Protocadherin-18b interacts with Nap1 to control motor axon growth and arborization in zebrafish

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ABSTRACT The proper assembly of neural circuits during development requires the precise control of axon outgrowth, guidance, and arborization. Although the protocadherin family of cell surface receptors is widely hypothesized to participate in neural circuit assembly, their specific roles in neuronal development remain largely unknown. Here we demonstrate that zebrafish pcdh18b is involved in regulating axon arborization in primary motoneurons. Although axon outgrowth and elongation appear normal, antisense morpholino knockdown of pcdh18b results in dose-dependent axon branching defects in caudal primary motoneurons. Cell transplantation experiments show that this effect is cell autonomous. Pcdh18b interacts with Nap1, a core component of the WAVE complex, through its intracellular domain, suggesting a role in the control of actin assembly. Like that of Pcdh18b, depletion of Nap1 results in reduced branching of motor axons. Time-lapse imaging and quantitative analysis of axon dynamics indicate that both Pcdh18b and Nap1 regulate axon arborization by affecting the density of filopodia along the shaft of the extending axon.

INTRODUCTION

The outgrowth, guidance, and arborization of axons are central to the development and assembly of the mature nervous system. Although several molecular pathways underlying axon guidance have been discovered, our understanding of the range of mechanisms contributing to axonal morphogenesis remains incomplete. The protocadherins are the largest group within the cadherin superfamily of cell surface receptors. They are broadly divided into the clustered (pcdhα, pcdhβ, and pcdhγ) and nonclustered (Nollet et al., 2000; Hulpiau and van Roy, 2009, 2011) genes. Human genetics reveals an involvement of nonclustered protocadherins in a variety of neurodevelopmental disorders (Hirano and Takeichi, 2012; Redies et al., 2012), indicating that they play essential roles in neural circuit assembly. Although functional data are lacking for many of these genes, accumulating evidence suggests that protocadherins play a variety of roles in cell migration and cell motility (Kim et al., 1998; Aamar and Dawid, 2008; Nakao et al., 2008; Emond et al., 2009; Biswas et al., 2010), including axon outgrowth and guidance (Uemura et al., 2007; Hasegawa et al., 2008; Piper et al., 2008; Katori et al., 2009; Leung et al., 2013). Inactivation of the pcdhα gene in mice disrupts the organization of both olfactory sensory axons and the axons of serotonergic neurons (Hasegawa et al., 2008; Katori et al., 2009). Similarly, deletion of pcdh10 (OL-pc) results in defects in thalamocortical axon trajectories (Uemura et al., 2007), and interference with Pcdh7 (NFPC) disrupts axon outgrowth in Xenopus retinal ganglion cells (Piper et al., 2008; Leung et al., 2013).

However, the specific developmental and cellular functions of protocadherins remain unknown, as do the underlying molecular mechanisms.

The spinal cord of the embryonic zebrafish contains three identified motoneurons that are repeated in each spinal hemisegment and are invariant among embryos (Myers, 1985; Myers et al., 1986; Eisen et al., 1986). The stereotyped axon development and trajectories of these primary motoneurons (rostral primary, middle primary, caudal primary [CaP]) make them excellent models for studying axon guidance and morphogenesis. To investigate the cellular roles of protocadherins in neuronal development, we identified a protocadherin expressed in the primary motoneurons of zebrafish. Protocadherin-18b (pcdh18b) is a δ2-protocadherin, consisting of six extracellular cadherin motifs, a single-pass transmembrane domain,
and a conserved intracellular domain (Kubota et al., 2008). Using antisense morpholino oligonucleotides, we show that depletion of Pcdh18b interferes with the Arborization of the CaP motor axon. In addition, Pcdh18b interacts with Nap1, an important regulator of actin dynamics. Antisense knockdown of Nap1 results in axon Arborization defects that are similar to those due to loss of Pcdh18b. Multiphoton imaging of CaP axon dynamics reveals that the primary effect of depleting either Pcdh18b or Nap1 is a reduction in the number of axonal filopodia. Thus both Pcdh18b and Nap1 may contribute to the elaboration of mature axonal arbors by regulating the rate of branch initiation during development.

RESULTS

Protocadherin-18b regulates motor axon Arborization

As reported previously (Kubota et al., 2008), pcdh18b is expressed in neurons of the ventral spinal cord (Figure 1A). Two-color in situ hybridization using riboprobes directed against pcdh18b and isl1 reveals that these ventral neurons are primary motoneurons (Figure 1, B–D). To determine the role of pcdh18b in motoneuron development, we designed antisense morpholino oligonucleotides against pcdh18b (Figure 2A). Injection of splice-blocking morpholinos into one-cell-stage embryos efficiently blocks the processing of pcdh18b transcripts (Figure 2, B and C). The misspliced transcripts are predicted to encode a truncated form of Pcdh18b that lacks the cytoplasmic domain, which was confirmed by cloning and sequencing of the misspliced products (Figure 2D).

To assess the role of Pcdh18b in motoneuron development, we used a transgenic line in which the hb9 promoter drives expression of green fluorescent protein (GFP), Tg(mnx1:0.6hsp70:GFP). In this line, GFP brightly labels primary motoneurons, as well as some secondary motoneurons and other spinal interneurons (Figure 2E). Transgenic embryos were injected with pcdh18b morpholinos, and two-photon image stacks of the ventrally projecting CaP axons were collected at 2 d postfertilization (dpf; Figure 2F). Compared to embryo injected with a mismatch control morpholino (pcdh18bMOS1-mis), CaP axons from pcdh18bMOS1-injected embryos exhibited a dose-dependent decrease in Arborization (Figure 2G). Comparable results were obtained with both splice-blocking morpholinos (pcdh18bMOS1 and pcdh18bMOS2) and a morpholino targeted to the translational start site (pcdh18bMOT). Axons from embryos injected with pcdh18bMOS1-mis had 39.9 ± 1.9 (mean ± SEM; n = 24 axons from eight embryos) total branches, whereas doses of 5 and 10 ng of pcdh18bMOS1 resulted in 27.1 ± 1.3 (n = 60 axons from 21 embryos, p = 8e^{-7}) and 20.3 ± 1.0 (n = 67 axons from 24 embryos, p = e^{-10}) total branches, respectively. To verify that the effects on motor axon Arborization are due to the loss of Pcdh18b, we performed rescue experiments using a bacterial artificial chromosome (BAC) clone that harbors the full pcdh18b gene (Figure 3). Compared to BAC-injected embryos (Figure 3A), the total number of axon branches and total branch length were reduced in morphant embryos (Figure 3, B, D, and E). Control embryos exhibited total branch length of 587 ± 14 μm (n = 66 axons) and total branch number of 24.9 ± 0.7, compared with 453 ± 12 μm and 20.6 ± 0.7 for morpholino-injected embryos, respectively. These branching defects were rescued in embryos injected both with morpholinos (MOS) and the pcdh18b BAC (Figure 3, C–E), which exhibited a total branch length of 549 ± 19 μm (p = 0.0006) and total branch number of 24.5 ± 0.8 (p = 0.003). To verify that BAC injection rescued Pcdh18b levels, we performed reverse transcription (RT)-PCR on embryos that had been injected with 8 ng of pcdh18bMOS2 or 8 ng of pcdh18bMOS2 and 100 pg of pcdh18b BAC DNA (Figure 3F). Coinjected embryos exhibited increased levels of wild-type transcripts.

To determine whether Pcdh18b is required cell autonomously, we performed cell transplantation experiments (Figure 4A). Cells from transgenic embryos were transplanted into wild-type embryos, and GFP-labeled CaP axons were imaged at 2 dpf (Figure 4, B–D). Motor axons of cells transplanted from pcdh18b-morphant embryos into wild-type embryos exhibited a decrease in Arborization compared with control transplants (Figure 4, B, E, and F). There was a decrease in total branch length (WT → WT, 359.8 ± 34.5, n = 7 axons, vs. MO → WT, 239.9 ± 20, n = 7 axons; p = 0.008) and branch number (WT → WT, 25.6 ± 2.1, vs. MO → WT, 18.5 ± 1.8; p = 0.009). In contrast, wild-type cells transplanted into morphant embryos exhibited normal axon arbors (Figure 4, D–F) in both total branch length (316.8 ± 25.4) and total branch number (27.9 ± 2.2). These results indicate that Pcdh18b is required cell autonomously for normal Arborization of CaP motor axons and is not required in the adjacent
Protocadherin-18b interacts with Nap1

Prior work showed that the cytodomains of both mouse Pcdh10 and chicken Pcdh19 interact directly with Nap1, a core component of the WAVE complex (Nakao et al., 2008; Tai et al., 2010). To determine whether Pcdh18b also interacts with Nap1, we performed coimmunoprecipitation with epitope-tagged Pcdh18b and Nap1 (Figure 6). When cotransfected into HEK293 cells, Nap1-Myc coimmunoprecipitates with full-length Pcdh18b-GFP (Figure 6A). Similarly, Nap1 coimmunoprecipitates with a soluble form of the Pcdh18b intracellular domain (Figure 6B). In contrast, a truncated version of Pcdh18b lacking the intracellular domain (Pcdh18bΔICD) fails to interact with Nap1 (Figure 6C). To determine the relative distributions of Pcdh18b and Nap1 in cells, we cotransfected HEK293 cells with Pcdh18b-GFP and Nap1-Myc (Figure 6, D–F). Nap1-Myc is present diffusely throughout the cytoplasm of transfected cells, including a strong presence in the nucleus and lamellipodia (Figure 6D). Pcdh18b-GFP is present diffusely along the plasma membrane, as well as in discrete puncta.
FIGURE 3: Rescue of axon branching defects by injection of pcdh18b BAC clone. (A–C) Maximum-intensity projection of a two-photon image stack from Tg(mnx1:0.6hsp70:GFP)~26 embryos at 48 hpf that were injected with BAC clone CH211-154p8 (A), pcdh18bMOS2 (B), or both (C). Scale bar, 25 μm. (D) Injection of BAC CH211-154p8 rescues the defect in total branch length. BAC (587 ± 14 μm, n = 66), MO (453 ± 12 μm, n = 56), rescue (549 ± 19 μm, n = 71; *p = 0.00056). Significance determined using Wilcoxon signed-rank test. (E) Injection of BAC clone CH211-154p8 also rescues the defect in branch number. BAC, 24.9 ± 0.7; MO, 20.6 ± 0.7; rescue, 24.5 ± 0.8; *p = 0.0033. Significance determined using Wilcoxon signed-rank test. (F) Injection of BAC clone CH211-154p8 results in a partial recovery of wild-type pcdh18b transcript levels. RT-PCR was performed on wild-type embryos (lane 2), embryos injected with 8 ng of pcdh18bMOS2 (lane 3), or 8 ng of pcdh18bMOS2 and 100 ng of CH211-154p8 BAC DNA (lane 4). (Figure 6E). An overlay of Nap1-Myc and Pcdh18b-GFP exhibits extensive overlap of fluorescence signal in the lamellipodia (Figure 6F).

Given that Nap1 is a regulator of actin dynamics and has been shown to participate in neurite outgrowth, we hypothesized that Nap1 could be involved in the axon growth defects exhibited by pcdh18b morphants (Figure 7). To test this, we designed morpholinos against nap1 (Figure 7A). Injection of splice-blocking morpholinos (nap1MOS1 or nap1MOS2) efficiently impairs the processing of nap1 transcripts (Figure 7B), resulting in truncated protein (Figure 7C). Injection of a morpholino directed against the translational start site (nap1MOT) resulted in a dose-dependent loss of Nap1 (Figure 7D). Depletion of Nap1 using either splice-blocking or translation-blocking morpholinos resulted in axon arborization defects similar to those of pcdh18b morphants (Figure 7, E–H). As shown for Pcdh18b, depletion of Nap1 resulted in a loss of total axon branch length (WT, 491 ± 53.7, n = 7, vs. nap1MOS2, 334.1 ± 28.3, n = 10; p = 0.006) and branch number (WT, 28.6 ± 3.0, vs. nap1MOS2, 17.8 ± 1.3; p = 0.02). These results demonstrate that Nap1 participates in the development of CaP motor axons and support the idea that the Pcdh18b–Nap1 interaction could be important for axon development.

**Protocadherin-18b and Nap1 control motor axon dynamics**

To investigate the effects of Pcdh18b or Nap1 depletion in more detail, we collected in vivo two-photon time-lapse image sequences of GFP-labeled CaP axons (Figure 8, A–C). Growing CaP axons are highly active, exhibiting dynamic filopodia along the axon shaft, as well as the growth cones (Figure 8, A–C). To determine the origins of the branching defects exhibited by pcdh18b-and nap1-morphant embryos, we quantified the filopodia dynamics in 1-dpf embryos, as the CaP axonal arbor is beginning to elaborate. Compared to control axons, the density of filopodia along the main shaft of the CaP axon was reduced in embryos depleted of either Pcdh18b or Nap1 (Figure 8D; WT, 0.6 ± 0.05 filopodia/μm, n = 8 axons from four embryos, vs. pcdh18b-MOs2, 0.47 ± 0.04, n = 8 axons from four embryos, p = 0.002; vs. nap1MOT, 0.42 ± 0.03, n = 10 axons from five embryos, p = 0.005). In contrast, loss of neither protein significantly affected the dynamic properties of filopodia, as the lifetimes (Figure 8E), rates of extension and retraction (Figure 8F), and lengths (Figure 8G) were comparable to those of control axons. These data indicate that both Pcdh18b and Nap1 may promote the formation of new filopodia in axons but do not affect the dynamic properties of those filopodia that do form.

**DISCUSSION**

Accumulating evidence implicates protocadherins in a range of neurodevelopmental disorders, including autism spectrum disorders, schizophrenia, intellectual disability, and epilepsy (Hirano and Takeichi, 2012; Redies et al., 2012). However, the molecular, cellular and developmental functions of protocadherins are poorly understood, as are the pathways linking molecular disruptions to brain disorders. Recently a genomic deletion that includes only the pcdh18b gene was associated with severe developmental disturbance that included seizures, developmental delay, microcephaly, and behavioral abnormalities, as well as broad morphological defects (Kasnauskiene et al., 2012). A previous study demonstrated a role for a related gene, pcdh18a, in cell migration during zebrafish gastrulation (Aamar and Dawid, 2008). However, the neuronal functions of PCDH18 were not previously investigated. Here we demonstrate that Pcdh18b associates with the actin regulator Nap1 and both proteins play a role in the arborization of motor axons in the embryonic zebrafish.

Prior studies implicated protocadherin family members in axon growth. Mice deficient in α-Pcdhs have defects in serotonergic axon tracts (Katori et al., 2009) and exhibit mistargeting of olfactory
sensory axons (Hasegawa et al., 2008). Deletion of mouse pcdh10/OL-pc causes a defect in thalamocortical tracts in the ventral telencephalon and impairs striatal axon outgrowth in vitro and in vivo (Uemura et al., 2007). Similarly, expression of Pcdh7/NFPC dominant-interfering constructs in Xenopus retinal ganglion cells also affects axon outgrowth (Piper et al., 2008). Our results differ in that axon initiation and extension appear to proceed normally. Instead, the branching patterns of CaP axons are sparser, exhibiting fewer branches and reduced total branch length. Time-lapse analysis suggests that the branching defects could be due to a reduced rate in the initiation of filopodia. This could reflect diversity in the specific functions played by different protocadherin family members. It is not clear whether the observed phenotype can be attributed entirely to the loss of Pcdh18b or may reflect a partial dominant-interfering activity of truncated Pcdh18b resulting from the use of splice-blocking morpholinos. However, the robust phenotypic rescue achieved indicates that the full-length protein can largely restore function, even in the presence of truncated Pcdh18b, arguing against a large dominant-interfering effect.

Nap1 is a core component of the WAVE complex, an important regulator of actin dynamics (Bear et al., 1998; Miki et al., 1998; Ismail et al., 2009; Chen et al., 2010). As such, Nap1 and the WAVE complex are ideally situated to regulate axon growth and arborization. Mice lacking Nap1 are defective in neurite outgrowth (Yokota et al., 2007), and Rac1 regulates axon growth through the WAVE complex (Tahirovic et al., 2010). Although it is not known what cell surface interactions or signals lead to the activation or suppression of WAVE-mediated actin dynamics, we show that Nap1 binds to the intracellular domain of Pcdh18b and depletion of either Pcdh18b or Nap1 yields very similar effects on axon branching and dynamics. Pcdh10/OL-pc was previously shown to bind to Nap1 and control contact-dependent cell motility in vitro (Nakao et al., 2008), and chicken Pcdh19 was also shown to interact with Nap1 (Tai et al., 2010). Thus Nap1 and the WAVE complex may be general downstream effectors of &-Pcdhs. Conversely, the &-Pcdhs appear to be major regulators of the WAVE complex and actin dynamics during nervous system development.

The interaction of Pcdh10/OL-pc with Nap1 promoted contact-dependent cell motility, suggesting a dependence on homophilic interactions between neighboring cells (Nakao et al., 2008). Our data are consistent with a contact-dependent promotion of filopodia extension, as loss of Pcdh18b or Nap1 reduces the density of filopodia and results in reduced axon arborization. Two observations argue against a homophilic mechanism in the case of Pcdh18b. First, we do not detect expression of pcdh18b in the somite, as was shown previously (Kubota et al., 2008). Thus there is no homophilic partner in the tissue adjacent to the extending axons. Second, Pcdh18b functions cell autonomously during the control of CaP axon branching, as wild-type axons transplanted into morphant embryos exhibit normal levels of arborization. It was similarly shown that the role of Pcdh10/OL-pc acts cell autonomously to promote axon outgrowth in striatal neurons and loss of Pcdh10 affects the guidance of axons that do not normally express it (Uemura et al., 2007). These data could be interpreted as evidence either for an indirect role for protocadherins or for heterophilic interactions. Although members of the cadherin superfamily are generally believed to function as calcium-dependent cell adhesion molecules, there is also evidence for diversity in the range of interactions mediated by cadherin superfamily members. Fat and dachsous proteins have been shown to interact heterophilically (Ishiiuchi et al., 2009), as have cadherin-23 and protocadherin-15 (Kazmierczak et al., 2007; Sotomayor et al., 2012). Moreover, classic cadherins exhibit heterophilic interactions, with homophilic specificity expressed only as a matter of a quantitative preference (Patel et al., 2006; Katsamba et al., 2009). Thus it is possible that protocadherins participate in both heterophilic and homophilic interactions.

In addition to the defect in axon growth and arborization, we observed a decrease in synaptic vesicle clusters, as represented by puncta of Syp-GFP. It is most likely that the decrease in

FIGURE 4: Pcdh18b acts cell autonomously in CaP motor axons. (A) Schematic of cell transplantation experiments. Cells are taken from Tg(mxnx1.0:0.6hsp70:GFP) donor embryos and transplanted into wild-type host embryos. Motoneurons derived from the donor embryos will be fluorescent. Pcdh18b can be knocked down either in the donor or in the host embryos. (B–D) Maximum-intensity projection of a two-photon image stack from 48-hpf host embryos that had been transplanted with cells from Tg(mxnx1.0:0.6hsp70:GFP) embryos in which both host and donor embryos were uninjected (B), donor embryos were injected with pcdh18bMOs2 (C), or host embryos were injected with pcdh18bMOs2 (D). Scale bar, 25 μm. (E) Morphant axons transplanted into a wild-type background exhibited a decrease in total branch length. WT → WT, 359 ± 35 μm, n = 7; MO → WT, 240 ± 20 μm, n = 7, *p = 0.008; n = 7; WT → MO, 317 ± 25 μm, n = 7. Significance determined using Wilcoxon signed-rank test. (F) Morphant axons transplanted into a wild type background exhibited a decrease in branch number. WT → WT, 25.6 ± 2.1, n = 7; MO → WT, 18.5 ± 1.8, n = 7, *p = 0.009; n = 7; WT → MO, 27.9 ± 2.2, n = 7, *p = 0.009. Significance determined using Wilcoxon signed-rank test.

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MATERIALS AND METHODS

Fish maintenance

Adult zebrafish (Danio rerio) and embryos of the Tübingen longfin and AB strains or Tg(mnx1:0.6hsp70:GFP)os26 (Hao le et al., 2012) were maintained at ∼28.5°C and staged according to The Zebrafish Book (Westerfield, 1995). Embryos were raised in E3 embryo medium (Westerfield, 1995) with 0.003% phenylthiourea (Sigma-Aldrich, St. Louis, MO) to inhibit pigment formation. Larvae were anesthetized in 0.016% ethyl 3-amino benzoate methanesulfonate (Sigma-Aldrich). All procedures were carried out in accordance with Institutional Animal Care and Use Committee–approved protocols.

Whole-mount in situ hybridization

The constant cytoplasmic domain of pcdh18b was cloned by RT-PCR. A T7 RNA polymerase binding site was added to the antisense strand by PCR. This PCR product was used as template for in vitro transcription (Promega, Madison, WI). Antisense riboprobe was labeled with digoxigenin-dUTP (Roche, Indianapolis, IN). Embryos were fixed at 4°C overnight in 4% paraformaldehyde in phosphate-buffered saline (PBS), dehydrated in 25, 50, 75% methanol, and stored in 100% methanol overnight at −20°C. They were rehydrated in decreasing concentrations of methanol and permeabilized using proteinase K (10 μg/ml; Roche). Embryos were refixed in 4% paraformaldehyde before hybridization. Digoxigenin-dUTP–labeled riboprobe was added to the final concentration of 200 ng/ml, and putative synapse number is a simple consequence of reduced arbor length, as the density of presynaptic puncta is comparable in control and Pcdh18b-deficient embryos. However, axon arbor exhibits synaptotropic growth (Meyer and Smith, 2006; Ruthazer et al., 2006). It is possible that the reduced extent of arborization could be due to inefficiency in forming or stabilizing synaptic contacts, which would impair the elaboration of a mature arbor. Our time-lapse data indicate that the effect of knocking down either Pcdh18b or Nap1 is not through an effect on synaptotropic growth. The primary effect is a reduction in filopodia density, consistent with a decreased rate in the formation of new filopodia. In contrast, neither the lifetimes of filopodia nor the rates of retraction are significantly affected. Given that previous time-lapse studies showed that new axon branches form from the stabilization and engorgement of filopodia, we expect that any role of Pcdh18b in stabilizing filopodia through adhesive contacts would manifest as an effect on filopodia lifetimes. It is interesting to note that despite the sizable differences in CaP axonal arbor at ∼48 hpf, the effects on axon dynamics at ∼24 hpf are relatively modest. It is possible that small quantitative changes in dynamics (e.g., a reduced rate of initiating filopodia) could have cumulative effects, leading to more pronounced consequences later in development. This could be important in understanding the relationship of molecular perturbations to the etiology of neurodevelopmental disorders.

FIGURE 5: Depletion of Pcdh18b results in arborization defects in single, labeled CaP motor axons. (A, B) GFP was expressed in single CaP motoneurons by coinjecting mnx1-3×125bp:Gal4-VP16 and 14xUAS-E1b:GFP into control (A) or embryos injected with pcdh18bMOs2 (B). Scale bar, 25 μm. (C, D) Coinjection of plasmids with pcdh18bMOs2 resulted in decreases in total branch length (C; control, 491 ± 54 μm, n = 8; pcdh18bMO, 291 ± 37 μm, n = 10, *p = 0.013) and total branch number (D; control, 28.6 ± 3, n = 8; pcdh18bMO, 16.1 ± 2.6, n = 7, *p = 0.014). Significance determined using Wilcoxon signed-rank test. (E, F) Synaptophysin-GFP was coexpressed with Kusabira orange in single CaP motoneurons by coinjecting mnx1-3×125bp:Gal4-VP16 and Syn-GFP:KO into control embryos (A) or embryos injected with pcdh18bMOs2 (B). (G) Coinjection of plasmids with pcdh18bMOs2 resulted in loss of Syn-GFP puncta (control: 69.5 ± 6.6, n = 6; pcdh18bMOs2: 46.9 ± 2.6, n = 26, *p = 0.003). Significance was determined using Wilcoxon signed-rank test. (H) Depletion of Pcdh18b had no effect on the size of Syn-GFP puncta along CaP axonal arbors (control: 1.83 ± 0.18 μm², n = 6; pcdh18bMOs2: 1.76 ± 0.1 μm²).
hybridization was carried out at 65°C overnight. Alkaline phosphatase–conjugated anti-digoxigenin Fab fragments (Roche) were used at 1:5000 dilution. Nitro blue tetrazolium/5-bromo-4-chloro-3'-indolyphosphate (Roche) was used for the coloration reaction. Transmitted light images were captured on a Leica MZ16F stereomicroscope (Leica Microsystems, Buffalo Grove, IL). For two-color in situ hybridization, commercially available TSA Kit (Invitrogen, Carlsbad, CA) was used to visualize digoxigenin-labeled riboprobe and fluorescein-labeled islet1 riboprobes. The two-color fluorescent in situ were collected on a custom-built two-photon microscope. The Ti:sapphire laser (Coherent, Santa Clara, CA) was tuned to 740 nm, and a Zeiss 20×/numerical aperture (NA) 0.5 water immersion objective was used. Image stacks were collected at 2-μm spacing.

**Plasmids, BACs, and DNA injections**

To drive transgene expression in individual CaP motoneurons, we used either of two Gal4-VP16 driver plasmids. The first was ~8 kb of genomic sequence upstream of the start codon of the neuropilin-1a gene, npr1a:Gal4-VP16. The second was mmx1-3×125bp:Gal4-VP16 (Zelenchuk and Bruses, 2011). These were injected into one-cell-stage embryos at a concentration of ~25 ng/μl. To express GFP, we coinjected 25 ng/μl 5xUAS:GFP with one of the Gal4 driver plasmids. For the synaptophysin-GFP experiments, we used a double-headed UAS plasmid to coexpress synaptophysin-GFP with Kusabira orange (pDual:Syp-GFP:KO).

For zebrafish pcdh18b constructs, PCR primers were used to amplify the pcdh18b coding sequence. The 5′ primer contained a EcoRI restriction site and a Kozak sequence, and the 3′ primer removed the stop codon and included codons for three glycine residues and a BamHI restriction site that placed pcdh18b in-frame with the N1 reading frame of Clontech vectors. This fragment was then subcloned into pEGFP-N1. The Pcdh18b-GFP fusion was sequenced and used as PCR template to generate both Pcdh18bΔICD-GFP and Pcdh18bICD-GFP.

To clone zebrafish nap1, PCR primers were designed against a nap1 sequence identified in a BLAST search. The 5′ primer contained a KpnI restriction site and a Kozak sequence, and the 3′ primer removed the stop codon and included codons for three glycine residues and an Agel restriction site that placed nap1 in-frame with the N1 reading frame of Clontech vectors. The nap1 fragment was then subcloned into a CMV:Myc-N1 vector.

The pcdh18b gene comprises four protein-encoding exons spread over ~8 kb. The entire pcdh18b gene is contained on the BAC clone, CH211-154p8. To facilitate broad expression, we used homologous recombination to insert the inverted Tol2 recognition arms (iTol2 cassette) into the BAC clone backbone. For the rescue experiments, 2 nl of 100 ng/μl pure BAC DNA was coinjected with 100 ng/μl mRNA encoding the Tol2 transposase.

**Imaging and analysis**

Labeled embryos were embedded in 1% agarose and imaged on a custom-built two-photon microscope. Excitation was provided by a Ti:sapphire laser tuned to 910 nm. For analysis of axonal arbors at 2 dpf, fluorescently labeled CaP motoneurons were imaged with a 20×/0.5 NA objective (pixel size, 0.4 μm). Image stacks of up to 30 optical sections were collected at 1.5-μm intervals. Individual sections were gamma adjusted (0.6), filtered (Gaussian blur, radius 0.7 pixel), and used to produce a maximum-intensity projection. Tracing and analysis of CaP axon arbors was performed in Fiji (Schindelin et al., 2012), using the Simple Neurite Tracer plug-in (Longair et al., 2011). Complete CaP axonal tracts were traced through three-dimensional image stacks. The sum of length of all the branches and total number of branch tips for each CaP axons were measured. Quantification of synaptic puncta was performed on unprocessed images using Fiji. A polygonal selection encompassing the branched region of the axon was made. The region within the polygonal selection was thresholded, and the number and size of puncta was determined using the Analyze particles command. The size of puncta was approximated as the area of...
Coimmunoprecipitation, Western blotting, and immunocytochemistry

Human embryonic kidney (HEK293) cells were maintained in DMEM supplemented with 10% fetal bovine serum and penicillin/streptomycin at 37°C with 5% CO₂. HEK293 cells were transfected with plasmids encoding Nap1-Myc and Pcdh18b-GFP, Pcdh18bΔICD-GFP, or Pcdh18bICD-GFP using calcium phosphate precipitation. After 24 h, cells were scraped into PBS, pelleted, lyzed on ice in cell lysis buffer (CLB; 20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM CaCl₂, 0.5% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, Complete Protease Inhibitor Cocktail [Roche]), and microcentrifuged at 4°C for 10 min. Supernatants were incubated with 2 mg of anti-GFP (Invitrogen) or anti-Myc (Sigma) primary antibody for 2 h at 4°C before the addition of protein A–Sepharose (GE Healthcare, Piscataway, NJ), and the coimmunoprecipitation was allowed to incubate overnight at 4°C. The beads were washed five times in CLB, resuspended in loading buffer, and boiled for 5 min. Samples were loaded onto 10% Bis-Tris NuPAGE gels (Invitrogen) and subjected to electrophoresis. Proteins were then transferred (Bio-Rad, Hercules, CA) to nitrocellulose (GE Healthcare), blocked with 5% nonfat milk in Tris-buffered saline/Tween 20, and incubated overnight with primary antibody (anti-GFP, 1:1000; anti-Myc, 1:5000). Horseradish peroxidase–conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA) were used at 1:5000, and the chemiluminescence signal was amplified using detected particles in pixels. Identical thresholds were used for all data sets.

For time-lapse analysis of filopodia dynamics, labeled CaP axons were imaged with a Zeiss 40×/0.8 NA objective (pixel size: 0.167 μm). Image stacks consisting of 13 optical sections at 1.5-μm spacing were collected at 1-min intervals for 1 h. The highest-quality time-lapse sequences were selected for filopodia analysis. Fiji was used to measure the lengths of filopodia in each frame of each time-lapse sequence. The resulting values were exported into Matlab, and quantitative data were extracted using custom scripts. Lengths were defined as the distance from the base of the filopodium to its tip in maximum-intensity projections. The rate of extension/retraction for a given filopodium was defined as the change in length between successive frames. Filopodia extension/retraction rates for a given motor neuron were calculated as the mean rate of extension or retraction for all filopodia observed on that neuron; these rates were averaged for four axons present in four separate embryos for both control and morphant embryos. The lifetime of an individual filopodium was defined as length of time for which its length was measurable. A filopodium extending from a position at which one had previously disappeared was counted as a second filopodium. The average filopodium lifetime was calculated for each axon, and these numbers were averaged for four axons from four control or morphant embryos. Filopodia density was calculated as the number of filopodia per unit length measured from the first time point from each of eight time-lapse sequences for control and morphant embryos. Statistical significance was determined in IGOR Pro (Wavemetrics, Lake Oswego, OR) using the Wilcoxon signed-rank test. Differences were deemed significant for p < 0.05.
Transplantation

To aid in visualization, we used Tg(hb9-hsp70:GFP) embryos with GFP-labeled motor neurons as donors and ABLF embryos as host. To ensure correct positioning of the labeled ectopic motor neurons into host embryos, we carried out gastrula-stage transplantation at 50% epiboly with the help of previously characterized fate map (Kimmel et al., 1990). The procedure was followed according to a published protocol (Deschene and Barresi, 2009).

Morpholino and DNA injections

Antisense morpholino oligonucleotides were purchased from GeneTools. The morpholinos were dissolved in dH2O at ~8 ng/µl, aliquoted, and stored at −80°C. For use, morpholinos were diluted to a working concentration of 2–8 ng/µl in dH2O, and 1–2 µl was...
injected into one-cell-stage embryos. The morpholinos used in this study are as follows:

- **pcdh18b-MOT:** 5'-AGTGTGCTCATATTTGGAAGCTGTG-3'
- **pcdh18b-MOS1:** 5'-AAGCTTGTGGTCTACCTCGACTG-3'
- **pcdh18b-MOS1mis:** 5'-AAGGATCTTGTTAGCTCAACTG-3'
- **nap1-MOT:** 5'-CCGAGACATGGCTCCAAACGACCGTC-3'
- **nap1-MOT-mis:** 5'-CCGAGACATGCTCCAAACGACCGTC-3'
- **nap1-MOS1:** 5'-GGTCAAAATTTACAGTCTGCATTGT-3'
- **nap1-MOS2:** 5'-AAgGTcTTGTCTTAgCTCcACTcGT-3'

The following amounts were used in this study: **pcdh18b-MOT,** 8 ng; **pcdh18b-MOS1,** 10 ng; **pcdh18b-MOS2,** 4 ng; **nap1-MOS1,** 8 ng; **nap1-MOT,** 8 ng. Control morpholinos were injected at 8 ng.

For rescue experiments, we used the BAC clone CH211-154p8, which harbors the complete **pcdh18b** gene. In addition, we inserted an iTol2 cassette to facilitate genome insertion and expression (Suster et al., 2011). Purified BAC DNA was used at 100 ng/μl and coinjected with 100 pg of mRNA encoding Tol2 transposase.

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