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A novel role for MuSK and non-canonical Wnt signaling during segmental neural crest cell migration

Santanu Banerjee[1], Laura Gordon[1], Thomas M. Donn[2], Caterina Berti[3], Cecilia B. Moens[2], Steven J. Burden[3] and Michael Granato[1,*]

SUMMARY

Trunk neural crest cells delaminate from the dorsal neural tube as an uninterrupted sheet; however, they convert into segmentally organized streams before migrating through the somitic territory. These neural crest cell streams join the segmental trajectories of pathfinding spinal motor axons, suggesting that interactions between these two cell types might be important for neural crest cell migration. Here, we show that in the zebrafish embryo migration of both neural crest cells and motor axons is temporally synchronized and spatially restricted to the center of the somite, but that motor axons are dispensable for segmental neural crest cell migration. Instead, we find that muscle-specific receptor kinase (MuSK) and its putative ligand Wnt11r are crucial for restricting neural crest cell migration to the center of each somite. Moreover, we find that blocking planar cell polarity (PCP) signaling in somitic muscle cells also results in non-segmental neural crest cell migration. Using an F-actin biosensor we show that in the absence of MuSK neural crest cells fail to retract non-productive leading edges, resulting in non-segmental migration. Finally, we show that MuSK knockout mice display similar neural crest cell migration defects, suggesting a novel, evolutionarily conserved role for MuSK in neural crest migration. We propose that a Wnt11r-MuSK dependent, PCP-like pathway restricts neural crest cells to their segmental path.

KEY WORDS: Zebrafish, Mouse, Trunk neural crest, Motoneuron, Planar cell polarity, Segmental cell migration, Muscle specific kinase, MuSK, wnt11r, Dishevelled, *unplugged*

INTRODUCTION

In the vertebrate embryonic trunk, neural crest cells delaminate along the entire length of the dorsal neural tube and then enter distinct migratory routes (Krull, 2010). In mammals, neural crest cells choose one of three distinct routes dependent on their time of delamination (Erickson and Weston, 1983). Neural crest cells of the 'early wave' migrate in the intersomitic space, along intersomitic blood vessels (Loring and Erickson, 1987), whereas those participating in the 'late wave' migrate along a dorsolateral trajectory between the dermomytome and the epidermis (Bronner-Fraser, 1986; Rickmann et al., 1985; Serbedzija et al., 1990). Neural crest cells of the 'intermediate wave' enter a narrow ventromedial route between the neural tube and somite. This ventromedial route is restricted to the anterior portion of each sclerotome, resulting in a segmentally restricted path (Bronner-Fraser, 1986; Rickmann et al., 1985; Serbedzija et al., 1990). This metameric organization of the migratory path is reflected in the localization of intermediate wave neural crest derivatives, such as dorsal root ganglia (DRGs), Schwann cells and sympathetic ganglia (Kasemeier-Kulesa et al., 2005; Kuan et al., 2004; Serbedzija et al., 1990; Teillet et al., 1987).

It is well known that signals from the somites control segmentally restricted neural crest cell migration (Bronner-Fraser and Stern, 1991; Kalcheim and Teillet, 1989). Several signaling systems, including the Ephrins and their Eph receptors (Krull et al., 1997; McLennan and Krull, 2002; Santiago and Erickson, 2002), F-spondin (Debby-Brafman et al., 1999), and extracellular matrix components such as chondriotin sulfate proteoglycans (Kubota et al., 1999) have been proposed to act as repulsive or inhibitory forces that signal from the posterior somite, thereby restricting neural crest cell migration to the anterior somite (Krull, 2010). Although the role of Eph signaling during segmental neural crest cell migration remains unclear (Adams et al., 2001; Davy et al., 2004; Wang et al., 1998), recent studies demonstrate multiple functional roles for Neuropilin (Nrp)/Semaphorin (Sema) signaling in this process. Nrp1/Sema3A signaling repels neural crest cells from the intersomitic space to the sclerotome, and Nrp2/Sema3F signaling functions to restrict neural crest cell migration to the anterior half of the sclerotome (Gammill et al., 2006b; Schwarz et al., 2009a; Schwarz et al., 2009b). Finally, combined loss of Nrp1 and Nrp2 signaling results in perturbation of DRG segmentation, whereas metameric organization of sympathetic ganglia remains unaffected (Roffers-Agarwal and Gammill, 2009). Combined, these studies have established a crucial role for Nrp/Sema signaling in segmental neural crest migration. Although Nrp2/Sema3F signaling is required for the initiation of segmental migration at the dorsal level of somite, its role in maintaining segmental migration is less clear. In embryos lacking Nrp2, neural crest cells migrate initially in an unsegmented manner but presumably reorganize and migrate in a segmental fashion at ventral somite levels where sympathetic ganglia form (Gammill et al., 2006b). Moreover, in Nrp1/Nrp2 double mutants, metameric organization of sympathetic ganglia still remains intact (Roffers-Agarwal and Gammill, 2009), suggesting that additional factors, and possibly additional signaling pathways, play a crucial role in keeping neural crest cells restricted to their segmental path. Finally, neural crest cells migrate in close proximity to spinal motor axons, and Nrp1 and Nrp2 are also

[1]Department of Cell and Developmental Biology, University of Pennsylvania School of Medicine, Philadelphia, PA 19104-6058, USA. [2]Division of Basic Science, Fred Hutchinson Cancer Research Center Seattle, WA 98109, USA. [3]Molecular Neurobiology Program, The Helen L. and Martin S. Kimmel Center for Biology and Medicine at the Skirball Institute of Biomolecular Medicine, New York University Medical School, 540 First Avenue, New York, NY 10016, USA.

*Author for correspondence (granatom@mail.med.upenn.edu)

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required for patterned spinal motor axon outgrowth (Roffers-Agarwal and Gammill, 2009), consistent with a potential role for motor axons in neural crest cell migration.

We have recently shown that in zebrafish the secreted glycoprotein Wnt11r binds muscle-specific kinase (MuSK) to initiate a Dishevelled-dependent signaling cascade that restricts the localization of acetylcholine receptors (AChRs) and the migration of growth cones to the center of adaxial muscle cells (Jing et al., 2009). Genetic ablation of adaxial muscle cells causes loss of segmental neural crest cell migration (Honjo and Eisen, 2005); therefore, we investigated whether the Wnt11r/MuSK/Dishevelled signaling module might also have a role in segmental neural crest cell migration. Here, we provide compelling genetic evidence that Wnt acts through the muscle-specific kinase MuSK and its downstream effector Dishevelled to maintain segmental neural crest cell migration. We find that in zebrafish *musk* (previously known as *unplugged*) mutants and in *wnt11r* mutants, neural crest cells are no longer restricted to their segmental path, and instead invade the entire somite territory. We show that these defects occur independently of motor axons and that perturbing Dishevelled function in adaxial muscle cells recapitulates the neural crest migration phenotype. Moreover, we show that MuSK modulates F-actin-based filopodia retraction in neural crest cells and that MuSK knockout mice display similar neural crest cell migration defects.

MATERIALS AND METHODS

Ethics statement

All experiments were conducted according to an animal protocol (protocol number 459800) fully approved by the University of Pennsylvania Institutional Animal Care and Use Committee (IACUC) on 15 February 2008. Veterinary care is under the supervision of the University Laboratory Animal Resources (ULAR) of the University of Pennsylvania.

Zebrafish genetics

All embryos used in this study were raised at 28°C for the required amount of time (see Mullins et al., 1994). Wild-type fish used for experiments were TLF, and mutants used were *sidetracked*/plexinA3^{p55emcf} (Palaisa and Granato, 2007), *unplugged*/MuSK^{tbr307} (Jing et al., 2009; Zhang and Granato, 2000; Zhang et al., 2004), *wnt11r*$^{fh224(G94*)}$. Tg*(mnx1:GFP)ml2* Tg*(mnx1:mCD8-GFP)p150*, Tg*(Sox10:mRFP)vu234* (Kirby et al., 2006), Tg*(mitfa:GFP)w47* (Curran et al., 2009) transgenic fish were used alone, in combination with each other or in combination with various mutant backgrounds.

Molecular biology

The mCherry-UtrCH in pCS2+ construct was a kind gift of Dr Mary Halloran (Andersen et al., 2010; Burkel et al., 2007) and was used to create I*Sce*I-*sox10(–4.9kb):*mCherry-UtrCH. pME-LifeAct-GFP was kindly provided by Darren Gilmour [The European Molecular Biology Laboratory (EMBL), Heidelberg, Germany] and was cloned under the control of the *sox10*(–4.9kb) promoter using standard Gateway (L-R) reactions.

Immunohistochemistry

Antibody staining was performed as described previously (Zeller et al., 2002). The following primary antibodies were used: znp-1 (1:200) (Trevarrow et al., 1990); Developmental Studies Hybridoma Bank, University of Iowa, IA, USA; SV2 (1:50, Developmental Studies Hybridoma Bank, University of Iowa, USA), myc (9E10, 1:1000, Covance), GFP (JL-8, 1:200, Clontech). Antibodies were visualized with Alexa-Fluor-594 conjugated secondary antibodies (1:500; Molecular Probes, Eugene, OR, USA). In situ hybridization with *crestin* probe (Luo et al., 2001) was performed as previously described (Schneider and Granato, 2006).

Live cell imaging

Embryos (16- to 20-somite stage) were briefly anesthetized using tricaine and then mounted laterally in 1% low melting agarose prepared in Ringer's solution containing tricaine. Images were captured over 1-10 minutes using a 63× water immersion lens in a spinning disc confocal microscope (Olympus) equipped with a 28°C temperature-controlled chamber. Appropriate numbers of *z* sections were used to create maximum intensity projection images using Slidebook (3i) or NIH ImageJ. Images were further processed using ImageJ and/or Photoshop.

Motor neuron ablation

Embryos were mounted as described for live imaging. Motor neuron cell bodies expressing green fluorescent protein (GFP) were ablated using a MicroPoint nitrogen pulsed laser (Photonic Instruments) mounted on a spinning disc microscope with a 63× water immersion objective lens. Ablations were carried out in up to four hemisegments per embryo. Ablations were verified after 30 minutes and after 3 hours. Segments with incomplete/partial ablation of motor neurons were not included in the analysis. Following ablation of motor neurons, migration of neural crest cells were analyzed either by live imaging for 3-5 hours or by fixing the embryos 3-5 hours post-ablation. Fixed embryos were subsequently analyzed by in situ hybridization and immunohistochemistry as described above.

Quantification of neural crest cell migration defect

Using ImageJ software, the widest extent of *crestin*-positive neural crest cells along the segmental path was measured and divided by the width of the segment to calculate the ratio of neural crest cell width with respect to segment width. A minimum of five motor neuron-ablated segments were scored for each genotype, averaged and plotted. Statistical significance ($P \leq 0.05$) was determined using *t*-test.

Quantification of stability of neural crest cell protrusions

For each migrating neural crest cell, the longest horizontal axis (along the anterior-posterior axis of the embryo) and vertical axis (along the dorsoventral axis of the embryo) were measured at each time point using Slidebook software. Each longest axis included at least one actin rich filopodial ending at one end of the cell. Ratios of horizontal axis/vertical axis were calculated and plotted against time. A ratio value greater than one corresponds with an elongated cell along the anterior-posterior axis and a ratio value less than one corresponds with a cell with an extended shape along the dorsoventral axis. Measurements were carried out over a 60-minute time window when cells approach the horizontal myoseptum area.

Mouse whole-mount fluorescent immunohistochemistry

Embryonic day (E) 9.5 and E10.5 embryos were dissected in ice cold PBS, fixed overnight at 4°C in 4% paraformaldehyde in PBS and incubated overnight with p75 antibody (a kind gift from M. Chao, Skirball Institute, NY, USA) diluted in PBS containing 1% Triton X-100, 10% fetal calf serum and 0.2% sodium azide, washed for several hours, incubated overnight with fluorochorome-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories), post-fixed in 4% paraformaldehyde for 20 minutes, mounted in 50% glycerol with Vectashield and viewed with a Zeiss 510 confocal microscope.

RESULTS

Migration of motor axons and neural crest cells is synchronized

In zebrafish, only two waves of trunk neural crest cell migration have been reported. During the second wave, neural crest cells enter a ventrolateral pathway between the somites and the epidermis whereas during the first wave, neural crest cells migrate along a ventromedial route between the neural tube and the somites (Raible et al., 1992). This migration route probably corresponds to the murine 'intermediate wave' and, like in the mouse, neural crest cells entering this route in the zebrafish coalesce from a broad region of the neural tube into a narrow, restricted path located in the center of the somite (Fig. 1A,B). As in mammals, pioneering motor growth cones share part of their trajectory through the somites with migrating neural crest cells (Pike et al., 1992; Rickmann et al., 1985). During all stages of their migration through

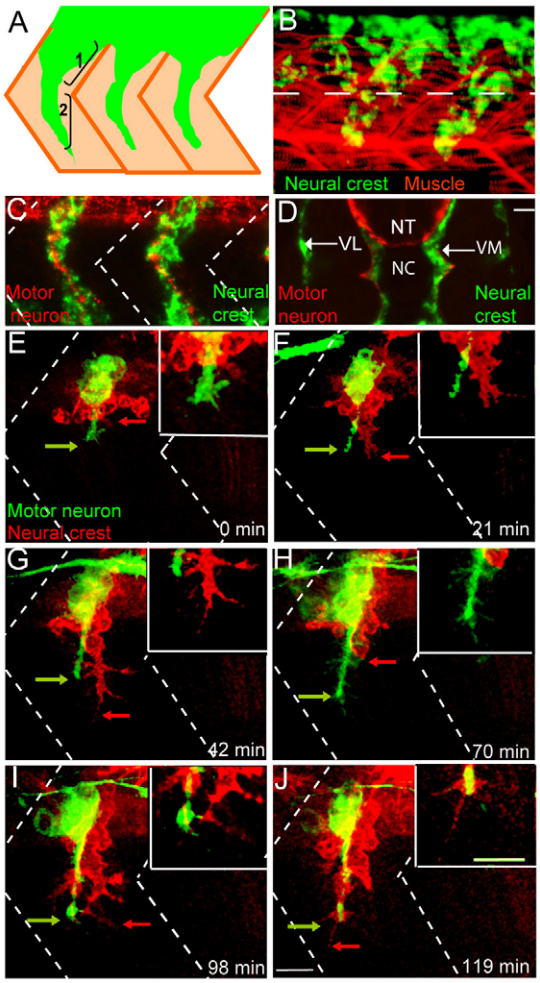

Fig. 1. Wild-type neural crest and motor axon migration. (**A**) Schematic showing two stages of zebrafish neural crest cell migration: (1) initiation and (2) maintenance of segmental migration. Neural crest cells (green) migrate through a central region of the somites (tan). (**B**) Lateral view of a 22-hour-old embryo stained with a *crestin* riboprobe (green) marking neural crest cells and with F59 antibody marking adaxial muscle cells. Dashed lines mark the ventral boundary of the neural tube. (**C**) Lateral view of a 28-hour-old zebrafish embryo revealing neural crest cells in green (*crestin* riboprobe) and motor axons in red (znp-1/SV2 antibody cocktail). Dashed lines indicate approximate locations of somite boundaries. (**D**) Cross-sectional view of a 28 hpf embryo stained for neural crest cells (*crestin*) in green and motor axons (znp-1/SV2) in red. NT, neural tube; NC, notochord; VL, ventrolateral (or lateral) pathway; VM, ventromedial (or medial) pathway. Scale bar: 10 μm. (**E-J**) Still images from a time-lapse movie showing early co-migration of neural crest cells (red) and motor axons (green). Arrows point to filopodial extensions. Dashed lines indicate approximate locations of somite boundaries. Scale bar: 10 μm. See also Movie 1 in the supplementary material.

the ventromedial somites, neural crest cells (*crestin* riboprobe, green) and motor axons (SV2 and znp-1 antibody cocktail, red) are in close proximity (Fig. 1C,D) (Eisen and Weston, 1993).

To investigate their spatiotemporal relationship in greater detail, we used live-cell imaging to follow membrane dynamics of neural crest cells using the neural crest cell-specific transgene Tg*(sox10:mRFP)vu234* (Kirby et al., 2006), and of motor axons using a membrane-tagged GFP, Tg*(mnx1:mCD8-GFP)p150*. In all the experiments (n=10) we found that motor growth cones entered the shared path first whereas neural crest cells slightly lagged behind growth cones (Fig. 1E and see Movie 1 in the supplementary material). However, once neural crest cells entered the shared path, their filopodial processes caught up with and extended as far as the leading growth cone protrusions (Fig. 1F). As they progressed further ventrally, neural crest cell filopodia sometimes extended ahead of growth cone filopodia (Fig. 1G), but retracted shortly thereafter (Fig. 1H). As they approached the end of their shared path, growth cone and neural crest cell migration appeared to be synchronized, with growth cone filopodia frequently extending slightly ahead of neural crest cell filopodia (Fig. 1I,J). Thus, motor axons enter the shared segmental path slightly ahead of neural crest cells, and migrate in close spatial and temporal proximity with neural crest cells, raising the possibility that motor axons might be important for neural crest cells to enter or migrate along their segmentally restricted path.

Motor neurons are dispensable for segmental neural crest cell migration

Given that their migrations are highly synchronized, we next asked whether misguided motor axons have the ability to direct neural crest cells, and whether they are required for neural crest cell migration. To determine whether motor axons have the ability to direct neural crest cell migration, we examined *plexin A3* (previously known as *sidetracked*) mutants. In zebrafish, the Plexin A3 guidance receptor is required for spinal motor axons to exit from the spinal cord through a mid segmental nerve root (Palaisa and Granato, 2007; Tanaka et al., 2007). Plexin A3 is only expressed in motoneurons, and in the absence of Plexin A3, a subset of motor axons exited through ectopic locations from the spinal cord, resulting in somitic hemisegments with multiple motor axons (Fig. 2A,B, arrows). Analysis of neural crest cell migration in *plexin A3* mutants revealed that neural crest cells follow ectopically exiting motor axons, suggesting that motor axons can influence neural crest cell migration (Fig. 2B, 82%, n=48 hemisegments).

Several studies have examined the role of motor axons in neural crest cell migration. Although surgical excision studies show that neural crest cells are dispensable for motor axon migration through the somites (Rickmann et al., 1985), the role of motor axons during neural crest migration is less clear. Removal of the neural tube prevents neural crest cell-derived Schwann cells from migrating into the limb (Noakes et al., 1988), yet other studies in which only the ventral spinal cord was removed have concluded that motoneurons and ventral roots are dispensable for initial neural crest cell migration (Bhattacharyya et al., 1994). However, neither of these studies selectively ablated motoneurons, nor did these studies specifically examine the segmental migration of neural crest cells. We, therefore, laser ablated identified motoneurons to examine segmental neural crest cell migration. We used Tg*(mnx1:GFP)ml2* [formerly known as Tg(*Hb9:GFP*)] (Flanagan-Steet et al., 2005) transgenic embryos to identify the cell bodies of pioneering motor axons (Fig. 2C, red asterisks). Using a pulsed nitrogen laser, we ablated pioneering motor neurons at 19 hours post-fertilization (hpf), as their axons exited from the spinal cord but before neural crest cells entered their shared path (Fig. 2C). Immediately following laser surgery, we observed swelling of the targeted cell bodies and shrinkage of their axons, suggesting that targeted motor neurons were undergoing cell death. This was further confirmed thirty minutes later by the absence of detectable GFP signal in the targeted neurons (data not shown). The cell

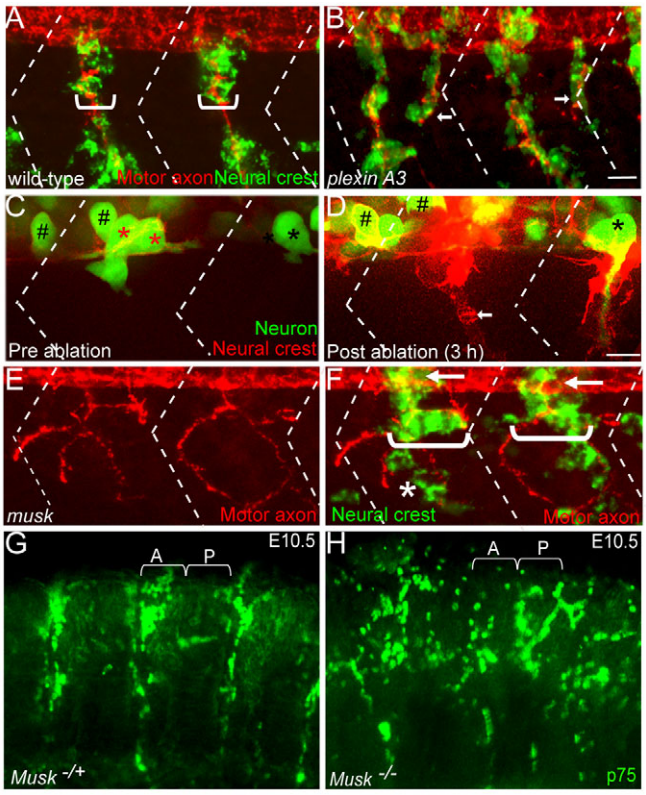

Fig. 2. The role of motor axons and MuSK signaling during segmental neural crest cell migration. (**A**,**B**) Lateral views of 28 hpf wild-type (A) and *plexin A3* mutant (B) zebrafish embryos with neural crest cells in green (*crestin*) and motor axons in red (znp-1/SV2). Brackets indicate width of neural crest cell stream. (**C**,**D**) Lateral views of a double Tg*(mnx1:GFP)ml2; (sox10:mRFP)vu234* embryo expressing GFP (green) in motor neurons (asterisks) and in interneurons (hash marks), and mRFP (red) in neural crest cells before laser ablation (C) and three hours following motor neuron ablation (D; arrow points to ventral migration of neural crest cells). Red asterisks in C indicate ablated motor neurons. (**E**,**F**) Lateral views of a 28 hpf *musk* mutant embryo stained to reveal neural crest cells (*crestin*, green) and motor axons (znp1+SV2, red). Arrows point to migrating neural crest cells before they join the path shared with motor axons. In the *musk* mutant embryo, neural crest cells and motor axons deviate from their central path and migrate over a wider region of the somite (compare brackets in F with A; asterisk marks neural crest cells taking a path independent of motor axons). Dashed lines in A-F indicate approximate positions of somite boundaries. Scale bars: 10 μm. (**G**,**H**) Lateral views of whole-mount E10.5 mouse embryos stained with p75 antibody labeling neural crest cells. Neural crest cells migrate through the anterior sclerotome in the wild-type sibling embryo (A), but migrate non-segmentally in the *Musk* knockout embryo. A, anterior somite; P, posterior somites.

bodies and axons of Tg*(mnx1:GFP)ml2*-positive interneurons, located adjacent to the ablated motoneurons, were present before and three hours after laser surgery, demonstrating the cell-type specificity and precision of the laser ablation (Fig. 2C,D). In most segments in which we ablated motor neurons and their axons, neural crest cells retained a wild-type-like, segmentally restricted path through the center of the somite (Fig. 2D, *n*=14/20). Taken together, this demonstrates that although motor axons can influence neural crest cell migration, they are dispensable for segmental neural crest cell migration.

The role of muscle-derived signals in segmental neural crest cell migration

Given that motor neurons are dispensable for segmentally restricted neural crest cell migration, we began to consider other cell types that might provide guidance information. One attractive candidate cell type, a subpopulation of somitic muscle known as adaxial cells, or slow-twitching muscle cells, has been shown to have a role in segmental neural crest cell migration (Honjo and Eisen, 2005). Removal of adaxial cells, either via surgery or via mutations affecting Sonic hedgehog signaling, affects segmental neural crest cell migration. In mutant or in surgically manipulated embryos, the streams of migrating neural crest cells are no longer restricted to the center of the somite, which suggests that adaxial muscle provides important signals for neural crest patterning (Honjo and Eisen, 2005). During embryogenesis, MuSK is exclusively expressed in somitic muscle, where it is required (Jing et al., 2009; Zhang et al., 2004). We have recently shown that both MuSK and the Wnt pathway effector Dishevelled (Dsh) function in adaxial muscles to restrict motor growth cones to the center of the somite (Jing et al., 2009).

We therefore investigated whether MuSK also has a role in segmental migration of neural crest cell. As previously reported, *musk* null mutant embryos exhibited excessive motor axon branching and strayed away from the center of the somite (Fig. 2E,F; 60% of hemisegment, *n*=80). Before they joined the path they share with motor axons, neural crest cells were restricted to the center of the somite, but once they entered the shared path, they streamed over a laterally expanded somite territory (Fig. 2F; *n*=53/80 hemisegments). Importantly, patterning of somitic muscle and specification of muscle pioneers are unaffected in *musk* mutants (Zhang et al., 2004). Thus, MuSK-dependent signals from adaxial muscle are crucial for segmental neural crest cell migration.

Musk knockout mice show neural crest migration defects

To our knowledge, this is the first evidence that MuSK is required for neural crest cell migration. To determine whether this is a teleost-specific function of MuSK or whether MuSK has an evolutionarily conserved role in trunk neural crest migration, we examined their migration in $Musk^{-/-}$ knockout mice. In E10.5 wild-type embryos, p75 staining revealed that neural crest cell migration was restricted to the anterior compartment of each somite (Fig. 2G, *n*=5). By contrast, in $Musk^{-/-}$ embryos, p75-positive neural crest cells were no longer restricted to the anterior somite and instead spread throughout the entire somite (Fig. 2H, *n*=5). Thus, zebrafish and mice lacking MuSK display identical neural crest cell migration phenotypes, strongly suggesting that MuSK plays an evolutionarily conserved role in restricting neural crest cell migration to a specific region of the somite. Given the ability of motor axons to direct neural crest cell migration, a key question is whether the migration defects observed in zebrafish and mice lacking MuSK are due to the axonal pathfinding defects observed in such mutants. In the subsequent experiments, we demonstrate that MuSK plays a crucial role in maintaining segmental neural crest cell migration, independently of motor axons (see below).

The role of Wnt/PCP signaling in segmental neural crest cell migration

Next, we asked whether neural crest cell migration is also dependent on the secreted glycoprotein Wnt11r, which we recently identified as a putative MuSK ligand (Jing et al., 2009). Knockdown of *wnt11r* recapitulates all aspects of the *musk* phenotype; in vitro, Wnt11r

protein binds to MuSK via the Frizzled cysteine-rich domain (CRD); and in vivo, Wnt11r protein binding to muscle cells depends on MuSK function (Jing et al., 2009). Using a TILLING approach (Moens et al., 2008), we identified a zebrafish *wnt11r* mutation that results in a premature stop codon (G94*). This eliminates the entire C-terminus, which has been shown to be necessary for Wnt activity (Du et al., 1995), and is likely to result in a *wnt11r*-null allele. As expected from our previous analysis of *wnt11r* morphant embryos, *wnt11r* mutant embryos displayed motor axon defects identical to those observed in *musk* mutants, including stalling and branching around the horizontal myoseptum (Fig. 3C; 25% of hemisegments, *n*=335 hemisegments). In these mutants, we found that a significant fraction of neural crest streams deviated from the central path and invaded more lateral somite territories (Fig. 3D, bracket; 20% of hemisegments, *n*=120). Interestingly, misguided neural crest streams frequently chose a trajectory separate from that of misguided motor axons (Fig. 3D, asterisk, 10% of the hemisegments), consistent with the idea that the migration defects of neural crest cells and motor axons are due to a common, primary defect.

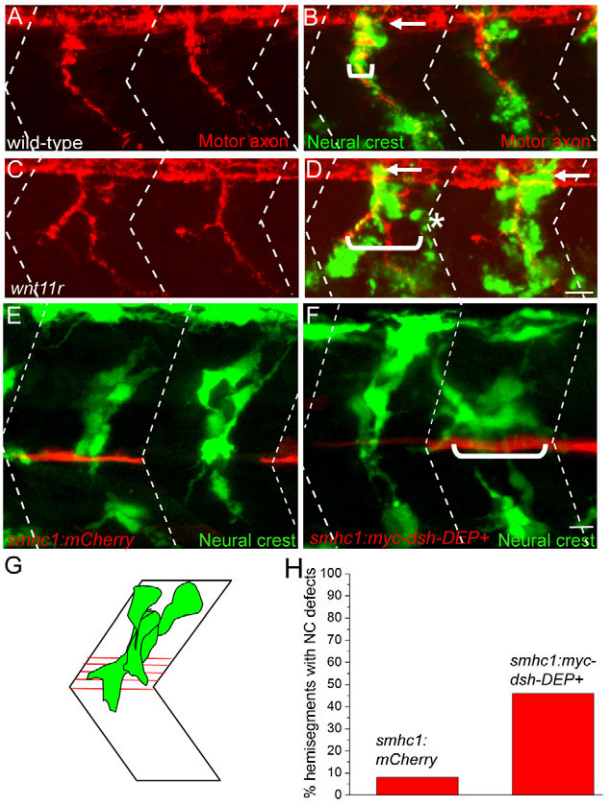

Fig. 3. Inhibition of non-canonical Wnt and Dishevelled signaling disrupts neural crest migration. (**A-D**) Lateral views of 28 hpf wild-type and *wnt11r* zebrafish embryos stained to reveal neural crest cells (*crestin*, green) and motor axons (znp1+SV2, red). Arrows point to migrating neural crest cells before they join the path shared with motor axons. In the *wnt11r* mutant embryo, neural crest cells and motor axons deviate from their central path and migrate over a wider region of the somite (compare brackets in D with B; asterisks mark neural crest cells taking a path independent of motor axons). (**E**) Stochastic expression of mCherry (red) in slow muscle fibers does not affect neural crest cell (green) migration through the center of the somite. (**F**) Stochastic expression of Myc-Dsh-DEP+ (red) in slow muscle cells located either at horizontal myoseptum or dorsal to the horizontal myoseptum causes aberrant neural crest cell migration. Neural crest cells in E and F were visualized using Tg*(mitfa:GFP)w47*. (**G**) Location of horizontal myoseptum cells used for quantification of the phenotype. (**H**) Quantification of neural crest migration defect phenotypes. Dashed lines in A-F indicate approximate positions of somite boundaries. Scale bars: 10 μm.

Finally, we investigated whether the MuSK effector Dishevelled (Dsh; Dvl – Zebrafish Information Network) is also required for segmental neural crest cell migration. Vertebrate MuSK interacts with Dishevelled (Jing et al., 2009; Luo et al., 2002), and we have recently shown that non-canonical Dishevelled signaling in adaxial muscle cells is critical to restrict motor growth cones to the center of the somite (Jing et al., 2009). To determine whether non-canonical Dishevelled signaling plays a similar role during neural crest cell migration, we expressed Myc-Dsh-DEP+, which specifically blocks non-canonical Wnt/PCP signaling in flies, fish and frogs (Axelrod et al., 1998; Heisenberg et al., 2000; Wallingford et al., 2000), under the control of an adaxial muscle-specific promotor (Elworthy et al., 2003). Analysis of transient transgenic zebrafish embryos expressing Myc-Dsh-DEP+ in a small subset of adaxial muscle cells recapitulated in ~46% of hemisegments examined the neural crest migration phenotype observed in *musk* and *wnt11r* mutant embryos (Fig. 3F,H).

We noticed that in embryos expressing Myc-Dsh-DEP+, the presence of individual Myc-Dsh-DEP+ adaxial muscle cells located at the horizontal myoseptum or immediately dorsal to the horizontal myoseptum resulted in neural crest cell migration defects (Fig. 3F,H; *n*=55). Interestingly, the location of this subset of adaxial muscle fibers is precisely where neural crest cells transition from an unsegmented to a segmented mode of migration. Taken together, our data demonstrate that *wnt11r-musk* and non-canonical *dishevelled* signaling functions to restrict neural crest cell migration to the somite center.

Wnt11r and MuSK signaling have a direct role in neural crest cell migration

The observation that misguided motor axons can direct neural crest cells away from their central path (Fig. 2F) prompted us to determine the requirement for Wnt11r and MuSK for neural crest cell migration in the absence of motor axons. For this, we ablated pioneering motor neurons in defined hemisegments of *wnt11r* and *musk* mutant zebrafish embryos at the time at which motor neurons start to extend their axonal processes. Like in wild-type embryos, neural crest cell migration in *musk* wild-type siblings was unaffected in the absence motor neurons (Fig. 4A-H). Just as in non-ablated *wnt11r* and *musk* mutants, the absence of motor neurons in *wnt11r* as well as in *musk* mutants resulted in neural crest cells straying away from their central path and invading lateral portions of the somites. To quantify this phenotype, we measured the width of the somitic segment covered by neural crest cells, and divided it by the total width of the somitic segment (Fig. 4I). In motor neuron-ablated wild-type siblings this ratio was ~0.25, whereas in motor neuron-ablated *wnt11r* or *musk* mutants this ratio was almost twofold (*musk*) or greater than twofold (*wnt11r*) higher (Fig. 4J). These results demonstrate that Wnt11r and MuSK function are crucial for confining neural crest cells to their segmental path, independently of motor axons.

MuSK functions to maintain segmental neural crest migration

We next investigated whether MuSK functions to organize neural crest cell migration into segmental streams as they enter the segmental path, or whether MuSK functions to maintain segmental neural crest cell migration once they have entered the segmental

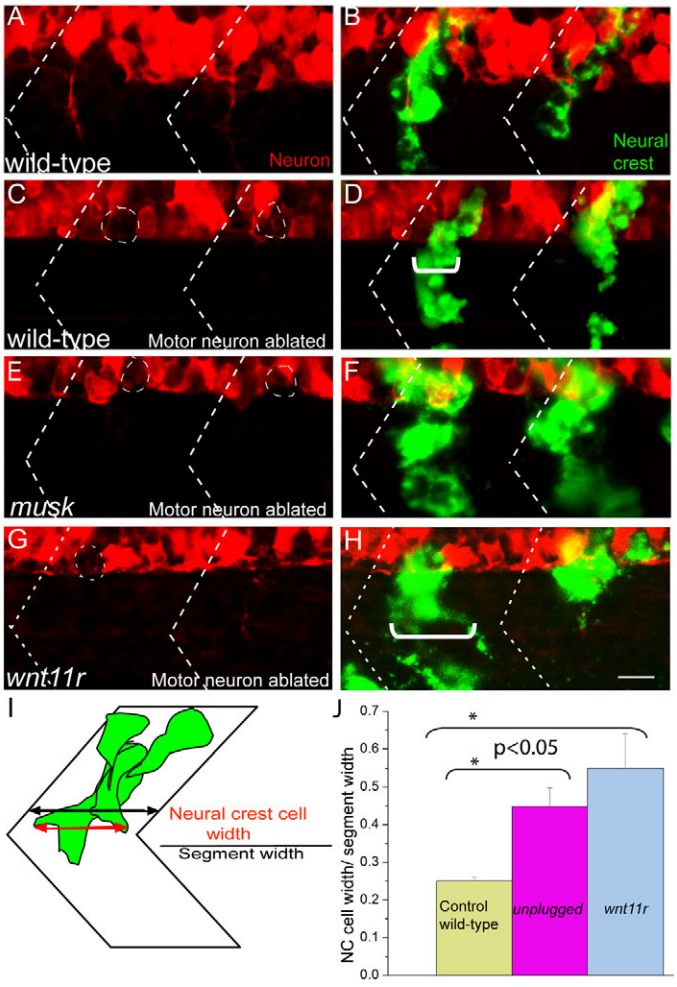

Fig. 4. MuSK and Wnt11r signaling influences neural crest cell migration independent of motor axons. (**A-D**) In wild-type zebrafish embryos in which motoneurons were ablated (C,D), segmental neural crest cell migration is indistinguishable from that of wild-type embryos with intact motoneurons (A,B). (**E-H**) Neural crest cells migrate through a wider region of the somite (marked with white bracket) in motoneuron ablated *musk* (F) and *wnt11r* (H) mutant embryos, compared with the motoneuron-ablated wild-type control (C). Dashed circles in C, E and G indicate approximate positions of ablated motoneuron cell bodies. Dashed lines in A-H indicate approximate positions of somite boundaries. (**I**,**J**) Quantification of the neural crest cell migration phenotype in wild type, *musk* and *wnt11r* mutants was carried out by calculating the ratio of neural crest cell width (red arrow) with respect to segment width (black arrow; for details see Materials and methods). A minimum of five segments in which motoneurons were ablated were scored for each genotype, averaged and plotted with s.e.m. Scale bar: 10 μm.

path. End-point analysis shows that in *musk* mutants neural crest cells initially migrate within a narrow stream, but subsequently spread over a wider somite territory, consistent with the idea that MuSK functions to maintain segmental neural crest migration (Fig. 2F). To determine at which point along the migration path MuSK activity is required to maintain segmental neural crest migration, we used live cell imaging of neural crest cells in *musk* sibling and mutant embryos. As shown in Fig. 5A and Movie 2 in the supplementary material, neural crest cells [Tg*(sox10:mRFP)vu234*, red] in *musk* wild-type sibling embryos entered the path they share with motor axon growth cones [Tg*(mnx1:GFP)ml2*, green], and migrated as a single stream in close proximity to motor axons (*n*=17 hemisegments, eight embryos). In *musk* mutant embryos, neural crest cells appropriately entered the common path (*n*=8 hemisegments, five embryos; Fig. 5B and Movie 3 in the supplementary material), but shortly thereafter (~40 minutes later), they started to stray away from the segmental path and frequently separated into two independent streams. Frequently, one stream stayed close to the motor growth cones and eventually spread over a wider territory (Fig. 5B, bracket). A second stream, without a motor axon nearby, invaded the lateral somite and eventually crossed into the neighboring somite territory (*n*=4/8 hemisegments, five embryos), a behavior never observed in wild-type embryos (Fig. 5B, arrows). Thus, live cell imaging reveals that MuSK in adaxial muscle functions non-cell-autonomously to maintain neural crest cell migration within a restricted region in the center of the somite.

One prediction from the analysis above is that if MuSK-dependent signals restrict neural crest cells to a narrow path at the center of the somite, then the absence of MuSK should lead to morphological changes at the leading edge of migrating neural crest cells. One dynamic structure at the leading edge of neural crest cells is the actin-rich filopodial protrusions (Berndt et al., 2008). To monitor cellular protrusions in neural crest cells in live embryos, we used the neural crest-specific *sox10* promoter (Wada et al., 2005) to express two F-actin biosensors in individual neural crest cells (Fig. 5C,D). The first biosensor, Lifeact-GFP, labels all F-actin (Riedl et al., 2008), and the second, mCherry-Utrophin (mCherry-UtrCH), labels stable F-actin (Burkel et al., 2007). In the early phase of their migration, when they converge into segmental streams, neural crest cells frequently project actin-rich filopodial protrusions laterally towards the somite boundaries. Consequently, neural crest cells often appeared extended perpendicular to the direction of migration (see Movie 4 in the supplementary material and Fig. 5C, quantified in Fig. S1 in the supplementary material, 1-38 minutes). Once neural crest cells had entered the segmental path, filopodial protrusions projecting laterally towards the somite boundaries were rarely observed. Instead, we observed filopodia projecting ventrally along the center of the somite and, consequently, neural crest cells appeared extended along the direction of their migration (Fig. 5C, 61-228 minutes, quantified in Fig. S1 in the supplementary material, 38-60 minutes).

By contrast, in *musk* mutants, neural crest cells extended filopodial protrusions towards the somite boundary throughout all stages of their migration (Fig. 5D and see Movie 5 in the supplementary material). Moreover, mutant cells failed to retract these F-actin positive filopodial protrusions and, instead, they persisted longer than those observed in wild-type embryos (Fig. 5D; 36 minutes, 60 minutes). Consequently, neural crest cells spread over a wider somite territory (see Fig. S1 in the supplementary material) and were delayed in their migration (*n*=5 cells, three embryos). Thus, MuSK function in adaxial muscle cells influences the dynamics of F-actin positive filopodial protrusions in adjacent neural crest cells, thereby modulating their migratory properties to favor a path through the center of the somite.

DISCUSSION

Genetic studies in mice and fish have shown that the induction, maturation and maintenance of postsynaptic acetylcholine receptor clusters, as well as the development of presynaptic structures requires signaling through the MuSK receptor tyrosine kinase (DeChiara et al., 1996; Hesser et al., 2006; Yang et al., 2001; Zhang and Granato, 2000; Zhang et al., 2004). MuSK signaling can

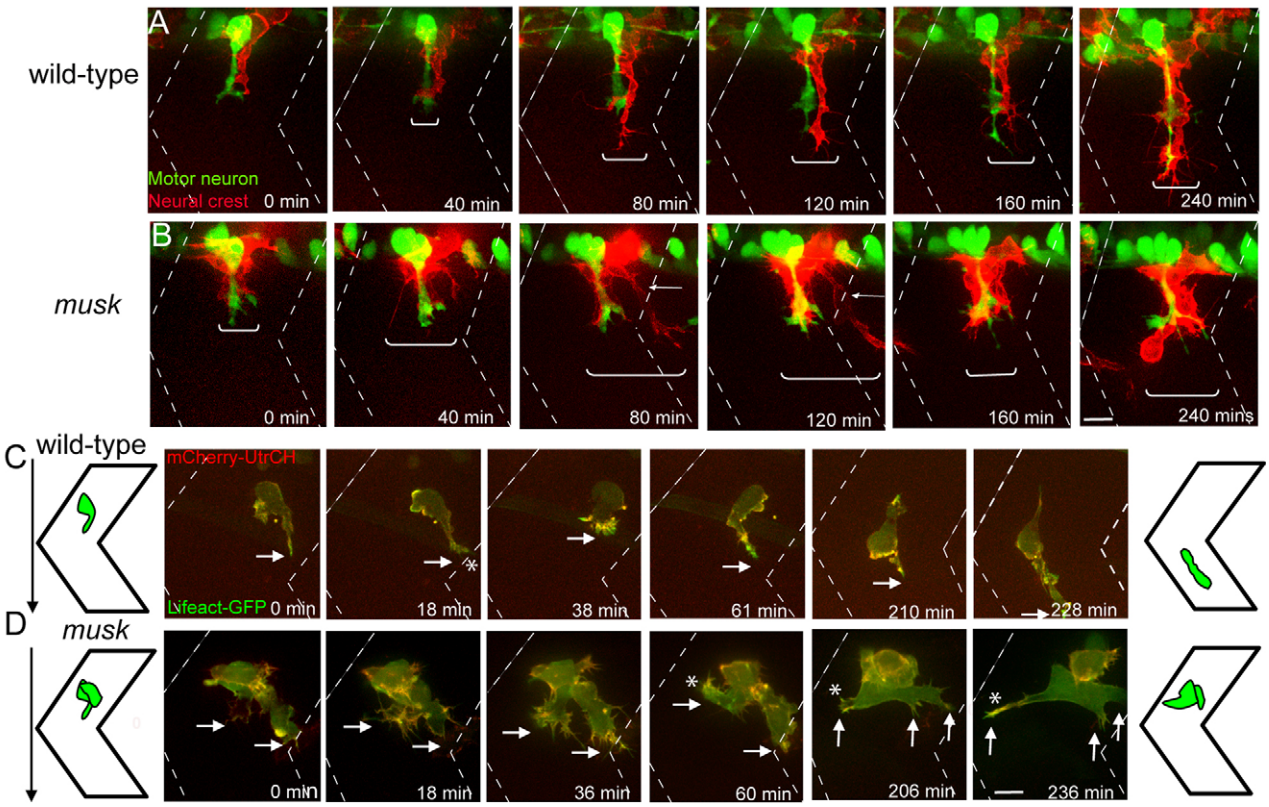

Fig. 5. MuSK regulates maintenance of segmental neural crest migration. (**A**,**B**) Still images from time-lapse movies showing neural crest cells expressing membrane bound RFP [red, Tg*(sox10:mRFP)vu234*] and motor axons expressing GFP (green, Tg*(mnx1:GFP)ml2*) in wild-type (A) and *musk* mutant embryos (B). In *musk* mutants, neural crest cells migrate over a wider area of the somite (compare brackets between A and B). Mutant neural crest cells frequently form two streams, one migrating in close proximity to the motor axons, and one straying away from the central path towards the somite boundary (arrow). Scale bar: 10 μm. (**C**,**D**) Wild-type (C) and *musk* mutant (D) embryos expressing bioprobes for stable F-actin (mCherry-UtrCH) and total F-actin (Lifeact-GFP) in individual neural crest cells. A strong F-actin signal is visible at the leading front (arrow) of wild-type (C) and *musk* mutant (D) neural crest cells. Asterisks indicate filopodial projections towards somite boundaries (at 18 minutes in C; at 60, 206 and 236 minutes in D). Filopodial protrusions near the somite boundary are stabilized in the *musk* mutant embryo (marked by asterisk in D at 60, 206 and 236 minutes). Dashed lines indicate approximate positions of somite boundaries. Scale bars: 10 μm. See also Movies 2, 3, 4 and 5 in the supplementary material.

be subdivided into two phases: a ‘later’ phase, when nerve-released Agrin induces neural synapses through MuSK and its co-receptor Lrp4 (Kim et al., 2008; Zhang et al., 2008); and an ‘early’ phase, prior to the arrival of motor axons and the formation of neural synapses (Yang et al., 2000). During this ‘early’ stage, MuSK is essential for generating a ‘pre-pattern’ of postsynaptic acetylcholine receptors and for restricting incoming motor growth cones to a central region of muscle cells (Jing et al., 2009; Lin et al., 2001; Yang et al., 2001). Our previous work in zebrafish suggests that in response to Wnt signals MuSK triggers a Dishevelled-dependent signaling cascade to establish polarity within the plane of the muscle cell, thereby generating a central zone to which AChR pre-patterning and growth cones are restricted (Jing et al., 2009). Here, we demonstrate that MuSK plays a novel, evolutionarily conserved role to maintain segmental neural crest cell migration independently of motor axons.

The role of motor axons in trunk neural crest cell migration

Since the initial observation that trunk neural crest cells migrate in very close proximity to spinal motor nerves, motor axons were thought to influence neural crest cell migration (Rickmann et al., 1985). We find that ablation of motoneurons does not impair segmental neural crest cell migration (Fig. 2), consistent with previous studies in avian embryos, in which trunk neural crest cells retain their ability to migrate along normal pathways after ventral spinal cord removal (Bhattacharyya et al., 1994). This strongly suggests that the signals directing neural crest cells along segmental streams are produced by non-neuronal cell types, and previous studies and our data identify adaxial muscle cells as a source for such signals (see below).

Even though motor axons are dispensable for segmental neural crest cell migration, we find that motor axons can nonetheless provide instructive cues to neural crest cells. For instance, the Plexin A3 guidance receptor is only expressed in motor axons, and loss of Plexin A3 causes rerouting of spinal motor axons, which, in turn, ‘guide’ neural crest cells into somite territories from which they are normally excluded (Fig. 2). This is consistent with the well-established instructive role of peripheral nerves on co-migrating glia cells, e.g. during zebrafish lateral line nerve migration (Gilmour et al., 2002). Thus, although spinal motor axons in the zebrafish can influence neural crest cell migration, they are dispensable for segmental neural crest cell migration through the center of the somite.

A novel role for MuSK in neural crest cell migration

Elegant manipulations have shown that neural crest cells enter two broad migration routes known as the dorsolateral and ventromedial pathways (Bronner-Fraser, 1993). A unique feature of the ventromedial pathway is that neural crest cells entering this route rearrange from a continuous sheet into narrow, segmentally restricted streams. Recent genetic evidence has demonstrated that Nrp2/Sema3F signaling is crucial for organization of neural crest cells into segmental streams (Gammill et al., 2006a; Roffers-Agarwal and Gammill, 2009; Schwarz et al., 2009a), yet its role in maintaining segmental migration is less clear. Our analysis of *musk* mutant embryos reveals that neural crest cells properly organize into segmental streams, but subsequently fail to maintain their narrow, segmental path (Fig. 2). This defect is not secondary to misguided motor axons, as ablation of motor neurons in the *musk* mutant recapitulates the neural crest migration phenotype (Fig. 4). Combined, these results are consistent with a simple model by which MuSK is dispensable for the initiation of segmental neural crest migration, but plays a crucial role in the maintenance of segmental migration. Despite its clear role in segmental neural crest migration, segmentation of dorsal root ganglia was only mildly affected (~10% of the hemisegments; data not shown) in *musk* mutant embryos.

One possible mechanism through which MuSK might exert its influence on migrating neural crest cells is through specific modifications of the extracellular matrix. In the zebrafish embryo, neural crest cells migrate through the central portion of the somite, along the center of adaxial muscle cells expressing MuSK. The central domain of these adaxial cells, which span the entire somite, is laterally delineated by the accumulation of two components of the extracellular matrix, chondroitin sulfate proteoglycans (CSPGs) and Tenascin C. In embryos lacking MuSK, overall somite and muscle patterning is unaffected, but both Tenascin C and CSPGs are no longer localized around the central zone of adaxial cells, and instead appear diffuse (Schweitzer et al., 2005; Zhang et al., 2004). CSPGs and Tenascins have well characterized activities known to inhibit or repel axonal growth cones (Becker et al., 2003; Masuda et al., 2004), and it is conceivable that they also destabilize or repel filopodia on neural crest cells, thereby restricting their path to the center of adaxial cells and, hence, to the center of the somite. For example, high resolution imaging shows that neural crest cells use their filopodia to sample the environment and thereby restrict their migration to a restricted path (Kasemeier-Kulesa et al., 2005). Indeed, our live cell imaging using F-actin biosensors reveals that when neural crest cells invade lateral somite territories, this is accompanied by dramatic changes in neural crest filapodia dynamics (Fig. 5). Thus, MuSK signaling influences filopodia dynamics through a non-cell-autonomous process, possibly through modifications of the extracellular matrix (ECM), thereby keeping neural crest cell migration focused to the center of adaxial muscle cells and somites.

A Wnt-dependent pathway maintains segmental neural crest cell migration

musk and *wnt11r* mutants display identical axonal and neural crest cell migration defects, suggesting that both proteins act as part of a common signaling pathway. In vitro, Wnt11r binds to the MuSK receptor, and in vivo, Wnt11r binding to the surface of adaxial muscle cells requires MuSK function (Jing et al., 2009). Moreover, blocking non-canonical Dishevelled signaling in adaxial muscle cells recapitulates the neural crest cell defects observed in *musk* and *wnt11r* mutant embryos, consistent with the idea that a Wnt11r-MuSK dependent, PCP-like pathway in adaxial muscle restricts neural crest cells to their segmental path, possibly through modification of ECM components (see above).

Alternatively, Wnt11r could act directly on neural crest cells, where it could activate a non-canonical Wnt/PCP pathway. In fact, Wnt signals have been shown to be important for generating polarized cell morphology that is sufficient to initiate directional migration of cephalic neural crest cells (De Calisto et al., 2005; Matthews et al., 2008a; Matthews et al., 2008b). Cephalic and trunk neural crest cell migration differs in many important aspects, and whether Wnt proteins influence migration of trunk neural crest cells directly has not been examined. Although we can not exclude a direct effect on neural crest cells, our finding that blocking the Dishevelled-mediated PCP pathway in individual adaxial muscle cells caused identical defects in segmental neural crest cell migration as those we observed in *musk* and *wnt11r* mutants strongly argues that Dishevelled and Wnt11r influence trunk neural crest cell migration indirectly, through somitic muscle cells.

Taken together, our results suggest a model in which Wnt11r, through the muscle-expressed MuSK receptor, initiates a Dishevelled-dependent signaling cascade that maintains segmental neural crest cell migration through the central region of zebrafish somites (Fig. 6A). We propose that MuSK signaling is required once neural crest cells reorganize into a segmental pattern established by Nrp2/Sema3F signaling pathway (Fig. 6B). Within

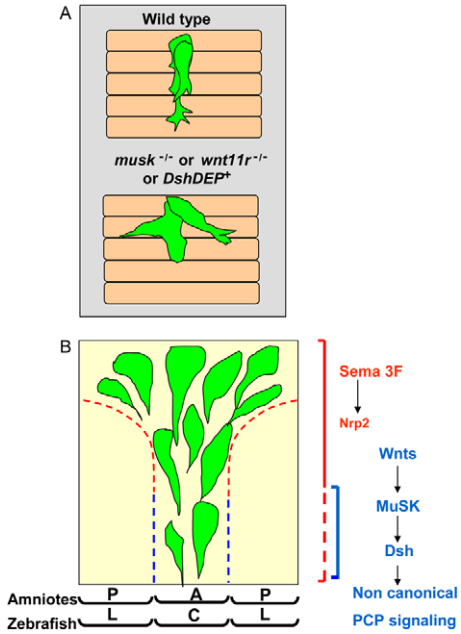

Fig. 6. Model of the role of MuSK in control of segmental neural crest cell migration. (**A**) In the absence of MuSK, Wnt11r and non-canonical Dishevelled signaling, neural crest cells stray from their central path. (**B**) Proposed model showing initiation and maintenance of segmental neural crest cell migration. First, Nrp2/Sema3F signaling acts to initiate neural crest cell migration into segmental streams. Second, Wnt signals act though MuSK present in the muscle to activate non-canonical Dishevelled/PCP signaling, which keeps neural crest cells organized into a restricted central region of the somite in zebrafish and in the anterior somite of amniotes. It is not known whether Nrp2/Sema3F also have a role in maintaining segmental neural crest cell migration. A, anterior somite; C, central zone; L, lateral zone; P, posterior somites.

muscle cells, MuSK signaling recruits components of the non-canonical Wnt/PCP pathway to establish a central zone along the anterior-posterior axis of each muscle (Fig. 6A). This can be visualized by the localization of CSPGs and Tenascin C flanking the central zone to which the segmental path of trunk neural crest cells is restricted. We have previously shown that CSPGs and Tenascin C localization is dependent on MuSK function, and that morpholino knockdown of Tenascin C leads to axonal pathfinding defects (Schweitzer et al., 2005; Zhang et al., 2004). By contrast, knockdown of Chondroitin synthase (Chsy1), a key enzyme in the biochemical synthesis of CSPGs was inconclusive, owing to the widespread requirements of CSPGs in many tissues and during earlier developmental events (Zhang et al., 2004). In the future, it will be interesting to determine the precise complement and arrangement of ECM components crucial for segmental neural crest cell migration. Nonetheless, work presented here reveals a previously unknown, yet evolutionarily conserved role for MuSK in maintaining segmental neural crest migration via a Wnt-PCP like pathway.

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Competing interests statement
The authors declare no competing financial interests.

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