Development/Plasticity/Repair

Notum Homolog Plays a Novel Role in Primary Motor Innervation

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To form complex neuronal networks, growth cones use intermediate targets as guideposts on the path to more distant targets. In the developing zebrafish (*Danio rerio*), the muscle pioneers (MPs) are intermediate targets for primary motor neurons (PMNs) that innervate the trunk musculature. The mechanisms regulating PMN axon guidance at the MPs are not fully understood. We have identified a new member of the Notum family in zebrafish, Notum 2, which is expressed exclusively in the MPs during primary motor innervation. While homologs of Notum, including zebrafish Notum 1a, negatively regulate the Wnt/β-catenin signaling pathway, we discovered a novel function of Notum 2 in regulating motor axon guidance. Knockdown of Notum 2 resulted in a failure of caudal primary (CaP) axons to migrate beyond the MPs, despite the proper specification of the intermediate target. In contrast, mosaic Notum 2 overexpression induced branching of PMN axons. This effect is specific to Notum 2, as overexpression of Notum 1a does not affect PMN axon trajectory. Ectopic expression of Notum 2 by cells contacting the growing CaP axon induced the highest frequency of branching, suggesting that localized Notum 2 expression affects axon behavior. We propose a model where Notum 2 expression at the MPs provides a cue to release CaP motor axons from their intermediate targets, allowing growth cones to proceed to secondary targets in the ventral muscle. This work demonstrates an unexpected role for a Notum homolog in regulating growth cone migration, separate from the well established functions of other Notum homologs in Wnt signaling.

Introduction

During development, the nervous system undergoes extensive wiring programs to generate a fully functional nervous system (Tessier-Lavigne and Goodman, 1996; Dickson, 2002). To direct the axon toward proper targets, the neuronal growth cone responds to various guidance cues. The path toward the target cell is not always linear, and the growth cone can migrate through several intermediate targets before reaching its final synaptic target (O'Connor, 1999). The identification of the signaling mechanisms directing axons to and beyond intermediate targets is an important subject in the study of nervous system development and may help create novel therapeutic strategies for traumatic brain and spinal cord injury (Benowitz and Yin, 2007; Yaron and Zheng, 2007).

The guidance of primary motor axons during muscle innervation in the zebrafish (*Danio rerio*) is a classic model used to study the role of intermediate targets. Each hemisegment of the developing zebrafish produces three to four primary motor neurons (PMNs): the rostral (RoP), medial (MiP), variable (VaP), and caudal (CaP) primary motor neurons (Myers et al., 1986; Westerfield et al., 1986; Eisen et al., 1990). The CaP axon is the first to exit, followed closely by axons from VaP (if present) then MiP and RoP. All axons migrate ventrally along a common path on the medial surface of the myotome toward the muscle pioneers (MPs), an intermediate target where the first synaptic contacts are made (Eisen, 1999). After a brief pause, the growth cones separate from the MPs to innervate ventral (CaP), dorsal (MiP), and lateral (RoP) muscle groups. Ablation of the MPs significantly increases the frequency of truncation of CaP axons (Melançon et al., 1997), suggesting the MPs are essential in promoting growth into the ventral muscle. However, the molecular nature of the MP signal(s) allowing separation from intermediate to final target muscles is yet to be determined.

Here we describe a novel gene, *notum 2*, expressed exclusively in the MPs. The Notum genes encode secreted α/β hydrolases shown to cleave glycosylphosphatidylinositol (GPI)-anchored glypicans, which bind and regulate diverse signaling molecules (Liang et al., 1999; Ronca et al., 2001; Topczewski et al., 2001; Gerlitz and Basler, 2002; Giráldez et al., 2002; Song et al., 2005; Rhiner and Hengartner, 2006; Gumienny et al., 2007; Beckett et al., 2008; Capurro et al., 2008; Filmus et al., 2008; Torisu et al., 2008; Traister et al., 2008; Ayers et al., 2010; Petersen and Reddien, 2011; Flowers et al., 2012). Unlike previously described homologs, Notum 2 does not play a role in tissue patterning, but instead plays a novel role in axon guidance. Knockdown of Notum 2 does not affect the specification of the MPs, but prevents

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 0270-6474/13/332177-11$15.00/0

Table 1. Primer and morpholino sequences

	Sequences
Primer 1	AGCATCAATCCGAAAACCAC
Primer 2	TGTATCTGGAATCGCAGGTC
Attb1-Notum 2	GGGGACAAGTTTGTACAAAAAAGCAGGCTGTCCTGTCAGGTTGGATATGAA
Attb2R-Notum 2	GGGGACCACTTTGTACAAGAAAGCTGGGTTTTAGCCACCATTGCTGACCAT
Attb2R-Notum 2 no stop	GGGGACCACTTTGTACAAGAAAGCTGGGTTGCCACCATTGCTGACCATTCC
AUG-MO	ATATTCATATCCAACCTGACAGGAC
5′UTR-MO	CCCTTTTAAGCAACTGGTCCTGGTC
Nrp1A-MO	CTTCTAGCCGACAGAGCCCAGTGCA (Lee et al., 2002)

the extension of CaP motor axons beyond the intermediate target into the ventral myotome. Furthermore, mosaic overexpression by cells along the medial surface of the myotome causes primary motor axon branching, demonstrating that Notum 2 can disrupt the path of motor axon growth. This effect requires an intact hydrolase catalytic triad (Ser-Asp-His) and is specific to Notum 2 as it cannot be recapitulated by Notum 1a, previously shown to inhibit the Wnt/β-catenin pathway (Flowers et al., 2012). We propose, that Notum 2 is a "release signal" that promotes CaP axon growth beyond the MPs to innervate the ventral myotome.

Materials and Methods

Fish strains and maintenance. Wild-type (AB) zebrafish (*D. rerio*) embryos of both sexes were maintained between 25°C and 28°C unless otherwise noted. Embryos were staged according to Kimmel et al. (1995).

Cloning. The *notum 2* was cloned from 26 h postfertilization (hpf) total zebrafish mRNA using primers 1 and 2 (Table 1). To generate overexpression constructs, the *notum 2* coding sequence was amplified using RT-PCR with Att-flanked primers Attb1-Notum 2 and Attb2r-Notum 2 or Attb1-Notum 2 and Attb2r-Notum 2 no stop (Table 1). The PCR product was recombined with pDONR221 entry vector using Gateway BP clonase (Invitrogen) to generate a pME-*notum2* middle entry vector and pME-*notum2nostop*. Using pME-*notum2* as a template, we used inverse PCR to mutagenize Serine-234 to alanine to generate enzyme-dead pME-*mutNotum2*. For mRNA overexpression and probe synthesis, the pME-*notum2* entry vector was recombined with pCSDest (Villefranc et al., 2007) using LR clonase II (Invitrogen) to generate pCS-*notum2*. For *notum 2-EGFP* overexpression constructs, pME-*notum2nostop* was recombined with *p5E-CMV/SP6*, *p3E-EGFP,* and *pDestTol2pA* to generate *CMV/SP6:notum2-egfp* (Kwan et al., 2007). For heat shock-inducible overexpression constructs, the pME-*notum1a* (Flowers et al., 2012), pME-*notum 2*, and pME-*mutnotum 2,* vectors were recombined with *p5Ehsp70l*, *p3EIRES-eGFPCAAX*, and *pDestTol2pA* from the Tol2Kit (Kwan et al., 2007) using LR Clonase II plus (Invitrogen) to generate *hsp:notum2*, *hsp:mutnotum2*, and *hsp:notum1a*. Constructs are flanked by Tol2 transposable elements that increase the frequency of genomic integration (Kwan et al., 2007).

In situ *hybridization*. Antisense DIG-labeled probes were transcribed using T7 polymerase from linearized pCS-*notum2* vector. Whole-mount *in situ* hybridization was carried out as described previously (Thisse and Thisse, 2008).

Gene knockdown. For knockdown of Notum 2, two antisense morpholino (MO) oligonucleotides were purchased from Gene Tools that target the AUG translation initiation site (AUGMO, Table 1) or the 5′-untranslated region (5UTRMO) of *notum 2* (Table 1). Neuropilin 1a (Nrp1a) was knocked down using a previously published Nrp1aMO (Table 1) (Lee et al., 2002). Each embryo was injected at the 1–2 cell stage and fixed at indicated stages in 4% paraformaldehyde. Axon truncation was scored when ventral innervation beyond the horizontal myoseptum was absent. An affected embryo was counted when more than two truncated motor axons were present in segments 5–15 over the yolk extension (20 hemisegments counted per embryo) (Feldner et al., 2007). Embryos were developmentally stage matched using the migration of the posterior lateral line (PLL) primordium. Embryos were scored when the PLL primordium was between segments 4 and 7 (~26 hpf). For single cell labeling, both wild-type and AUGMO-injected embryos were-injected with *mnx1*:*egfp* (Flanagan-Steet et al., 2005). Embryos containing GFP-positive PMNs at 24 hpf were raised at 28°C and fixed at 48 hpf.

Transplantations. Blastula stage transplantations were performed as described previously (Kimmel et al., 1990). Host embryos were injected with 6 ng of AUGMO at the one cell stage. Donor embryos were injected with an equal part mixture of rhodamine/biotinylated dextran (total dextran 5%, Invitrogen, catalog nos. D1816/D1956) in 0.2M KCl. Cells were transplanted from the donor embryo to the margin of the host embryo. The following day, host embryos were screened for rhodamine-positive muscle fibers in the trunk and fixed at 26 hpf. For immunohistochemical analysis, transplanted cells were labeled with Alexa Fluor 647-conjugated streptavidin (Invitrogen, catalog no. S21374).

Immunohistochemistry. Primary antibodies used were SV2 (Developmental Studies Hybridoma Bank, DSHB, University of Iowa, Iowa City, IA), anti-GFP (Invitrogen catalog no. A11122) 4d9 (DSHB), and anti-Prox-1 (Millipore catalog no. AB5475). Fluorescent secondary antibodies used were anti-mouse Alexa Fluor 488 and 546 (Invitrogen catalog nos. A10667 and A10036) and anti-rabbit: Alexa Fluor 568 and 647 (Invitrogen catalog nos. A11011 and A21244). Following fixation, embryos were washed in PBS with 0.05% Tween 20 (PBST), permeabilized with 0.05% trypsin (CellGro catalog no. 25-052), washed with PBST, placed in 100% acetone, and incubated at −20°C for 10 min. Embryos were again washed with PBST and incubated at 25°C for at least an hour in AB Block (PBST with 2% goat serum and 0.01% DMSO). Antibodies were diluted in AB Block. Embryos were incubated in diluted antibody overnight at 4°C or on the bench top (~22°C) for 2–3 h, washed, and stored in PBST.

Gene overexpression. For global overexpression, capped mRNA was transcribed *in vitro* from linearized plasmid using mMessage mMachine (Invitrogen). Embryos were injected at the one cell stage with 100–300 pg of mRNA. For mosaic overexpression, 25–50 pg of DNA plasmid was coinjected with 100 pg *in vitro* transcribed *transposase* mRNA (Kwan et al., 2007). Gene expression was induced by placing DNA-injected embryos in a warm water bath (37°C) for 1 h. Embryos were then returned to the 28°C incubator for recovery and fixed at the appropriate stage.

Behavioral analysis. Behavioral analysis was performed on a Photron FastCam 1024PCI camera (100K Model) mounted on a dissecting scope. Zebrafish larvae at 2 days postfertilization were mounted dorsal side up in 1.5% low-melting agarose in egg water. Once cooled, agarose was removed from the trunk, tail, and surrounding region, allowing the trunk and tail full range of movement. The electrical pulse was provided by a pair of tungsten electrodes (AM Systems catalog no. 575500) controlled by an AM Systems Isolated Pulse Stimulator (2100 model) that were placed at the tip of the tail. Images were captured at 1000 frames/s, and all measurements were made using ImageJ software. Each larva was treated with a series of test pulses to determine the lowest threshold that could induce an escape response between 5.8 and 8.0 V. Behavior was analyzed using the shape of the trunk and tail to indicate individual behaviors. Latency was measured as the time between the stimulus and the first movement of the tail. Coiling was measured as the duration of C-shaped trunk and tail movements. Swimming was measured as the duration of repetitive low-amplitude, S-shaped trunk and tail movements. For these behavioral tests, a subset of embryos was fixed at 26 hpf to determine the efficiency of MO knockdown (6/16 morphant embryos analyzed displayed >5 truncated axons).

Results

Notum 2 belongs to a novel subfamily of Notum homologs in fish

To identify homologs of Notum in the zebrafish, we used the Basic Local Alignment Search Tool, BLAST, at NCBI to search nonredundant zebrafish protein sequences with homology to the *Drosophila* Notum peptide (Altschul et al., 1990; Giráldez et al., 2002). Our search identified a novel homolog we designated *notum 2*. To better understand its relationship to other *notum* genes, we compared *notum 2* to homologs in zebrafish and other vertebrate species. The novel gene encodes a mature protein sharing

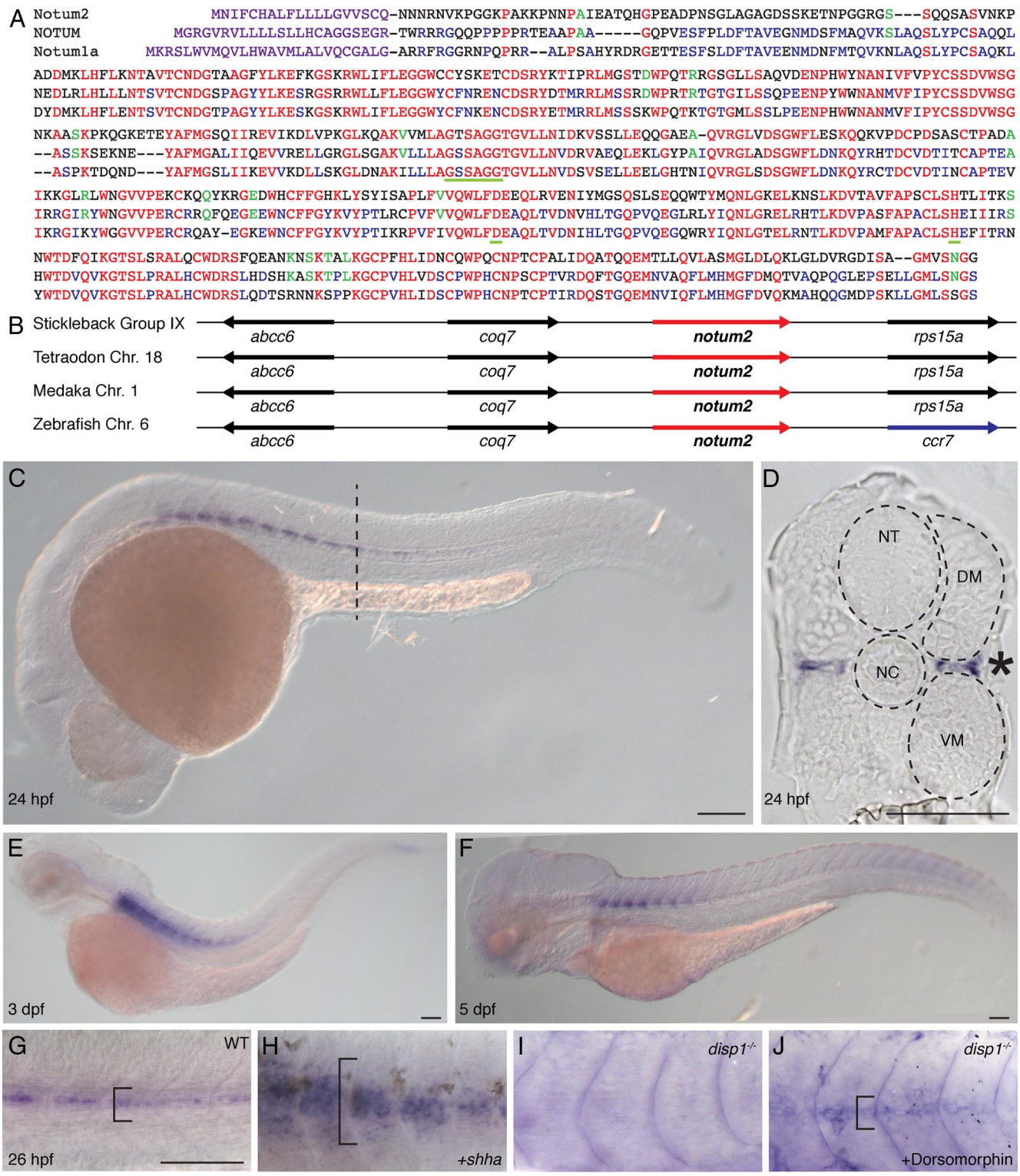

Figure 1. Sequence and expression of Notum 2. ***A***, Protein sequence alignment with human NOTUM and zebrafish Notum 1a shows a predicted signal peptide sequence (purple letters) and conservation between homologs in human and zebrafish including Ser-Asp-His catalytic triad and the GXSXG active site motif (green bars). Red letters indicate identical amino acids shared by all three homologs, green letters are shared by Notum 2 and NOTUM, blue letters are shared between NOTUM and Notum 1a. ***B***, Conserved synteny of Notum 2 with orthologs in stickleback, tetraodon, and medaka. ***C***, Expression of *notum 2* at 24 hpf is detected along the horizontal myoseptum. ***D***, Cross section of trunk at 24 hpf, at the level indicated by dashed line in ***C***, shows *notum 2* expression in the MPs. Asterisk indicates level of HM separating dorsal myotome (DM) and ventral myotome(VM); NT is neural tube and NC is notochord. ***E***, Expression of *notum 2* at 3dpf is expanded in anterior segments. ***F***, At 5 dpf, expression of *notum 2* is reduced to the HM and surrounding slow muscle in the most anterior segments. Lateral views of zebrafish trunk stained by whole-mount *in situ* RNA hybridization of *notum 2* at 26 hpf (***G***–***J***) are shown. ***G***, Expression of *notum 2* in wild-type embryos is restricted to the MPs (black bracket). ***H***, Overexpression of Shha causes expansion of *notum 2* expression domain (black bracket). ***I***. Expression of *notum 2* is lost in Hh-deficient *disp1*^{tm15a} mutants. ***J***. Expression of *notum 2* can be restored by treatment with Bmp inhibitor Dorsomorphin (black bracket). Scale bars (in ***C***, ***E***–***G***), 100 μm; (in ***D***), 50 μm.

51% amino acid identity with human NOTUM and 54% identity with zebrafish Notum 1a (Fig. 1*A*) (Torisu et al., 2008; Flowers et al., 2012). The *notum 2* gene contains a conserved Ser-Asp-His catalytic triad and the G-X-S-X-G active site motif that are necessary for α/β hydrolase activity (Fig. 1*A*) (Nardini and Dijkstra, 1999; Giráldez et al., 2002). While genomic synteny among *notum 1* genes is conserved among vertebrates (Flowers et al., 2012), *notum 2* genes are absent in mammals but share conserved synteny in all fish (Fig. 1*B*). Thus, the designation *notum* 2 distinguishes this novel homolog from other known *notum* genes and indicates it is a homolog, but not a paralog, of *notum 1a.*

Muscle pioneers express *notum 2*

To begin our investigation of *notum 2* in zebrafish, we examined its expression. The *notum 2* transcript was first detectable by RT-PCR and *in situ* hybridization at mid-segmentation stages (15 somites stage) along the horizontal myoseptum (HM) (data not shown). *In situ* mRNA hybridization at 24 hpf shows *notum 2* expression along the trunk and tail (Fig. 1*C*). Transverse sections through the trunk at this stage reveal that *notum 2* is expressed in the muscle pioneers, a distinct class of slow muscles adjacent to the notochord between the dorsal and ventral myotomes (Felsenfeld et al., 1991) (Fig. 1*D*). At 3 days postfertilization, dpf, *notum 2* expression expands dorsally and ventrally into cells immediately surrounding the MPs in the most anterior segments but remains restricted to the MPs in more posterior segments (Fig. 1*E*). Along with dorsal–ventral expansion in anterior trunk segments, transverse sections in the anterior trunk at 3 dpf show that *notum 2* is expressed in superficial slow muscles and is no longer expressed in muscle cells immediately adjacent to the notochord (data not shown). At 5 dpf, *notum 2* expression is similar with broad expression in the anterior segments but is undetectable in the posterior HM (Fig. 1*F*).

At 24 hpf, slow muscles in the trunk can be divided into two groups: superficial slow muscles and MPs. Specification and maintenance of the MP fate requires high levels of Hedgehog (Hh) signaling from the notochord to antagonize bone morphogenic protein (Bmp) (Hatta et al., 1991; Currie and Ingham, 1996; Du et al., 1997; Dolez et al., 2011). To test whether Notum 2 is regulated similarly to other markers of MPs, we analyzed the *notum 2* expression pattern in embryos with differ-

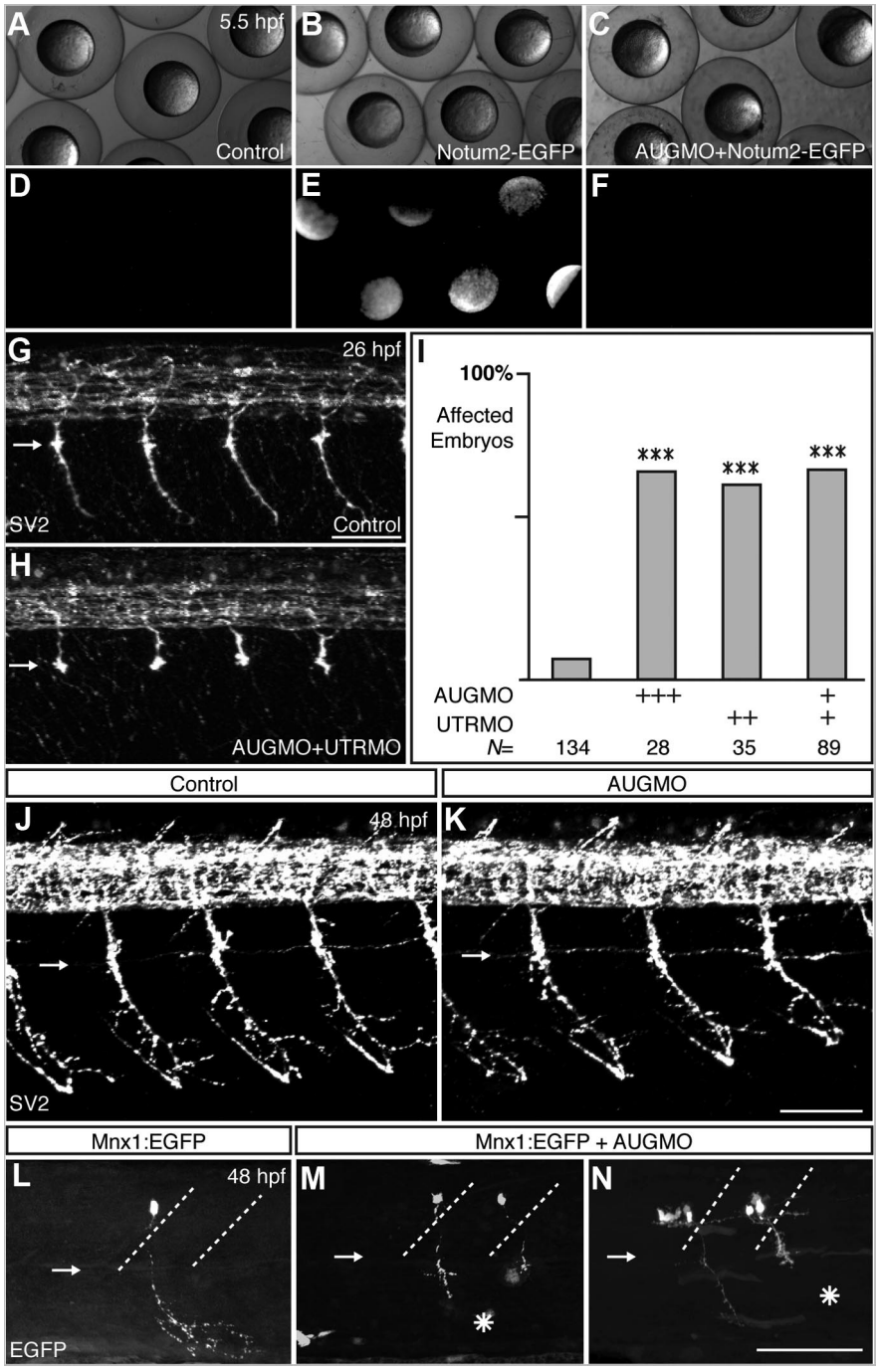

Figure 2. Notum 2 knockdown causes CaP axon truncation. Zebrafish embryos at 5.5 hpf in bright-field (***A–C***) or GFP channel (***D–F***). ***A***, ***D***, Uninjected, wild-type controls do not express EGFP. ***B***, ***E***, Embryos injected with *notum 2-EGFP* mRNA show bright EGFP signal. ***C***, ***F***, Coinjection with AUGMO effectively blocks translation of the Notum 2-EGFP protein. ***G***, ***H***, Lateral views of motor axons stained with SV2 antibody at 26 hpf; arrows indicate position of the horizontal myoseptum. ***G***, CaP motor axons in uninjected wild-type controls have crossed the HM to innervate the ventral myotome. ***H***, Knockdown of Notum 2 using a combination of AUG and 5′UTR antisense Morpholino oligonucleotides causes axon stalling at the HM. ***I***, Percentage of truncated axons in uninjected control or injected with 6 ng AUG-MO, 4 ng 5′UTR-MO, or 2 ng/AUG-MO + 2 ng/5′UTR-MO. ***$p < 0.001$; + corresponds to 2 ng of MO per embryo. ***J***, ***K***, Lateral views of 48 hpf zebrafish trunk stained with SV2 antibody show no difference in motor nerves between wild-type (***J***) and AUGMO (***K***). Lateral views of Hb9:EGFP-positive motor neurons in 48 hpf zebrafish are shown (***L–N***). Dashed lines indicate the segmental borders; arrows mark the position of the horizontal myoseptum. ***L***, In wild-type CaP, axons innervate the ventral myotome. ***M***, Truncated CaP axons in Notum 2 morphants do not appear to recover (asterisk), while labeled RoP in the adjacent segment shows the relative position of the cell body in reference to the segmental border. ***N***, Two clusters of Hb9:GFP-positive neurons show one innervating CaP and one truncated CaP (asterisk). Scale bars: 50 μm.

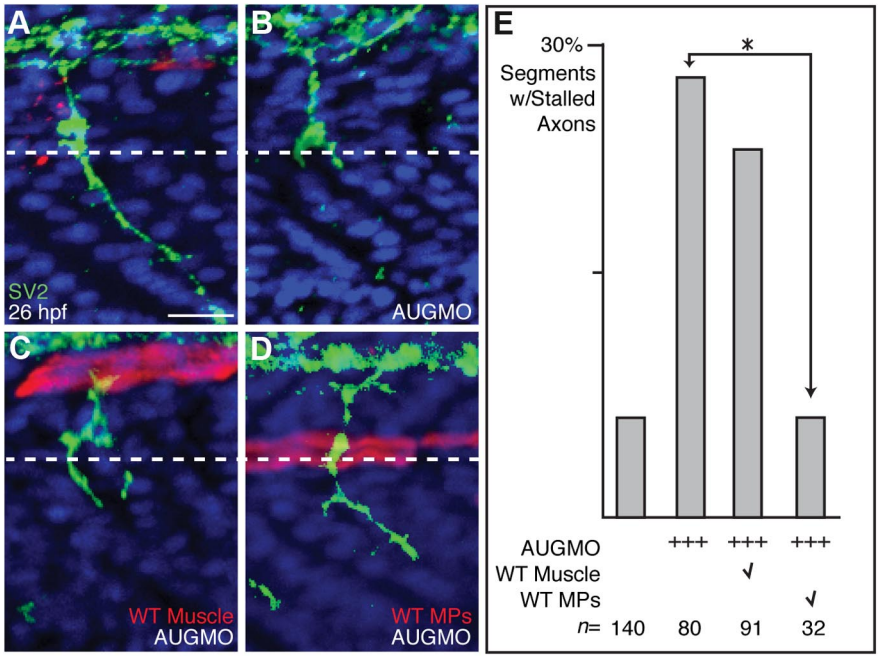

Figure 3. Wild-type MPs rescue morpholino-induced CaP axon truncation. ***A***, Normal innervation of ventral myotome by CaP motor axon in uninjected control embryo. ***B***, Truncated CaP axon in 6 ng AUG-MO-injected host embryo. ***C***, Truncated CaP axon in AUG-MO host segment with transplanted WT fast muscle cells (red). ***D***, Rescued CaP axon in AUG-MO host with uninjected WT MPs. ***E***, Percentage of segments with truncated axons in uninjected controls, AUG morphants with no transplanted cells, AUG morphants with transplanted non-MP muscle fibers, and AUG morphants with transplanted MPs are shown. The *p* value compares AUG-MO-injected segments with no transplanted cells and segments with transplanted WT MPs. The *p* value calculated from Fisher's exact test; $*p < 0.05$; + corresponds to 2 ng of MO per embryo; *n* = number of segments observed. Scale bar (in ***A***), 50 μm.

ent levels of Hh signaling. As compared to wild-type (WT) controls (Fig. 1*G*), embryos injected with *shha* mRNA (Krauss et al., 1993) have an expanded domain of *notum 2* expression (Fig. 1*H*). By contrast, Hh signaling deficient mutants, *disp1*$^{-/-}$ and *gli2*$^{-/-}$, lack MPs (Karlstrom et al., 1999; Nakano et al., 2004) and *notum 2* expression (Fig. 1*I* and data not shown). Inhibition of BMP signaling by treatment with the small molecule inhibitor, dorsomorphin, is able to restore MPs in Hh-deficient embryos (Dolez et al., 2011). We determined that similar treatment with dorsomorphin could restore expression of *notum 2* in *disp1*$^{-/-}$ mutants (Fig. 1*J*). To our knowledge, *notum 2* is the first gene identified with truly restricted expression in the MPs. This observation suggests that Notum 2 may play a role in MP-dependent developmental processes.

Notum 2 is necessary for normal growth of caudal primary motor axon

Given the role of MPs as intermediate targets during innervation of the trunk by primary motor neurons (Melançon et al., 1997), we wanted to determine whether Notum 2 was necessary for proper motor axon growth. We used antisense morpholino oligonucleotides (Summerton and Weller, 1997; Nasevicius and Ekker, 2000) to knock down Notum 2. To ensure effective protein knockdown, we coinjected AUGMO with mRNA encoding Notum 2-EGFP and assessed EGFP expression at 5.5 hpf (Fig. 2*A–F*). As compared to uninjected control (Fig. 2*A,D*), embryos injected with mRNA alone showed bright EGFP signal (Fig. 2*B,E*) while embryos coinjected with MO showed no EGFP (Fig. 2*C,F*), demonstrating that translation of the Notum 2-EGFP fusion protein could be effectively blocked by AUGMO. Morpholino injection was well tolerated at doses up to 6 ng and did not cause morphological defects or significant developmental delay. We next assessed PMN axon morphology at 26 hpf in MO-injected embryos. At this stage of development, the caudal primary (CaP) motor axons of the trunk have exited the spinal cord, crossed the horizontal myoseptum, and reached the most ventral portion of the myotome (Fig. 2*G*). By contrast, CaP axons frequently failed to migrate beyond the MPs in stage-matched Notum 2 morphant embryos (6 ng of AUGMO = 67.9% affected embryos; 4 ng of UTRMO = 63.6% affected embryos). To ensure that this phenotype was a specific result of Notum 2 knockdown, we tested whether injection of both morpholinos at suboptimal doses could enhance the observed phenotype. Injection of a single morpholino at low concentrations induced a weak axon-stalling phenotype (2 ng of AUGMO = 22.2% defects; $N = 9$ embryos or 2 ng UTRMO = 54.5% defects, $N = 11$ embryos). By contrast, simultaneous injection of both MOs at the same concentration resulted in an increased number of stalled motor axons (2ng of ATGMO:2ng of UTRMO = 68.5% affected embryos) (Fig. 2*H,I*).

At 48 hpf, both primary and secondary motor axons innervate the ventral trunk. Using the SV2 antibody, which detects both axon subtypes, we did not observe any significant difference between wild-type and Notum 2 morphants ($N = 10$ wild-type, 10 AUG-MO embryos)(Fig. 2*J,K*). To discriminate between axon subtypes and assess whether primary motor axons remained truncated at 48 hpf, we injected a *mnx1*:*egfp* DNA to randomly label individual motor axons (Flanagan-Steet et al., 2005). At 48 hpf, the GFP-labeled CaP neurons are easily identified by the location of the cell body in the ventral spinal cord, just rostral to the anterior vertical myoseptum (Myers et al., 1986). In wild-type embryos, single labeled CaPs (3/3) display a highly arborized, ventrally innervating axon (Fig. 2*L*). In contrast, we observed several cases (3/6) where CaP axons in Notum 2 morphants fail to extend beyond either the MPs or the border of the ventral notochord (Fig. 2*M,N*). In summary, these results suggest that expression of Notum 2 by the MPs is necessary for proper PMN axon path finding and promotes the growth of CaP axons beyond the level of the intermediate target, a defect that is not corrected at later stages of development.

Wild-type muscle pioneers can rescue Notum 2 knockdown

If Notum 2 acts as a permissive signal to allow CaP axon growth beyond the MPs, transplantation of wild-type MPs, but not other muscle subtypes, should rescue the axon truncation phenotype in Notum 2 morphants. Individual segments in control embryos display a low frequency of truncated CaP axons (8.6%) (Fig. 3*A*) as compared to CaP axons in morphant segments (27.4%, $p < 0.0001$, Fisher's exact test) (Fig. 3*B*). Similarly, CaP axons in morphant segments with transplanted wild-type fast muscle and non-MP slow muscle fibers continued to stall at the HM at a higher than normal rate (20.1%, $p = 0.0174$) (Fig. 3*C*). By contrast, morphant segments containing transplanted MPs were less likely to have truncated CaP axons compared to morphant segments without transplanted cells (6.9%, $p = 0.0247$) (Fig. 3*D,E*).

Indeed, transplantation of wild-type MPs into morphant embryos reduces the frequency of axon truncations to that observed in wild-type embryos at 26 hpf ($p = 1.00$). The ability of wild-type MPs to restore CaP axon growth to the ventral myotome presents further evidence that the phenotype of Notum 2 knockdown results from the loss of a permissive signal that promotes axon migration beyond the intermediate target, and not unspecific toxicity caused by morpholino injection.

Notum 2 is not necessary for muscle specification

Previously studied Notum homologs have the ability to cleave GPI-anchored glypicans that modulate both Hh and BMP-type signaling pathways (Grisaru et al., 2001; Gumienny et al., 2007; Beckett et al., 2008; Capurro et al., 2008; Ayers et al., 2010). Thus, Notum 2 could play an essential role in specifying the intermediate target, the MPs. To test whether Notum 2 activity is needed for the specification of the MPs and slow muscles, we compared the number of double-labeled, Prox-1/Engrailed (4d9)-positive cells in Notum 2 morphants (Ekker et al., 1992; Glasgow and Tomarev, 1998). Despite defects in CaP axon growth, we observed no significant difference in the number of differentiated MPs in Notum 2 morphant segments (mean = 4.3 MPs, $n = 75$ segments) compared to WT (mean = 3.9 MPs, $n = 71$ segments). Similarly, the overall number of differentiated slow muscles, identified by single Prox-1 labeling, did not differ between wild-type (mean = 25.19 slow muscles, $n = 98$ segments) and Notum 2 morphant (mean = 25.2 slow muscles, $n = 98$ segments) embryos. These data indicate that Notum 2 is not necessary for specification of the intermediate target and likely directly affects axon guidance.

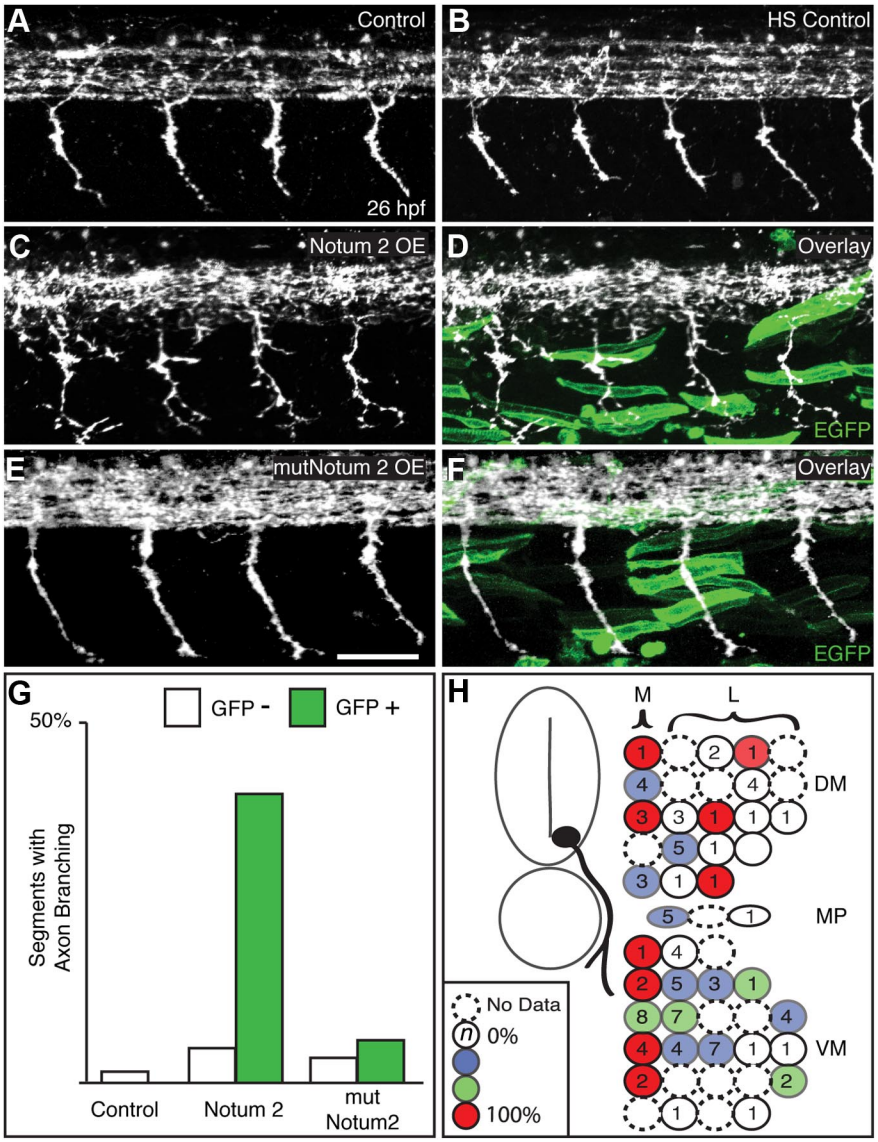

Figure 4. Notum 2 overexpression causes motor axon branching. ***A***, Uninjected, non-heat-shocked control. ***B***, Uninjected embryo, heat-shocked control. ***C***, Notum 2 overexpressing embryo shows extensive axon branching. ***D***, GFP-positive cells (green) indicate Notum 2-expressing cells. ***E***, Hemisegments overexpressing enzyme-dead Notum 2 do not induce motor axon branching. ***F***, GFP-positive cells (green) indicate position of mutNotum 2-expressing cells. Lateral views of primary motor axons stained with SV2 antibody. Scale bar (in ***E***) ***A***–***F***, 50 μm ***G***, Percentage of segments with motor axon branching in heat-shocked control, Notum 2-overexpressing and mutNotum 2-overexpressing embryos. White columns indicate GFP-negative hemisegments; green columns indicate GFP-positive hemisegments. ***H***, Schematic of data collected from segments with a single Notum 2 overexpressing cell. The numbers represent the number of observations in this relative location in the myotome. The color of the circle represents the frequency of branched axons induced by overexpressing cells in this position (bins: dashed circle, no data for this position; white, 0–24%, blue, 25–49%; green, 50–74%; red, 75–100%).

Notum 2 overexpression causes ectopic branching

To further explore Notum 2's role in PMN guidance, we carried out gain-of-function experiments. In contrast to overexpression of Notum 1a, which strongly anteriorized and dorsalized embryos, global overexpression of Notum 2 following mRNA injection did not produce a noticeable phenotype ($n = 109$ embryos), showing that Notum 2 does not affect early developmental patterning associated with negative regulation of Wnt/β-catenin (Flowers et al., 2012). Because Notum 2 is expressed in a discrete position along the path of migrating PMN axons, we tested whether ectopic expression could misroute CaP axons. To locally misexpress Notum 2, we injected single cell embryos with a DNA plasmid encoding heat shock-inducible *notum 2* (*hsp:notum2*) and induced expression immediately before PMN axons begin to exit the spinal cord (~15 somites). Segments of uninjected and heat-shocked controls (Fig. 4*A*,*B*) displayed few abnormal or branched axons (1.2%, $n = 500$ axons). In contrast, segments containing Notum 2-overexpressing cells (Fig. 4*C*,*D*) displayed excessive axon branching (40.3%, $n = 134$ axons). To test whether axon branching requires Notum 2 enzymatic activity, we generated an enzyme-dead version of Notum 2 (mutNotum 2), by using site-directed mutagenesis to change a critical serine residue in the active site to alanine, disrupting the α/β hydrolase catalytic function (Giráldez et al., 2002). Contrasting with segments ectopically expressing wild-type Notum 2, segments misexpressing mutNotum 2 (Fig. 4*E*,*F*) had decreased frequency of axon branching (6.0% $n = 151$ segments, Fig. 4*G*), showing that Notum 2 catalytic activity is required to induce ectopic motor

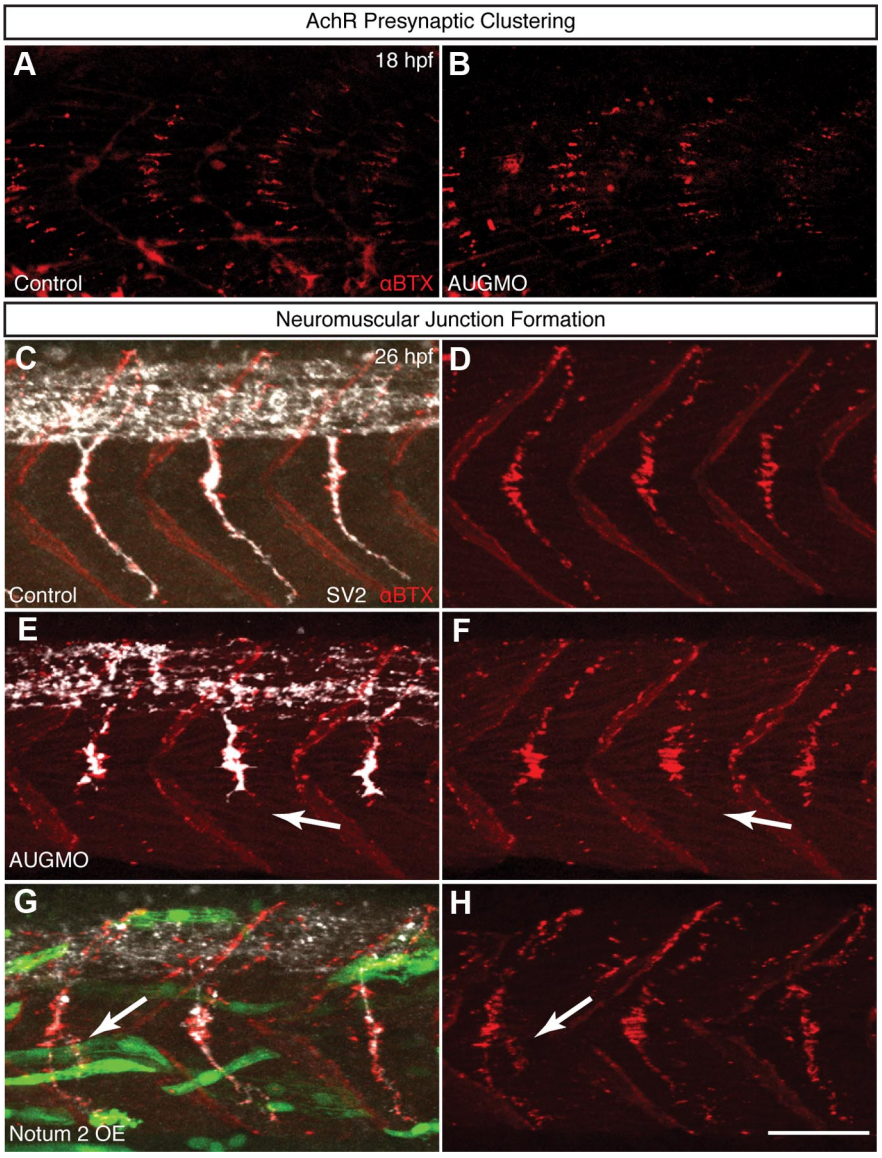

Figure 5. Notum 2 does not affect acetylcholine receptor (AchR) clustering. ***A***, ***B***, Presynaptic patterning in uninjected control (***A***) and Notum 2 morphant (***B***) embryos at 18 hpf. ***C***, ***D***, Synaptic vesicles (***C***) and AchRs (***D***) colocalize to form NMJs in wild-type embryos at 26 hpf. ***E***, Knockdown of Notum 2 does not affect NMJ formation (arrow). ***F***, Segments with truncated axons do not form ectopic AchR clusters (arrow). ***G***, ***H***, Branched axons in Notum 2 overexpressing embryos form ectopic NMJs (arrows). Scale bar (in ***H***), 50 μm.

axon branching. We next tested whether the ability to affect axon path finding is specific to Notum 2 by misexpressing zebrafish Notum 1a. In contrast to our previous experiments with Notum 2, ectopic mosaic expression of Notum 1a did not cause motor axon branching (1.6% $n = 128$ segments). Together, these results show that Notum 1a and Notum 2 have different activities, and only Notum 2 possesses a unique ability to induce axon branching.

Global, in contrast to mosaic, Notum 2 overexpression does not cause motor axon branching at 26 hpf (N = 38 embryos injected with 150 ng of *notum 2* mRNA). This result suggests that localized expression of Notum 2 is necessary to promote axon growth, and thus we determined whether axon branching was affected by the location of the ectopic Notum 2-expressing muscle. We performed detailed three-dimensional analysis of segments containing a single Notum 2-overexpressing cell. This analysis revealed that ectopic motor axon branching was more likely to occur when a Notum 2-expressing cell was located in the medial surface of the myotome (75% $n = 28$ segments, 50 = embryos) than when a Notum 2-expressing cell was located more laterally in the myotome (35% $n = 63$ segments, 50 = embryos) (Fig. 4*H*). As motor axons are most sensitive to contact with Notum 2-overexpressing cells, the target of Notum 2 is likely localized on the membrane of either the CaP growth cone or the medial muscle.

Notum 2 is not necessary for acetylcholine receptor clustering

Axon defects have been observed in embryos with defective acetylcholine receptor (AchR) clustering. For example, knockdown of Agrin, a secreted proteoglycan necessary for AchR clustering, causes abnormal branching, axon truncations, and defective formation of neuromuscular junctions (NMJs) along motor axons (Kim et al., 2007). Similarly, mutations in the *unplugged/muscle-specific kinase* (*MusK*) result in the absence of presynaptic clustering and lead to abnormal axon branching and defective NMJ formation in a Wnt/PCP-dependent manner (Zhang and Granato, 2000; Zhang et al., 2004; Lefebvre et al., 2007; Jing et al., 2009). To test whether Notum 2 is involved in AchR clustering, we used α-bungarotoxin to label AchRs in Notum 2 morphants during presynaptic patterning. Before innervation (18 hpf), individual segments display presynaptic clustering between the vertical myoseptum, along the middle of the myotome (Fig. 5*A*). Presynaptic clustering appears unaffected in Notum 2 morphants ($n = 10$ embryos) (Fig. 5*B*). After primary innervation (26 hpf), colocalization of both presynaptic boutons and AchR clusters show that primary motor axons have formed NMJs in both the dorsal and ventral myotomes (Fig. 5*C*,*D*). While knockdown of Notum 2 causes stalling of ventrally innervating axons, AchR clusters do form at the MPs and along the trajectory of MiP axons in the dorsal myotome (Fig. 5*E*,*F*). Similarly, segments with ectopic Notum 2-expressing cells display AchR clusters along abnormal branches (Fig. 5*G*,*H*). These results show that Notum 2 does not affect Wnt/PCP signaling and indicate that axon defects following modulation of Notum 2 levels are not caused by defective AchR clustering or NMJ formation.

Knockdown of Nrp1A does not rescue CaP axon truncation

After exiting the spinal cord, the CaP axon migrates along a common path toward the MPs. This migration is mediated by the guidance receptor Neuropilin 1a. The CaP neuron expresses Nrp1a, a receptor for the repulsive guidance cue Sema3a1 (Lee et al., 2002; Feldner et al., 2005). Sema3a1 is expressed in a graded fashion with high levels in the dorsal and ventral myotomes and low levels at the HM (Sato-Maeda et al., 2006). Upon exiting the spinal cord, the growth cone is repelled from the dorsal myotome

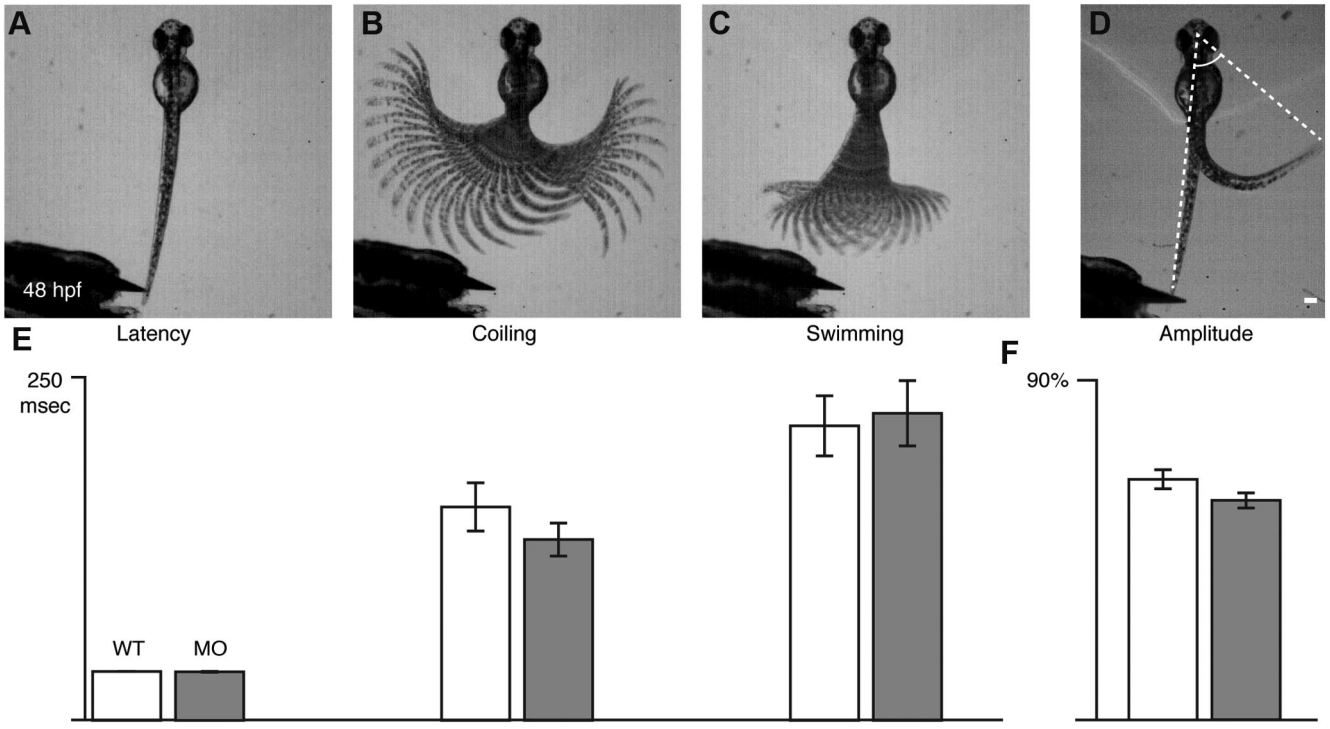

Figure 6. Knockdown of Notum 2 does not affect escape behavior. ***A***, Zebrafish in resting position. ***B***, ***C***, Composite image of 30 frames over 90 ms illustrate the coiling and swimming phase of the escape response. ***D***, Composite image of two frames at rest and peak coiling (42 ms); angled dashed lines show the amplitude of initial bend of the escape response. Dorsal view of zebrafish larvae at 48 hpf with agarose-restrained head is shown (***A***–***D***). ***E***, ***F***, White columns are uninjected controls; gray columns are Notum 2 morphants injected with 6 ng AUG-MO. ***E***, Average time in each phase of the escape response. ***F***, Amplitude of initial c-bend. $N = 10$ wild-type, 9 morphants, 10 trials for each embryo. Scale bar (in ***D***): 100 μm.

toward the MPs. As migration beyond the MPs requires the growth cone to travel into an increasing concentration of Sema3a1 in the ventral myotome, transcriptional downregulation of the Nrp1a receptor must occur, making the growth cone insensitive to the Sema3a1 repellent (Sato-Maeda et al., 2006). If Notum 2 were regulating Nrp1a-dependent signaling pathways to promote growth beyond the MPs, we would expect knockdown of Nrp1a to override CaP axon stalling in Notum 2 morphants. In this experiment, a single dose of Notum 2 morpholino caused axon truncation in 66.6% embryos ($N = 21$), while a single dose of Nrp1A morpholino caused axon truncation in 33.3% of embryos ($N = 15$). Double injection showed that Nrp1a knockdown could not rescue the axon truncation phenotype in Notum 2 morphants (55.6% affected of $N = 9$ embryos), suggesting that Notum 2 and Nrp1A/Sema3a act on separate CaP guidance pathways.

Notum 2 knockdown does not result in behavioral deficits

The escape response is a sensory-gated motor behavior that recruits both primary and secondary motor neurons to generate alternating rhythmic trunk muscle contractions (Kimmel et al., 1974; Liu and Westerfield, 1988; Fetcho and O'Malley, 1995). However, this behavior has not been studied in fish with compromised primary motor innervation. At 48 hpf, the escape response can be observed using a tactile stimulus. The fish responds to the stimulus with a series of coiling, or c-bend movements followed by s-shaped swimming before coming to a stop and resting laterally on the bottom of the dish (Saint-Amant and Drapeau, 1998). We used high-speed video capture to monitor the escape response. We restrained the head of the zebrafish in low-melting agarose, allowing free movement of the trunk and tail. To elicit the escape response, we used a small electrical pulse at the tip of the tail. We did not observe a difference in the escape response between wild-type and Notum 2 morphants ($n = 10$ uninjected controls, 9 injected with 6 ng of AUG-MO, 10 trials/embryo). Analysis of siblings at 26 hpf showed that morphant embryos from this series of injections contain up to 11 truncated CaP axons. Wild-type embryos responded to the electrical stimulus within 33 ± 2 ms (latency) with 158 ± 38 ms of coiling and 218 ± 43 ms of swimming (Fig. 6*A*–*C*). Notum 2 morphants did not differ significantly from wild-type siblings and responded to the stimulus at 33 ± 2 ms (latency) with 134 ± 26 ms of coiling and 226 ± 50 ms of swimming (Fig. 6*D*). To determine whether the strength of the coiling was affected by the loss of Notum 2, we measured the angle of the initial c-bend (Fig. 6*E*). Again, we did not observe a significant difference in the angle of the initial c-bend between wild-type and Notum 2 morphants (Fig. 6*F*). These results show that Notum 2 morphants behave normally, with no gross developmental abnormalities. Furthermore, these results suggest that secondary innervation is sufficient to overcome CaP motor axon deficits in the escape response. It is difficult to ascertain whether this apparently normal behavior is a result of incomplete penetrance of the Notum 2 knockdown phenotype, as no previous behavioral studies have specifically tested the loss of primary innervation on the escape response.

Discussion

Intermediate targeting has been described in grasshopper, worms, flies, birds, mammals, and fish (Tessier-Lavigne and Goodman, 1996; O'Connor, 1999; Dickson, 2002). To use the intermediate targets, growth cones must be capable of changing responsiveness to a finite set of guidance cues. Very few target-derived molecules have been identified that facilitate the transition between intermediate and final targets, so mechanisms

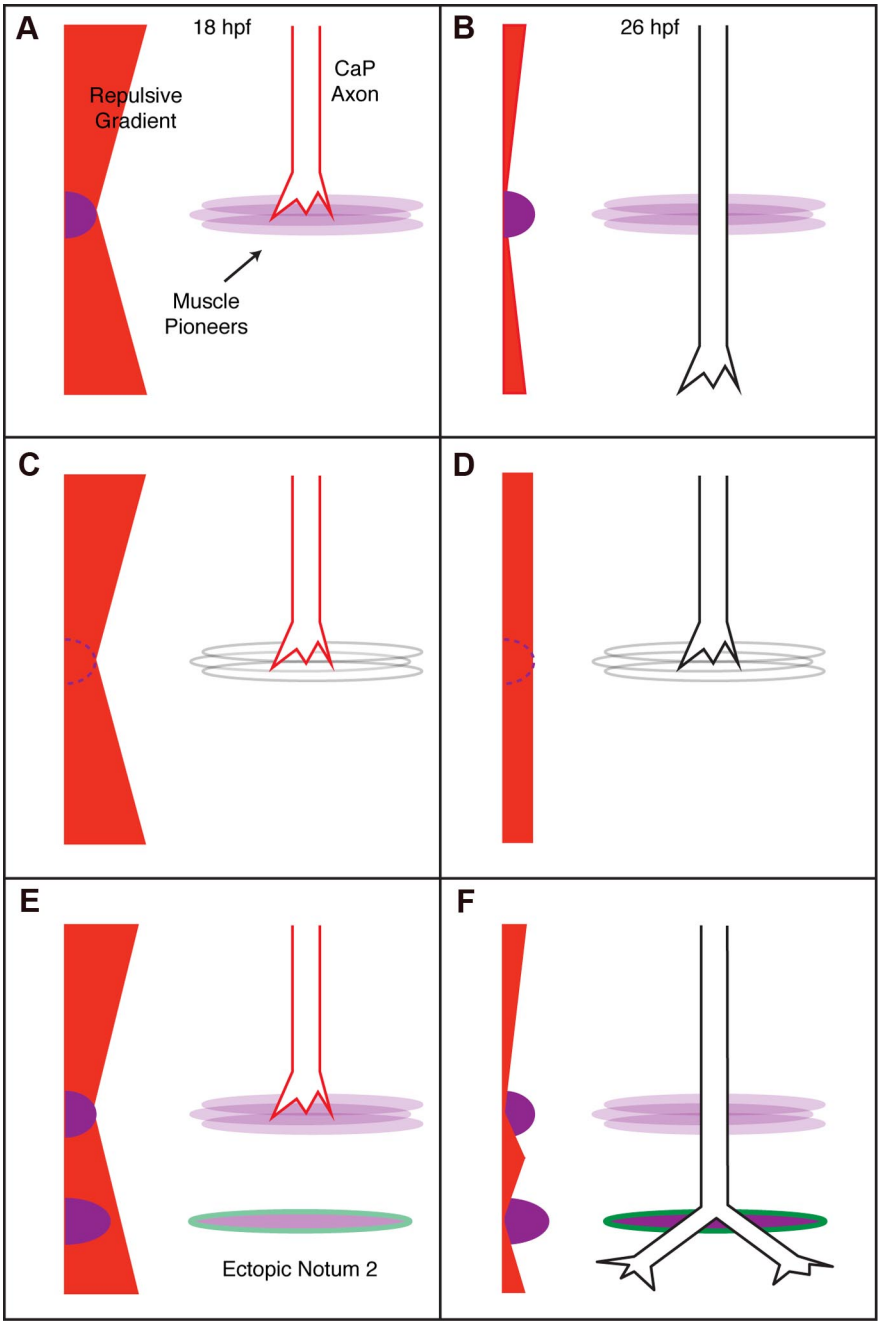

Figure 7. Notum 2 guides caudal primary axon through intermediate target. Schematic representation of CaP growth cone migrating through the MPs is shown. Red color indicates the gradient of Nrp1a/Sema3a1 repulsion. Purple marks the Notum 2 signaling center. ***A***, CaP axon at 18 hpf uses Nrp1a/Sema3a1 repulsion to reach intermediate target. ***B***, CaP axon at 26 hpf requires desensitization to Sema3a1 and Notum 2 expression to migrate beyond the MPs. ***C***, At 18 hpf, CaP axon growth to the MPs is unaffected following Notum 2 knockdown. ***D***, At 26 hpf, CaP axon growth beyond the MPs is inhibited following Notum 2 knockdown. ***E***, At 18 hpf, ectopic Notum 2 (green and purple cell) is induced in the ventral myotome. ***F***, At 26 hpf, contact with ectopic Notum 2 causes branching in CaP axons.

describing this transition have focused on genes expressed by the growth cone. For example, the CaP axon is initially guided from the spinal cord to the MPs by responding to repulsive signals from Sema3a1 in the dorsal myotomes (Yee et al., 1999; Sato-Maeda et al., 2006). To migrate beyond the MPs, the CaP growth cone downregulates the Nrp1a receptor, becoming desensitized to Sema3a1, and migrates into the ventral myotome (Feldner et al., 2005; Sato-Maeda et al., 2006). While this work explains how the growth cone is permitted to migrate away from the MPs, the mechanism responsible for promoting the CaP axon to enter the ventral myotome is unclear.

Notum 2 is a target-derived molecule that modifies axon trajectory and is necessary for axon migration past the intermediate target at the MPs. We postulate that secreted Notum 2 is necessary for primary innervation of the ventral muscle tissue by altering the sensitivity of the growth cone to different sets of guidance molecules (Fig. 7*A*,*B*). Knockdown of Notum 2 removes this signal and stalls axon growth at the MPs (Fig. 7*C*,*D*). Muscle fibers overexpressing Notum 2 in the ventral myotome appear to act as ectopic "choice points" affecting the trajectory of the CaP axon (Fig. 7*E*,*F*). Together, these results demonstrate an unexpected role for Notum 2 in axon guidance and identify a novel molecule that promotes migration through the intermediate MP target. Identification of the molecular targets of Notum 2 will enhance our understanding of intermediate targeting and provide great insight into the mechanisms by which axon paths are shaped.

Studies on Notum homologs in both vertebrates and invertebrates have identified the GPI anchor as the cleavage site for Notum hydrolases (Kreuger et al., 2004; Traister et al., 2008). The role of glypicans in motor axon guidance is not clear. Evidence from other model systems provides clues as to their function as guidance cues. Studies in *Drosophila* show that Dally-like protein, a glypican cleaved by Notum, is necessary for guiding retinal projections (Rawson et al., 2005). In *Caenorhabditis elegans*, the glypican LON-2 helps guide the migration of motor axon projections, and modifications to heparan sulfate (HS) side chains on LON-2 change its axon guidance potential (Bülow et al., 2008). Studies in both flies and mammals have shown that HS side chains on glypicans, such as *Drosophila* Dally-like or rat glypican 1, bind and may help regulate the distribution of Slit ligands (Liang et al., 1999; Ronca et al., 2001; Johnson et al., 2004). Further illustrating the importance of HS in axon guidance, mutations affecting the synthesis of HS result in aberrant axonal projections from retinal ganglion cells in the zebrafish (Lee et al., 2004).

Although no direct evidence has demonstrated a role in zebrafish trunk innervation, HS has been shown to interact with a number of guidance molecules involved in primary motor axon guidance. Recent evidence has shown that Netrin 1a and its receptor DCC are necessary for blocking innervation of the ventral muscle tissue by the variable fate (VaP) PMN and breaking its equivalence with CaP (Hale et al., 2011). Evidence from mouse shows that HS is required for the Dcc receptor to respond to the

Netrin-1 guidance signal in commissural neurons (Matsumoto et al., 2007), but it is not known whether HS is required for Netrin/DCC signaling in zebrafish. Similar to Netrin/DCC, semaphorin/neuropilin signaling utilizes HS as a cofactor in axon guidance (Kantor et al., 2004). Semaphorin 5A contains a domain of thrombospondin repeats (TSRs) that have been shown to bind HS (Kantor et al., 2004). In zebrafish, knockdown of semaphorin 5A causes CaP axons to either branch extensively or stall at the MPs (Hilario et al., 2009). Interestingly, expression of the heparin-binding TSR fragment of Sema5a is sufficient to rescue stalling CaP axons at the MPs following semaphorin 5a knockdown (Hilario et al., 2009). Similarly, collagen XVIII and collagen XIXa1 contain a thrombospondin-like N-terminal (TSPN) domain capable of binding heparin (Schneider and Granato, 2006; Tan et al., 2006; Hilario et al., 2010). Knockdown of either collagen XVIII or collagen XIXa1 cause primary motor axon stalling at the MPs; however, it is not known whether the heparin-binding TSPN domain is sufficient to rescue this effect (Schneider and Granato, 2006; Hilario et al., 2010). Further studies on these HS-binding proteins are necessary to elucidate the full capability of HS in axon guidance.

The identification of Notum 2 target(s) is further complicated by a recent study which demonstrate that mammalian Notum, in addition to glypicans, is able to cleave multiple GPI-anchored proteins in a cell culture system (Traister et al., 2008). Given the numerous GPI-anchored proteins that have been shown to participate in axon guidance, like Ephrin-A, Tenascin-C, Sema7, and NCAMs, the list of Notum 2 targets can become quite extensive. Yet our results from Notum 2 overexpression suggest that Notum 2 is not a promiscuous GPI-cleaving enzyme. Thus far, all Notum homologs characterized in planarians, insects, fish, and mammals have been shown to negatively regulate the Wnt/β-catenin pathway (Gerlitz and Basler, 2002; Giráldez et al., 2002; Torisu et al., 2008; Traister et al., 2008; Petersen and Reddien, 2011; Flowers et al., 2012). Early global overexpression of Notum 2 does not result in any overt phenotype indicating no change in Wnt/β-catenin-dependent tissue patterning. By contrast, overexpression of its homolog Notum 1a causes a clear Wnt/β-catenin inhibition phenotype, with truncation of posterior structures and enlargement of the eye and forebrain (Flowers et al., 2012). Analysis of the Glypican 4 mutant provides further evidence that Notum 2 does not act on all GPI targets. Loss of Glypican 4 affects Wnt/planar cell polarity and causes defective gastrulation movements (Topczewski et al., 2001). Again, global overexpression of Notum 2 does not induce abnormal gastrulation movements, showing that Notum 2 does not cleave Glypican 4. The zebrafish genome encodes a total of nine glypicans, and it is likely that Notum 2 acts on an uncharacterized glypican expressed by either CaP or the muscles. Further studies will be necessary to identify the molecular target(s) of Notum 2 responsible for its function in axon guidance.

Here we describe a homolog of Notum in zebrafish with a novel function in regulating primary motor axon growth. While glypicans, the GPI-anchored targets of Notum, have previously been implicated in axon pathfinding (Liang et al., 1999; Ronca et al., 2001; Johnson et al., 2004; Rawson et al., 2005; Bülow et al., 2008), this is the first indication that regulation by Notum hydrolases serves as a mechanism of axon guidance. Furthermore, the potential to regulate axon guidance by inducing axon branching is not shared by Notum 1a, suggesting that Notum homologs in fish do not share molecular targets. Thus, this function in axon guidance may be divergent of all other Notum homologs that have conserved roles as Wnt inhibitors (Gerlitz and Basler, 2002; Giráldez et al., 2002; Torisu et al., 2008; Traister et al., 2008; Ayers et al., 2010; Petersen and Reddien, 2011; Flowers et al., 2012). Identification of the molecular targets of Notum in fish is necessary to understand how Notum 2 regulates axon guidance, and further study of the mammalian homologs will determine whether this mechanism is conserved in mammals, or if this ability was lost in evolution.

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