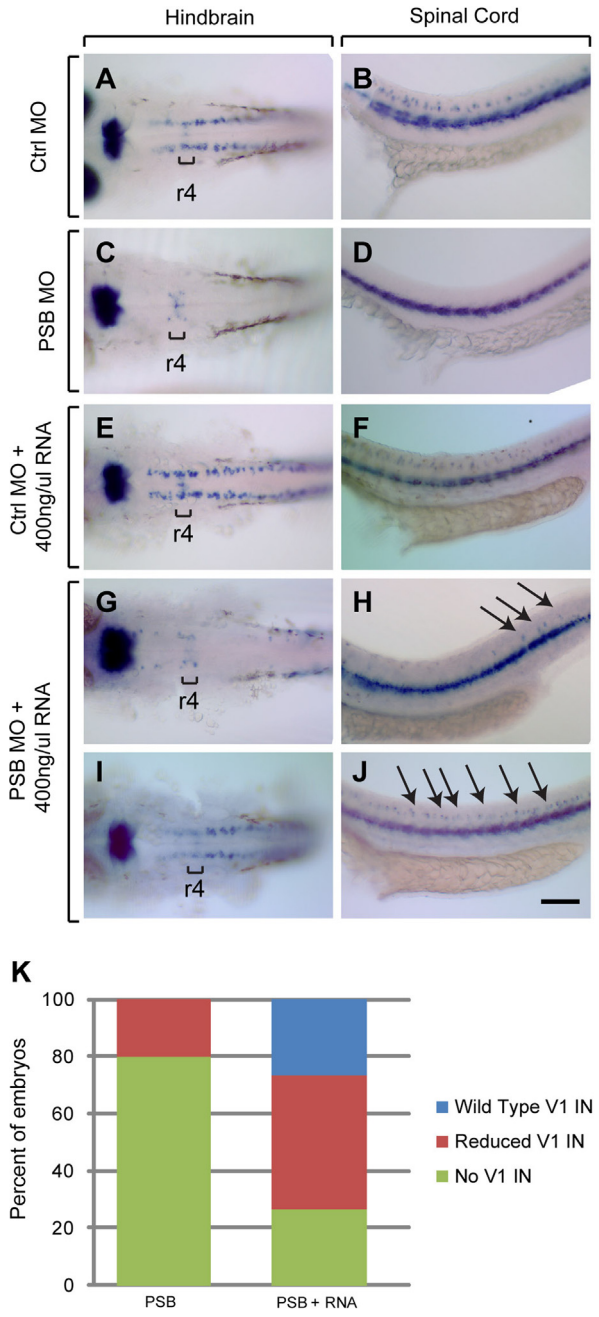

Fig. 4. *prdm12b* mRNA rescues *eng1b* expression. (A–J) *eng1b* Expression in embryos injected with control MO (A, B), PSB MO (C, D), Ctrl MO+400 ng/μl RNA (E, F), or PSB MO+400 ng/μl RNA (I, J). (K) Quantification of rescue. Arrows indicate rescued V1 interneurons, brackets mark r4. Scale bars are 100 μm.

However, *nkx2.2* expression in the p3 domain is unaffected by the PTB MO (Fig. 3T and U). Similarly, expression of *evx1*, which is restricted to the p0 domain (Burrill et al., 1997) and *vsx2*, which is restricted to the p2 domain (Ericson et al., 1997b), is unaffected by injection of PTB MO (Fig. 3V–Y). We conclude that *prdm12b* regulates specification of *eng1b*-expressing V1, but not V0, V2 or V3 interneurons.

prdm12b Defines the dorsal limit of nkx6.1 expression

Transcription factors expressed in one progenitor domain often play a role in establishing and/or maintaining boundaries of adjacent progenitor domains, thereby affecting dorsoventral patterning of the neural tube. We therefore examined how loss of *prdm12b* affects gene expression along the dorsoventral axis of the neural tube, starting with *dbx1a* (a marker of the p0 domain; (Pierani et al., 1999)) and *nkx6.1* (expressed in a ventral domain from the floor plate into the p2 domain; (Qiu et al., 1998; Briscoe et al., 2000)). In wild type embryos, *dbx1a* and *nkx6.1* expression flank the *prdm12b*-expressing p1 domain (Fig. 5A), but in PTB MO-injected embryos the *dbx1a* and *nkx6.1* domains are closer together and ectopic *nkx6.1* expression is observed dorsally (arrow in Fig. 5H). Similarly, when embryos are co-stained for *nkx6.1* together with *pax3* (that labels dP6–dP1; (Goulding et al., 1991)), the gap between *nkx6.1* and *pax3*, which corresponds to p1 and p0, is reduced in PTB MO-injected embryos and ectopic *nkx6.1* expression is observed dorsally (Fig. 5B and I). Approximately 90% of sections from PTB MO-injected embryos stained for *dbx1a/nkx6.1* or *pax3/nkx6.1* reveal a reduction in the p1 domain and 10–20% also display ectopic patches of *nkx6.1* expression in the dorsal neural tube (Fig. 5O). Specifically, the average distance between *dbx1a* and *nkx6.1*, or *pax3* and *nkx6.1*, was significantly reduced (4.8–1.6 μm [$p=2.37E-07$] and 7.5–4.3 μm [$p=6.84E-07$], respectively; Fig. 5Q). Examination of embryos co-stained for *nkx2.2* (that labels the p3 domain; (Ericson et al., 1997b; Briscoe et al., 2000)) and *evx1* (that labels the p0 domain; (Burrill et al., 1997; Matise and Joyner, 1997; Pierani et al., 1999)) reveals an unstained region corresponding to the p1, p2 and pMN domains in wild type embryos (bracket in Fig. 5C). In PTB MO-injected embryos, this region is reduced in approximately 75% of sections (reduced from 9.8 μm to 6.3 μm [$p=8.21E-04$] between wild-type and PTB embryos; Fig. 5J, O, and Q). Lastly, co-staining with *pax3* and *nkx2.2* – that are separated by the p0, p1, p2 and pMN domains reveals a reduction of the intervening region from 12.5 μm to 9.4 μm ($p=3.91E-04$; Fig. 5D, K, and Q). Thus, in accordance with the loss of *eng1b* expression observed in Fig. 3, the p1 domain appears to be reduced or lost upon disruption of *prdm12b* function. This effect is most noticeable by examination of marker genes separated only by the p1 domain (*dbx1a/nkx6.1*) and becomes less dramatic as the marker genes are separated by two (*pax3/nkx6.1*), three (*evx1/nkx2.2*) or four (*pax3/nkx2.2*) progenitor domains. Accordingly, expression of *dbx2*, a progenitor marker expressed in p1-dP6 domains (Pierani et al., 1999), is only mildly affected in PTB MO-injected embryos (Fig. S2A–J). We conclude that *prdm12b* plays a role in establishing the dorsal limit of *nkx6.1* expression.

prdm12b is not required for specification of the spinal cord pMN domain

We next determined if disrupting *prdm12b* has an effect on the pMN domain. An examination of embryos co-stained with *nkx2.2* (expressed in p3) and *vsx2* (expressed in p2) reveals an intervening unlabeled region, corresponding to the pMN domain, that is very slightly reduced in PTB MO-injected embryos (decreases from 7.2 μm to 6.0 μm [$p=0.026$]; Fig. 5E, L, and Q), suggesting that the pMN domain forms even in the absence of *prdm12b*. However, embryos co-stained with *irx3* (that has its ventral limit in p2; (Briscoe et al., 2000)) and *nkx2.2*, reveal that *irx3* expression expands ventrally to partially include the pMN domain upon disruption of *prdm12b* (the distance between *irx3* and *nkx2.2* is reduced from 5.4 μm to 3.1 μm [$p=6.54E-06$]; Fig. 5F, M, and Q). In spite of this ventral *irx3* expansion, *olig2* expression is only mildly affected in the hindbrain (Fig. 5G and N) and is normal in the spinal cord (Fig. 6E–H) of PTB MO-injected embryos. Accordingly, spinal motor neurons form normally (as detected by Islet1/2 expression; brackets in Fig. 6A–D) and project their axons correctly (arrowheads in Fig. 6I and J) in PTB MO-injected embryos, while the hindbrain exhibits a modest reduction in the number of abducens (nVI) neurons (derived from *olig2* expressing precursors in r5 and r6) in a subset of embryos (4/16; Fig. 6O–Q) and a slight

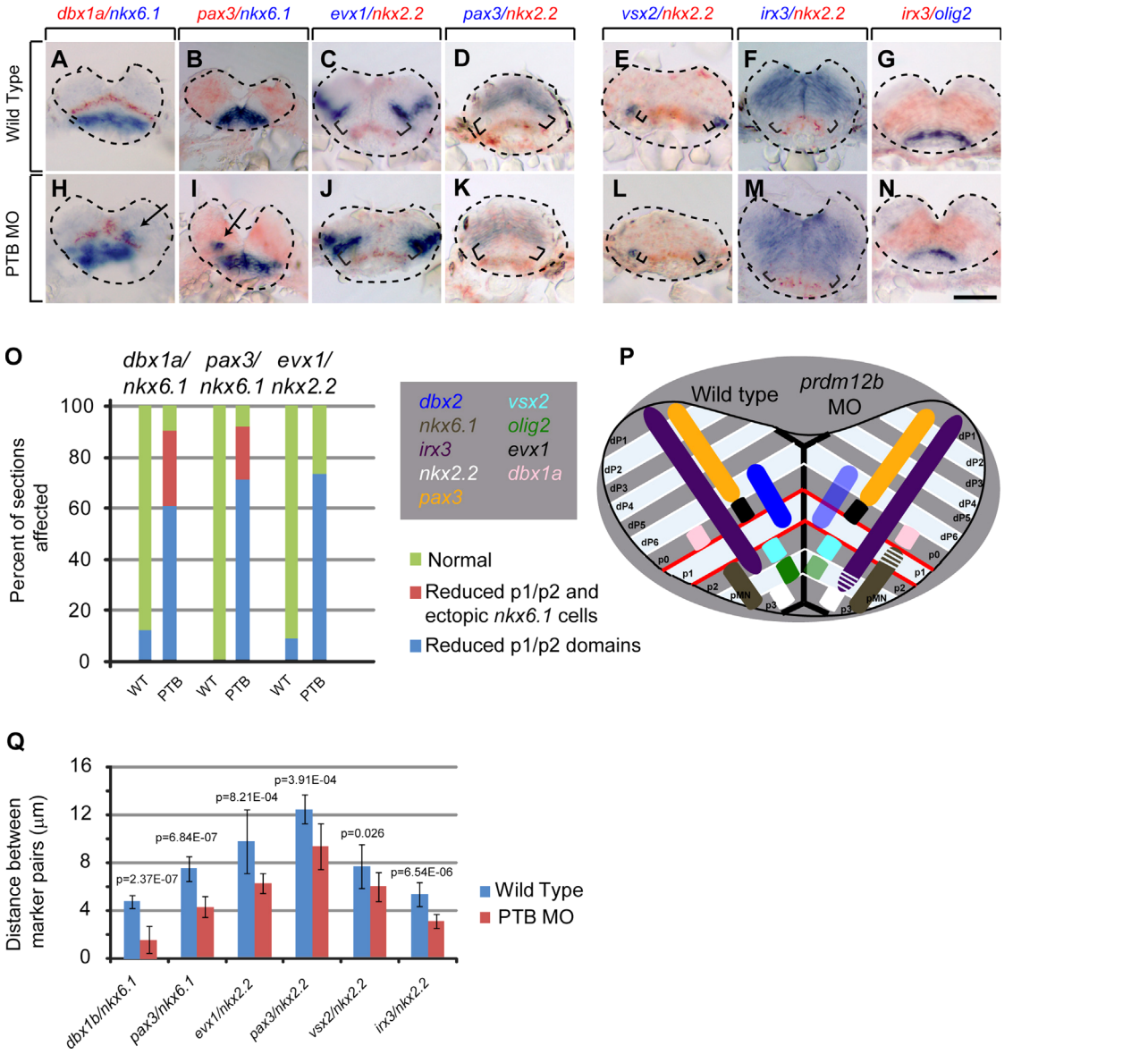

Fig. 5. *prdm12b* Defines the dorsal boundary of *nkx6.1* expression and regulates the size of the p1 domain. (A–N) Expression of *dbx1a/nkx6.1* (A, H), *pax3/nkx6.1* (B, I), *evx1/nkx2.2* (C, J), *pax3/nkx2.2* (D, K), *vsx2/nkx2.2* (E, L), *irx3/nkx2.2* (F, M) and *irx3/olig2* (G, N) in wild-type (A–G) or PTB MO-injected (H–N) 24 hpf embryos. Panels represent cross sections through the hindbrain with dorsal to the top. (O) Quantification of dorsoventral patterning defects using ~100 sections from 16 embryos for each gene pair. (P) Diagram of dorsoventral gene expression in wild type (left half) and *prdm12b* MO-injected (right half) embryos. (Q) Quantification of the distance between various gene expression domains using 10 representative sections for each gene pair. Arrows indicate ectopic *nkx6.1*$^{+}$ cells and brackets indicate unlabeled tissue between markers. Scale bar is 100 μm.

delay in the migration of facial (nVII) neurons (that form in r4 and migrate caudally) in a similar fraction of embryos (8/28; Fig. 6K–N). Thus, spinal motor neurons and most hindbrain motor neurons develop normally in PTB MO-injected embryos.

We also tested if oligodendrocyte precursor cells (OPCs), which form from the hindbrain pMN domain in r5 and r6 and differentiate into myelinating oligodendrocytes, are affected by *prdm12b* disruption. We find that the number of *olig2* expressing OPCs is reduced at 40 hpf and 50 hpf in PTB and PSB MO-injected embryos (Fig. 7A–F). This effect is observed in 75–90% of injected embryos and can be detected as early as 24 hpf (Fig. 7S). Similarly, expression of *sox10*, the first marker of specified OPCs in zebrafish (Kuhlbrodt et al., 1998), is reduced in 60–80% of PTB and PSB MO-injected embryos (arrows in Fig. 7G–L and T), while other domains of *sox10* expression, such as neural crest cells, are unaffected (arrowheads in Fig. 7G–L). Importantly, OPCs of PTB MO-injected embryos go on to express *dm20*, a marker for cells producing myelin (Timsit et al., 1995), suggesting that they differentiate properly into myelinating oligodendrocytes, but the number of *dm20*-expressing cells is reduced in MO-injected embryos (Fig. 7M–R and U). To better visualize *olig2*-expressing cells, we made use of the *Tg*(*olig2*:*egfp*) transgenic line. Consistent with the results in Fig. 7, the number of *olig2* positive cells is reduced at 36 hpf and 50 hpf also in PTB MO-injected *olig2*:*egfp* embryos (Fig. 8A, B, D, E, G,H, and J; note that the stability of eGFP protein causes labeling of motor neurons in r5/r6 to persist longer in the *olig2*:*egfp* line than in embryos analyzed by in situ hybridization). Strikingly however, the number of *olig2* positive cells appears to be recovering at later stages (Fig. 8C, F, and I). In particular, the number of affected embryos is reduced to ~20% (Fig. 8J) and PTB MO-injected embryos have ~90% the number of OPCs compared to control embryos by 72 hpf (Fig. 8K). Accordingly, BrdU labeling reveals an increase in proliferating *olig2*-positive cells at 72 hpf in PTB MO-injected embryos (Fig. 8L–P),

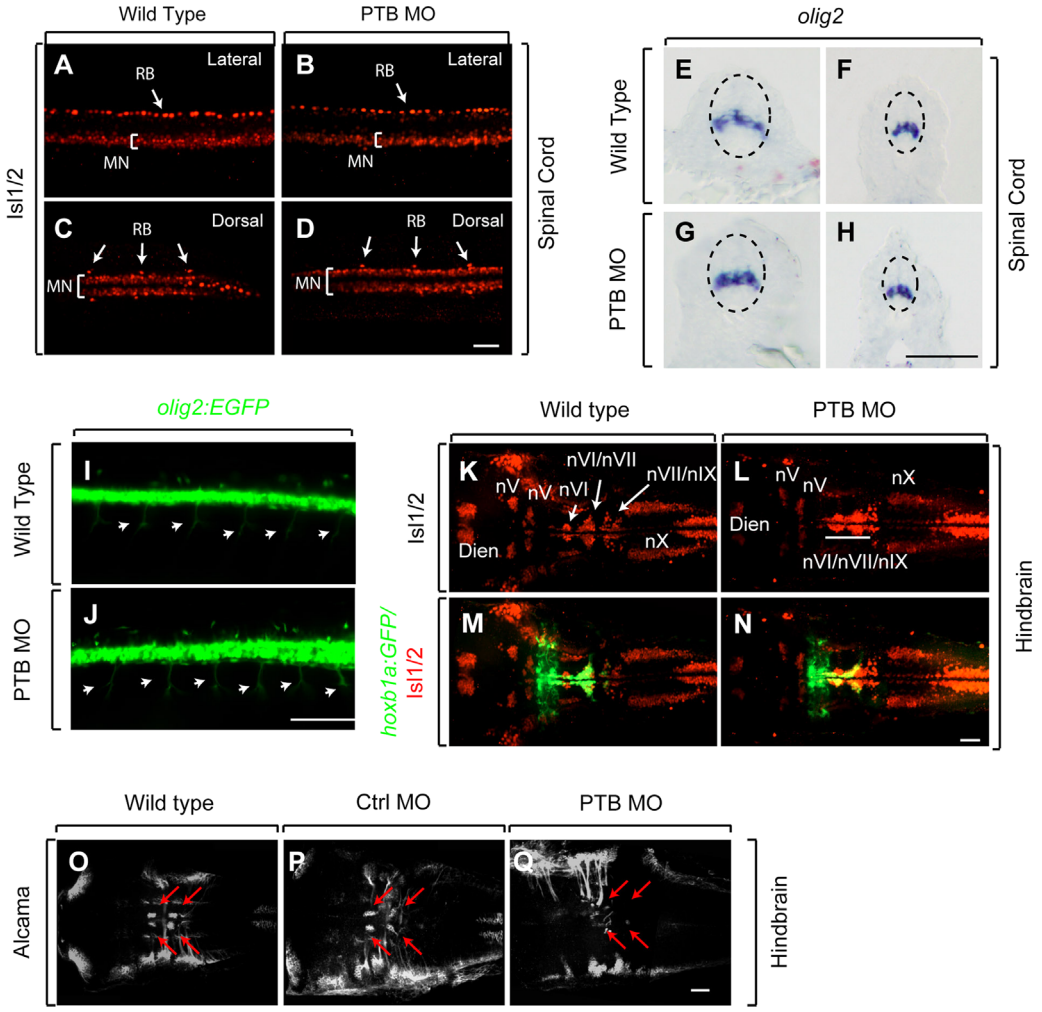

Fig. 6. Loss of *prdm12b* has a modest effect on hindbrain, but not spinal, motor neurons. (A–Q) Expression of Isl1/2 (A–D, K, L), *olig2* (E–H), *olig2:egfp* (I, J), Isl1/2 together with *hoxb1a:egfp* (M, N) or Alcama (O–Q) in wild-type (A, C, E, F, I, K, M, O), PTB MO-injected (B, D, G, H, J, L, N, Q), or control MO-injected (P) embryos at 24 hpf (A–H) or 50 hpf (I–Q). Embryos are shown in lateral (A, B, I, J) or dorsal (C, D, K–Q) view with anterior to the left, or in cross section (E–H) with dorsal to the top. All scale bars are 35 μm, except I, J are 100 μm and E, H are 50 μm. RB – Rohon-Beard cells, MN – motor neurons, and (n)=cranial nerves.

indicating that increased proliferation compensates for the reduced number of OPCs formed upon disruption of *prdm12b*. Notably, while the number of *dm20* expressing cells remains somewhat reduced at 6dpf (Fig. 7O and R), the extent of myelination appears comparable between PTB MO-injected and control embryos (Fig. 7V–Y).

Lastly, given that *prdm12b* has an early dynamic expression phase in the hindbrain, we tested if *prdm12b* plays a role in anteroposterior patterning of the hindbrain, but we do not find any changes in the expression of *hoxb1a*, *krox20*, *hoxb3a* or *neurogenin* upon disruption of *prdm12b* function (Fig. S3A–F). Hence, we conclude that *prdm12b* plays only a minimal role in the specification of motor neurons and oligodendrocytes and that *prdm12b* is not involved in anteroposterior patterning of the neural tube.

Loss of prdm12b leads to impairment of the zebrafish touch-evoked escape response

Electrophysiological recordings in fish and tadpoles suggest that V1 interneurons act as inhibitory interneurons in the modulation of motor circuits (Higashijima et al., 2004; Li et al., 2004), but this has not been tested functionally by assaying fish lacking V1 interneurons. Hence, we made use of the touch escape response, which represents a classical means of testing the function of motor circuits in aquatic species (Eaton et al., 2001), to test *prdm12b* and V1 interneuron function. In this response, a touch stimulus causes the fish to first undergo a large amplitude body bend (termed a C-bend) that brings the tail close to the head and causes the animal to turn away from the stimulus (Fig. 9A). The C-bend is followed by several lower amplitude undulations in a coordinated left-right pattern that allow the fish to swim away. Sensory input from a touch to the head is mediated via reticulospinal interneurons (primarily the Mauthner cells) that are unaffected by *prdm12b* disruption (Fig. 9C and D). To determine the behavioral effect of loss of *prdm12b* function, we recorded the escape response of control MO and PTB MO-injected 4-day old fish. Control MO-injected fish demonstrate a normal escape response with an initial C-bend followed by a burst of swim movements (Fig. 9A, Movie 1). PTB MO-injected fish also undergo an initial C-bend (Fig. 9B, Movie 2). However, *prdm12b*-deficient fish spend more time in the C-bend (an average of 0.034 s compared to 0.024 s for control embryos, $p=8.07E-03$). The initial C-bend is also more exaggerated in *prdm12b*-deficient fish with 4/10 fish extending their tails past the head, which is never observed in control fish. In addition, while control fish follow the C-bend with 3–4 lower amplitude undulations to swim away from the stimulus, PTB MO-injected fish undergo repeated large amplitude bends (an average of six large amplitude bends for PTB MO-injected fish compared to one

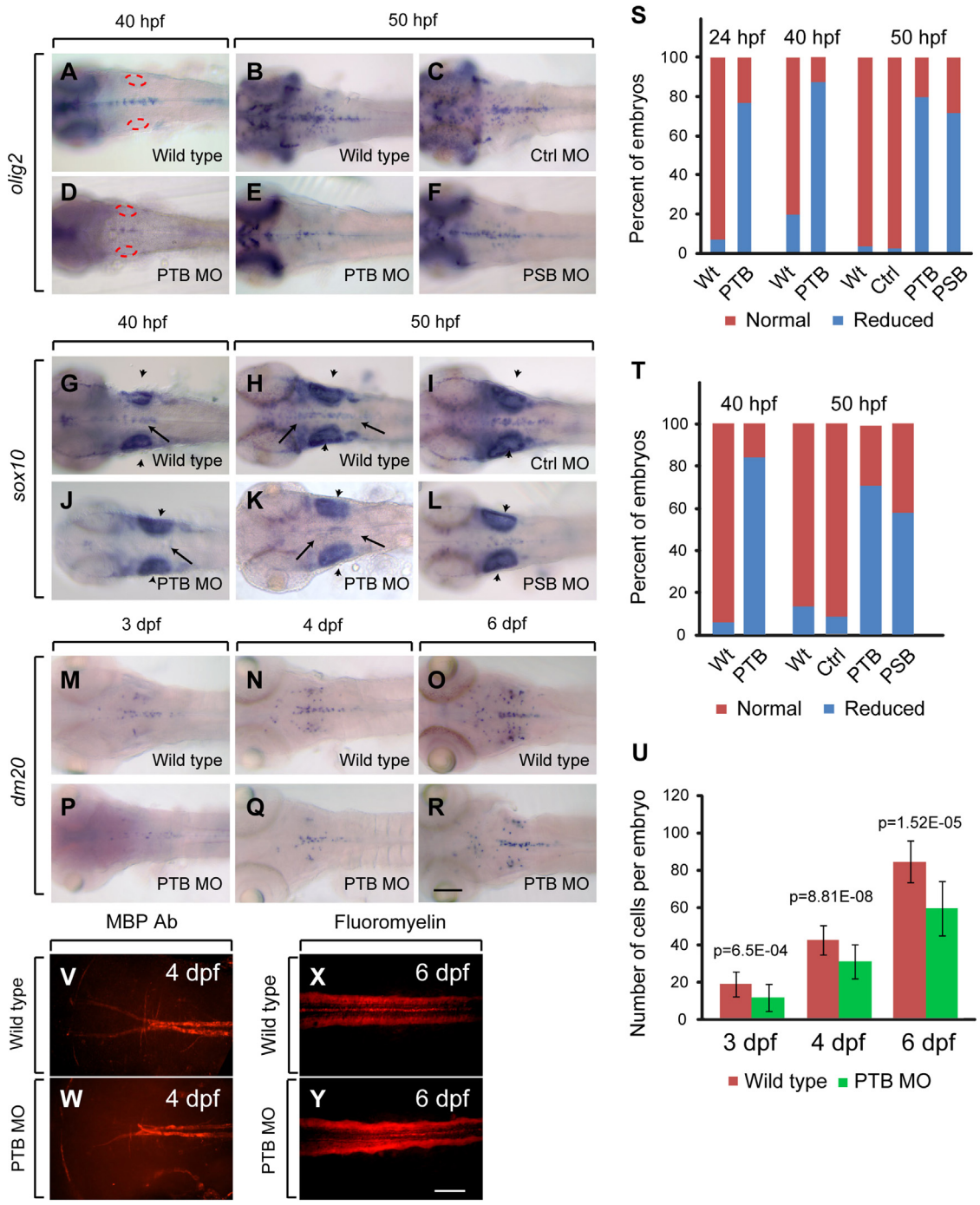

Fig. 7. Loss of *prdm12b* has a transient effect on the number of OPC cells. (A–R, V–Y) Expression of *olig2* (A–F), *sox10* (G–L), *dm20* (M–R), MBP (V, W) or fluoromyelin (X, Y) in wild type (A, B, G, H, M–O, V, X), control MO-injected (C, I), PTB MO-injected (D, E, J, K, P–R, W, Y) and PSB MO-injected (F, L) embryos. All embryos are in dorsal view with anterior to the left. Scale bars are 100 μm (A–R) or 35 μm (V–Y). Arrowheads indicate unaffected regions of *sox10* expression, while arrows indicate affected regions. Red circles demark the otic vesicles. (S–U) Quantification of reduction in *olig2* (S), *sox10* (T) and *dm20* (U) expression.

bend for control fish; Fig. 9E). Furthermore, the escape response in PTB MO-injected fish is active considerably longer than in control fish (an average of ~500 ms for PTB MO-injected fish compared to ~280 ms for control fish; Fig. 9F). Strikingly, the increased number of bends and prolonged response does not result in productive swim movements, as control fish travel significantly further than PTB MO-injected fish during the escape response (an average of ~5 mm in control fish as compared to ~1.5 mm in PTB MO-injected fish; Fig. 9G–I). Hence, *prdm12b*-depleted fish do undergo a touch response – consistent with reticulospinal neurons, spinal motor neurons and myelinating oligodendrocytes being largely normal in 4dpf *prdm12b*-deficient animals – but display exaggerated activity for several key components of the escape response, indicating an essential function for *prdm12b* and V1 interneurons in the modulation of zebrafish motor circuits.

Loss of V1 interneurons in the mouse leads to an increase in the step cycle period (Gosgnach et al., 2006). Since swimming and walking movements differ in many respects, it is difficult to identify a directly comparable measure in the fish. Nevertheless, we measured the time elapsed between consecutive tail bends

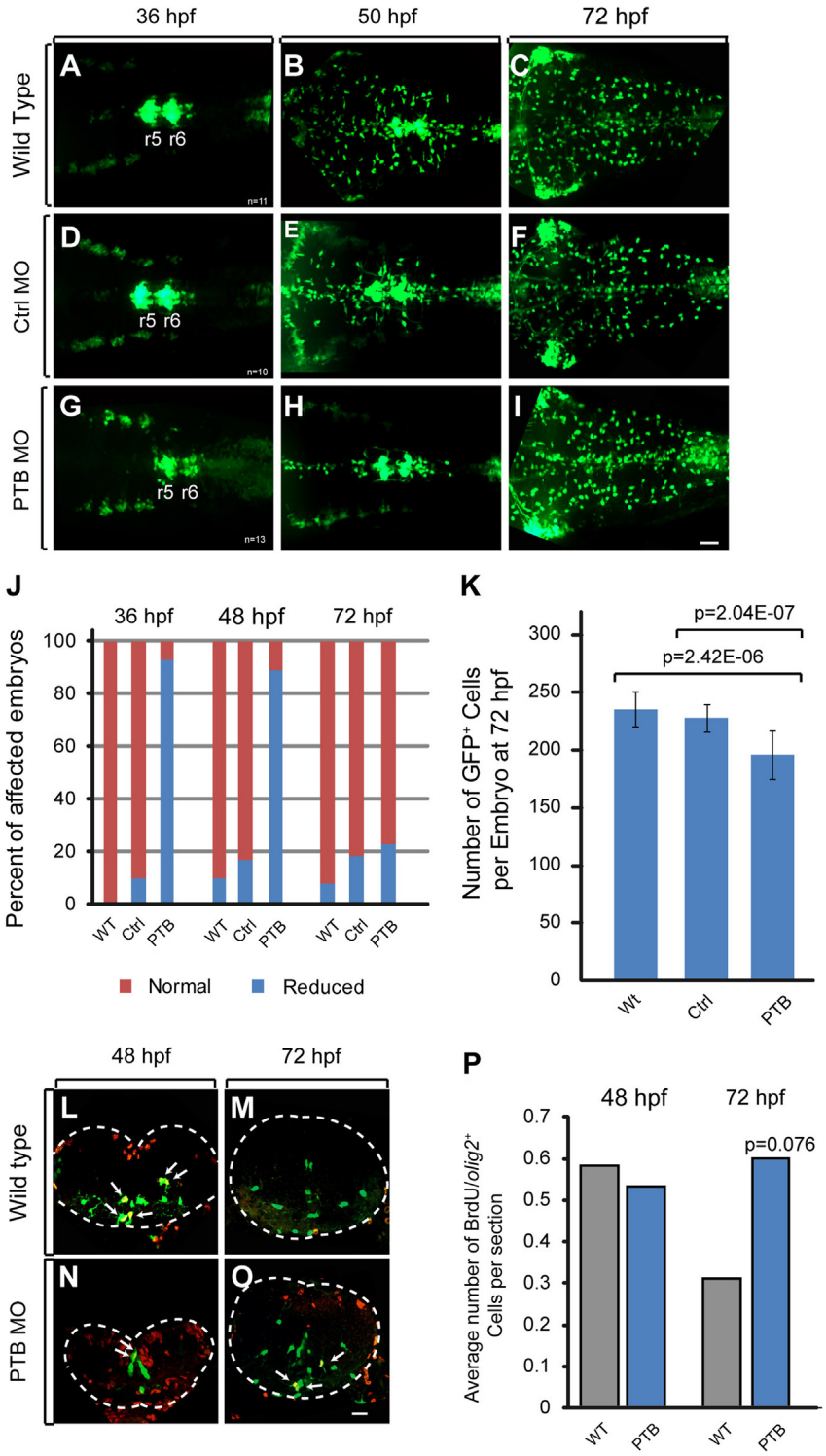

Fig. 8. Increased replication of OPCs in *prdm12b* deficient embryos. (A–I) *olig2*:*egfp* Expression in wild type (A–C), control MO-injected (D–F), or PTB MO-injected (G–I) embryos. (J) Quantification of embryos with reduced numbers of *olig2*:*egfp*$^+$ cells. (K) Quantification of *olig2*:*egfp*$^+$ cells per embryo at 72 hp. (L–O) Cross sections of wild type (L, M) and PTB MO-injected (N, O) *Tg(olig2*:*egfp)* embryos labeled with anti-BrdU. (P) Quantification of the number of *olig2*:*egfp*$^+$/BrdU$^+$ cells per section. Scale bars are 35 μm (A–I) or 17 μm (L, M). The neural tube is outlined with dotted line in L, M. Arrows indicate *olig2*:*egfp*/BrdU double positive cells.

during the escape response. We find that PTB MO-injected fish have a longer delay between tail bends than control fish (Fig. 9J). This delay is most pronounced for the interval between the first and second bends (23 ms in control versus 61 ms in PTB MO-injected fish) and between the second and third bends (26 ms in control versus 58 ms in PTB MO-injected fish), but is less apparent for subsequent intervals.

Discussion

Patterning of the neural tube into progenitor domains is essential for proper development of CNS neurons, as well as for their integration into functional circuits. Our analysis reveals that *prdm12b* is expressed in the p1 progenitor domain, at least partially in response to Shh signaling. Loss of *prdm12b* leads to inappropriate

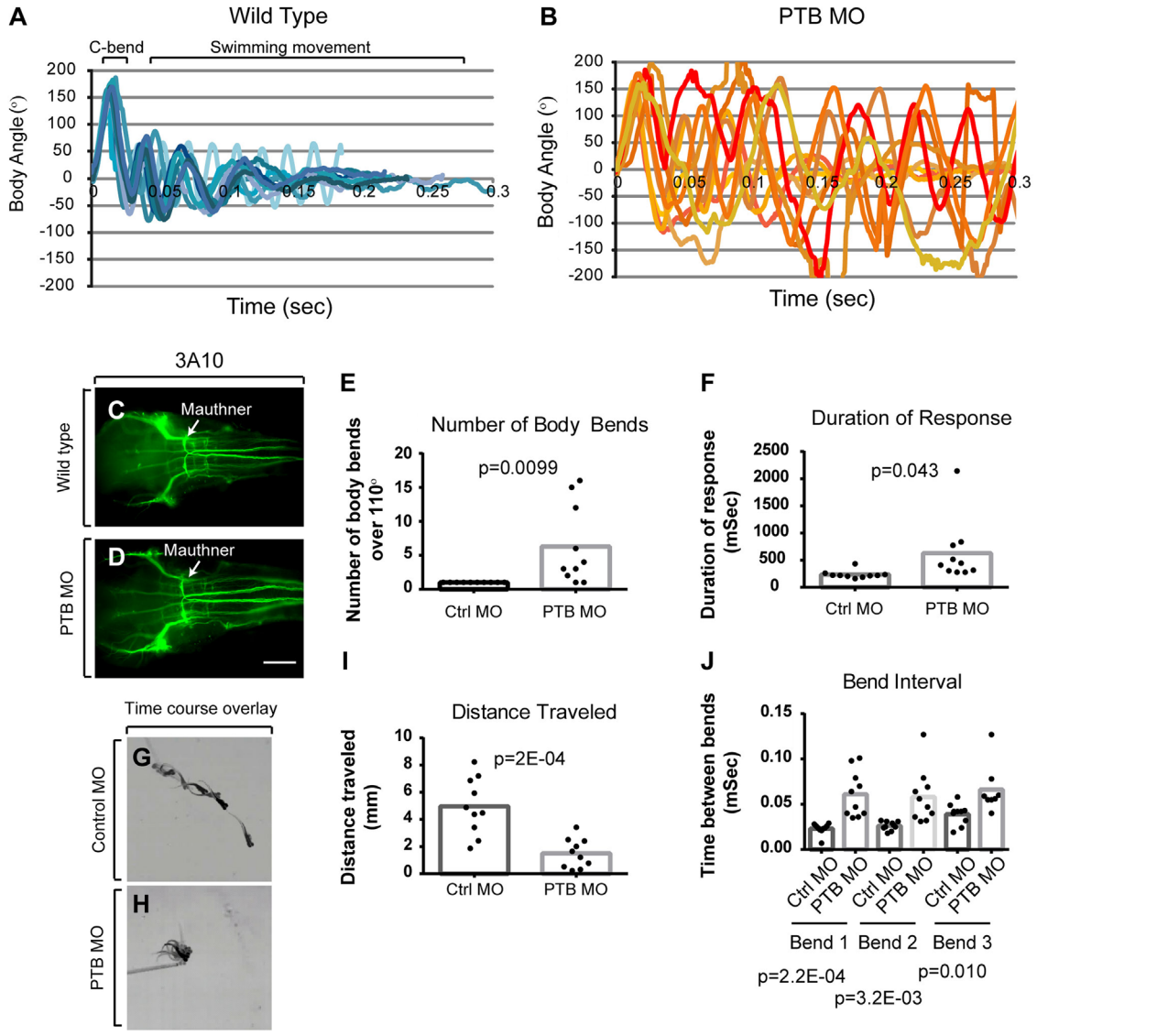

Fig. 9. Fish lacking *prdm12b* function display an abnormal touch response. (A, B) Kinematic traces for 10 control MO-injected (A) and 10 PTB MO-injected (B) 4dpf fish from one of three separate experiments. Zero degrees on the *y*-axis indicate a straight body while positive and negative angles represent body bends in opposite directions. (C, D) Anti-3A10 labeling of Mauthner neurons in wild type (C) and PTB MO-injected (D) embryos in dorsal view. (E) Quantification of body bends with amplitude similar to the C-bend (defined as exceeding 110°). (F) Quantification of escape response duration in milliseconds. (G, H) Overlay of frames taken every 20 ms from a high-speed recording of a touch response in control MO-injected (G) and PTB MO-injected (H) fish. (I) Quantification of distance traveled during the escape response. (J) Quantification of time interval between the first and second bend (bend 1), second and third bend (bend 2), third and fourth bend (bend 3) during the escape response.

Video S1. Wild type touch-evoked escape response. A control MO-injected zebrafish was tapped on the head and its movements recorded at 1000 frames/s. The movie is played back at 15 frames/s. A video clip is available online. Supplementary material related to this article can be found online at http://dx.doi.org/10.1016/j.ydbio.2014.02.025.

dorsoventral patterning of the neural tube, depletion of the V1 interneurons and an impaired escape response in zebrafish. Hence, *prdm12b* is a key component of the genetic program required for motor circuit formation.

Mechanism of prdm12b function in V1 interneuron formation

The mechanism of *prdm12b* function in dorsoventral patterning and V1 interneuron formation is not clear, but Prdm proteins likely act as transcription factors (reviewed in (Fog et al., 2012; Hohenauer and Moore, 2012)). In particular, some Prdm proteins bind DNA primarily, but not exclusively, via their zinc finger domain – for instance, Prdm1 binds a GAAAG motif (Kuo and Calame, 2004). However, other Prdm proteins (including Prdm12b) have not been shown to bind DNA and may instead be recruited to regulatory elements by other transcription factors, as recently demonstrated for Prdm13 (Chang et al., 2013). Similarly,

Video S2. Exaggerated touch-evoked escape response in *prdm12b*-deficient fish. A PTB MO-injected zebrafish was tapped on the head and its movements recorded at 1000 frames/s. The movie is played back at 15 frames/s. A video clip is available online. Supplementary material related to this article can be found online at http://dx.doi.org/10.1016/j.ydbio.2014.02.025.

while the PR domains of some Prdm proteins have methyltransferase activity, most Prdm proteins (including Prdm12b) appear to lack such activity and instead rely on recruitment of co-activators and co-repressors to regulate transcription (reviewed in (Fog et al., 2012; Hohenauer and Moore, 2012)). It is therefore plausible that Prdm12b regulates dorsoventral patterning of the neural tube by repressing expression of *nkx6.1*, although this effect need not be direct. It is less clear why *irx3* expression expands ventrally from the p2 domain into the pMN domain upon *prdm12b* disruption. Possibly, this is an indirect effect stemming from loss of the p1 domain. Lastly, it is not clear if *prdm12b* is required only to repress *nkx6.1* or if it is also directly required to induce expression of *eng1b* or other genes required for the formation of V1 interneurons. In the latter case, *prdm12b* would likely need to cooperate with other factors since over-expression of *prdm12b* did not lead to ectopic V1 neuron formation in our experiments.

An emerging role for prdm family genes in dorsoventral patterning of the neural tube

With our demonstration that *prdm12b* is required for V1 interneuron formation, there are now several reports of roles for Prdm proteins in specification of progenitor zones in the neural tube. In particular, *prdm13* is expressed in a broad domain dorsal to p0 (Kinameri et al., 2008) where it represses the formation of excitatory glutamatergic neurons and promotes the formation of inhibitory GABAergic neurons that function in somatosensory pathways (Chang et al., 2013). Prdm13 accomplishes this by interacting with the Ascl1 transcription factor to directly repress the expression of *Tlx1* and *Tlx3*. This activity of Prdm13 does not require the PR domain and Prdm13 does not bind DNA directly, but instead binds Ascl1 to switch it from a transcriptional activator to a repressor. In addition, *prdm14* is expressed in several types of neurons in the neural tube and disruption of *prdm14* affects motor neuron formation (Liu et al., 2012). Interestingly, *prdm14* does not control motor neuron fate, but is required for axonal extension and loss of *prdm14* leads to impaired motor circuits and diminished swimming. Prdm14 reportedly binds DNA directly via its zinc-finger domain (Ma et al., 2011) and ChIP analyses revealed Prdm14 binding upstream of the *islet2* gene (Liu et al., 2012), suggesting that Prdm14 directly regulates *islet2* expression to control motor axon extension. Lastly, *prdm1a* is required for the formation of sensory Rohon-Beard (RB) neurons in the dorsal neural tube (Hernandez-Lagunas et al., 2005). Accordingly, *prdm1a*-deficient zebrafish lack a touch response when prodded on the flank (RB-mediated response), but respond when touched on the head (Mauthner neuron-mediated response). Prdm1 proteins bind DNA directly (Kuo and Calame, 2004) and appear to regulate the expression *islet1* and *islet2* during RB neuron formation (Olesnicky et al., 2010). Hence, *islet2* expression is regulated by Prdm1a in RB neurons and by Prdm14 in motor neurons. Taken together, these data demonstrate that Prdm proteins play an important role in the formation of neuronal subtypes along the dorsoventral axis of the neural tube. Indeed, since additional Prdm proteins are expressed in the neural tube (Kinameri et al., 2008), it is possible that this family will turn out to be a major regulator of the specification and differentiation of neuronal subtypes.

The role of V1 interneurons in locomotion

Previous work suggested that V1 interneurons modulate activity in a central pattern generator (CPG) that produces the rhythmic output required for locomotion (reviewed in (Goulding, 2009)). In particular, recordings of V1 interneurons in zebrafish (CiA neurons) reveal rhythmic oscillations in membrane potential that are in phase with the activity of ipsilateral motor neurons during swimming episodes (Higashijima et al., 2004). Similarly, the V1 interneurons of *Xenopus* tadpoles (aIN neurons) are active during swimming episodes and can be shown to inhibit the activity of CPG motor neurons by whole-cell patch recordings (Li et al., 2004). V1 interneurons in the mouse share features with those in fish and amphibians in that they also project to motor neurons (Saueressig et al., 1999), but appear to be more complex such that they give rise to multiple types of inhibitory interneurons (including Renshaw cells and Ia neurons; (Sapir et al., 2004; Alvarez et al., 2005)). Nevertheless, both Renshaw cells and Ia interneurons inhibit the activity of spinal motor neurons (reviewed in (Goulding, 2009)). Hence, cell recordings indicate a role for V1 interneurons in regulating the CPG, but understanding the role of V1 interneurons in locomotor behaviors requires the depletion of this class of neurons.

We find that disruption of *prdm12b* function leads to loss of V1 interneurons, making *prdm12b*-deficient fish a useful model to study the role of these neurons. While we do observe additional phenotypes in *prdm12b*-deficient fish, these involve cells not directly implicated in locomotion (such as facial and abducens neurons) and are mild (observed in ~25%, while V1 interneurons and the escape response are affected in >75%, of *prdm12b*-deficient fish). Similarly, while OPC numbers are reduced at early stages, they have largely recovered by the stage the behavioral analysis is carried out. Accordingly, other areas involved in locomotor behaviors – such as the hindbrain – appear structurally unaffected. Although we cannot fully rule out functional defects in these areas, we do not observe known phenotypes associated with hindbrain dysfunction such as the unilateral C-bends observed in the *twitch twice* mutant where Mauthner projections are disrupted by *robo3* dysfunction (Granato et al., 1996; Burgess et al., 2009). Lastly, it remains possible that hitherto undefined neurons originating in p1 may also be affected by *prdm12b* depletion. Nevertheless, our results suggest that loss of V1 interneurons underlies aspects of the abnormal escape response and the ineffective swimming movements displayed by *prdm12b*-deficient animals.

There have been previous locomotor mutants identified in zebrafish genetic screens (Granato et al., 1996). By comparing the phenotypes of such mutants to those of *prdm12b*-deficient fish, we can further delineate the role of V1 interneurons in zebrafish. For instance, the *technotrouser* (*tnt*) mutation disrupts a glutamate transporter (*eaat2b*) expressed in glial cells, thereby causing excess glutamatergic stimulation. *tnt* Mutants show repeated large amplitude C-bends and a prolonged escape response (McKeown et al., 2012), similar to what we

observe upon disruption of *prdm12b*. Hence, excess excitatory stimulation produces the same C-bend phenotype as loss of V1 interneurons, consistent with these neurons having an inhibitory function in the escape response. However, *tnt* fish are able to swim and travel a greater distance than control fish during the escape response, while *prdm12b*-depleted fish undergo movements that do not lead to productive swimming. Therefore, V1 interneurons likely also function in the synchronization of muscle contractions required for productive swimming. Additionally, the *bandoneon* (*beo*) mutation affects glycine receptor b2 (*glrb2*), thereby interfering with inhibitory synaptic transmission (Hirata et al., 2005). *Beo* fish display defective coordination of left–right muscle contractions such that they fail to produce C-bends in response to touch and instead simultaneously contract muscles on both sides of the body (known as the "accordion" phenotype (Granato et al., 1996)). While V1 interneurons are glycinergic, *prdm12b*-deficient animals differ from *beo* mutants in that they are able to coordinate left–right muscle contractions during the C-bends, but are unable to subsequently achieve forward propulsion. Hence, V1 interneurons appear to play very specific roles both in the modulation of C-bend amplitude and in the organization of productive swimming movements.

Elimination of V1 interneurons has been achieved in the mouse by either depleting or inactivating *En1* positive neurons (Gosgnach et al., 2006). These experiments suggested a requirement for V1 interneurons in controlling the speed of locomotion – specifically fast locomotor outputs. Walking in mammals differs from swimming in fish, making it somewhat difficult to carry out direct comparisons. Nevertheless, in addition to the exaggerated escape response noted above, we also find that fish lacking V1 interneurons spend more time in each body bend and that the interval between body bends is increased, consistent with V1 interneurons being required to achieve fast responses also in fish. In summary, we conclude that *prdm12b* is essential for V1 interneuron formation and that V1 interneurons act as inhibitory neurons in zebrafish locomotor circuits.

Acknowledgments

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2014.02.025.

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