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RESEARCH ARTICLE

Migrating cells mediate long-range WNT signaling

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ABSTRACT

In amniotes, it is widely accepted that WNTs secreted by the dorsal neural tube form a concentration gradient that regulates early somite patterning and myotome organization. Here we demonstrate in the chicken embryo that WNT protein is not secreted to act at a distance, but rather loaded onto migrating neural crest cells that deliver it to somites. Inhibiting neural crest migration or ablating their population has a profound impact on the WNT response in somites. Furthermore, we show that a central player in the efficient delivery of WNT to somites is the heparan sulfate proteoglycan GPC4, expressed by neural crest. Together, our data describe a novel mode of signaling whereby WNT proteins hitch a ride on migratory neural crest cells to pattern the somites at a distance from its source.

KEY WORDS: Somite, WNT, Dermomyotome, Chick embryo

INTRODUCTION

Somites are blocks of cells sequentially generated through the epithelialization of the segmental plate (Christ and Ordahl, 1995; Scaal and Christ, 2004). Shortly after their formation, somites differentiate along their dorsoventral axis into a ventral mesenchyme, the sclerotome and a dorsal epithelium termed the dermomyotome. The dermomyotome is then patterned along its mediolateral axis into a medial compartment termed the dorsomedial lip (DML), a central dermomyotome and a ventrolateral lip (VLL), each with distinct function and fate.

The DML is the most extensively studied dermomyotome subdomain. It plays a crucial role during the initial stage of muscle morphogenesis, during which the primitive muscle (the primary myotome) expands solely from the generation of muscle cells (myocytes) originating from the DML (Denetclaw et al., 2001; Gros et al., 2004; Kahane et al., 1998; Venters and Ordahl, 2002). This production of myocytes at the DML is dependent upon the transient activation of NOTCH signaling in selected epithelial progenitors present in this structure (Rios et al., 2011). Finally, the DML also plays a crucial role as a myotome-organizing center, as it expresses a secreted factor, WNT11, which acts as a directional cue for the elongation of early myocytes in the anteroposterior axis of the chick embryo. WNT11 mediates this effect through the evolutionarily conserved planar cell polarity (PCP) pathway (Gros et al., 2009).

The signals crucial for DML formation have been identified. Using *WNT11* as a specific molecular marker of the DML, it was shown in the chick embryo that its expression is dependent upon WNT1 and/or WNT3a expressed by the dorsal neural tube, which trigger *WNT11* expression through a WNT/β-catenin-dependent pathway (Gros et al., 2009; Marcelle et al., 1997). These results were confirmed and extended in mouse, where it was demonstrated that WNT1 and WNT3a exert their inducing activity on *Wnt11* redundantly (Ikeya and Takada, 1998).

In chick and mouse embryos, the distance between the WNT1/3a source and the DML is considerable (~120 µm and ~90 µm, respectively; Fig. 1A-E,K-O). How WNT can act at such a distance from its source to pattern the medial somite compartment is unknown. A widely accepted view in the field is that WNTs form a concentration gradient from the dorsal neural tube that acts to pattern the somites at a distance (Capdevila et al., 1998; Fan et al., 1997; Marcelle et al., 1997; Munsterberg et al., 1995). However, the existence of this gradient has never been demonstrated experimentally. Importantly, WNT proteins are highly hydrophobic because of post-translational modifications by lipid adducts on highly conserved amino acid residues (Port and Basler, 2010; Takada et al., 2006; Willert et al., 2003) and as such are notoriously poorly mobile. These modifications attach WNTs to the lipid bilayers of membranes, restricting them to the secreting cells. In a number of cellular contexts, however, WNTs are known to act at a considerable distance from their source. This is accomplished through several molecular and cellular mechanisms. Heparan sulfate proteoglycans (HSPGs) of the glypican family, such as Dally and Dally-like protein (Dlp), allow the transfer of WNT from cell to cell, acting as a co-receptor for the ligand, thus allowing the formation of a gradient of WNT activity at a distance from the secreting cells (Yan and Lin, 2009). WNTs can also travel on membranous particles, called argosomes (Panáková et al., 2005), or within exosome-like vesicles (Korkut et al., 2009). Finally, although not formally demonstrated for WNTs, it is also possible that WNTs are transported at a distance similarly to Sonic hedgehog and the TGFβ family member Decapentaplegic, in which the receiving cells grow long cytoplasmic extensions that directly contact morphogen-secreting cells (Hsiung et al., 2005; Sanders et al., 2013).

Here we investigated the mode of signaling of WNTs from the dorsal neural tube to the DML, and uncovered a novel mechanism for long-range WNT signaling, whereby WNT expressed in the dorsal neural tube is loaded onto migrating cells (the neural crest cells) that physically transport the signal to the receiving cells of the DML. We also demonstrate the central role that the Dally-like molecule GPC4 plays in the transfer of the WNT signal from the presenting to the receiving cells.

RESULTS

WNT1 decorates the cell surface of migrating neural crest cells

Epithelial cells of the DML display long filopodia at their basal end that extend mediodorsally towards the dorsal neural tube (Rios et al., 2010, 2012). Although they may participate in the capture of signals from the environment, their length (~9-12 µm; our unpublished observations) is insufficient to reach the source of WNT in the roof plate. We therefore investigated alternative ways of WNT transfer from the dorsal neural tube to the DML.

The expression patterns of *WNT1* and *WNT3a* have been extensively characterized during mouse and chicken embryonic

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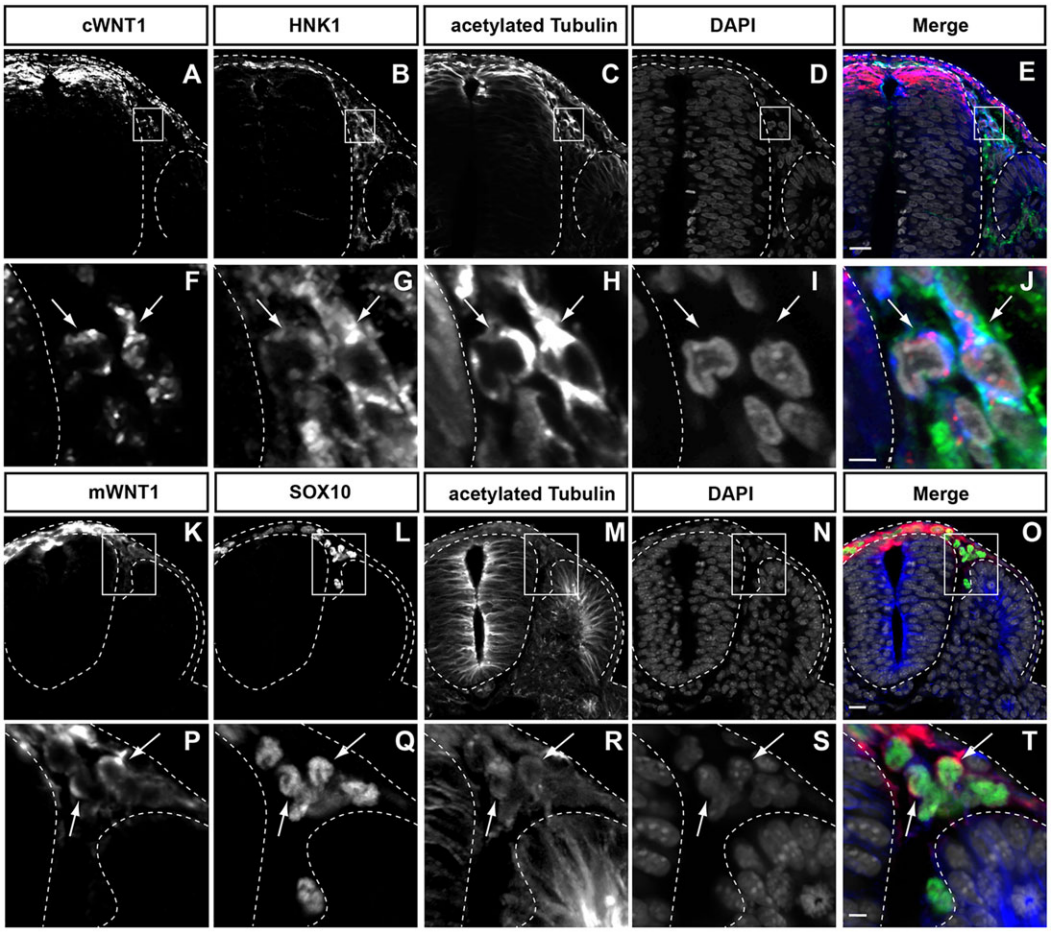

Fig. 1. WNT1 protein is present at the cell surface of migrating neural crest cells. (A-J) Transverse sections of an HH16 chicken embryo (A-J) immunostained for the chick WNT1 protein (cWNT1, A,F; red in merge), the neural crest-specific marker and membrane-bound epitope HNK1 (B,G; green in merge), acetylated tubulin (C,H; blue in merge) and with DAPI (D,I; white in merge). (K-T) Transverse sections of an E9.5 mouse embryo immunostained for mouse WNT1 protein (mWNT1, K,P; red in merge), the neural crest-specific SOX10 (L,Q; green in merge), acetylated tubulin (M,R; blue in merge) and with DAPI (N,S; white in merge). F-J and P-T are magnifications of the boxed areas in A-E and K-O, respectively, showing WNT1 at the surface of the neural crest cells (arrows). Ectoderm, neural tube and somite are outlined (dashed lines). Scale bars: 20 µm in E,O; 5 µm in J,T.

development (Hollyday et al., 1995; Marcelle et al., 1997; Parr et al., 1993). In mouse E9.5 embryos and in chick embryos at the equivalent developmental stage (HH16), *WNT1* and *WNT3a* mRNAs are specifically expressed in the roof plate of the neural tube. However, their transcripts are absent from migrating neural crest cells.

It is possible that, despite the lack of WNT transcript, WNT protein could be present on migrating neural crest cells. Indeed, at E9.5 in mouse and HH16 (E2.5) in chick embryos, WNT1 was detected, not only in the roof plate cells, but also at the cell membrane of early migrating neural crest cells, recognized by the expression of the neural crest-specific markers SOX10 in mouse and HNK1 in chick. WNT1 staining was also observed in the dorsal ectoderm, close to the roof plate (Fig. 1A-T). As neural crest cells migrate across the medial border of the dermomyotome and invade the sclerotome, WNT1 immunostaining decreases significantly. We attempted to confirm this finding for WNT3a; however, the specificity of the commercially available WNT3a antibodies was not sufficient to confidently assess its presence on migrating neural crest cells (data not shown).

Neural crest migration is required for WNT11 expression and myotome organization

This unexpected result prompted us to test whether neural crest cells play a role in the transport of WNT to somites. *WNT11* mRNA is strongly expressed by all DML cells. Its expression is induced and maintained by WNT1/3a from the neural tube and it is therefore a very reliable readout of WNT1/3a activity in this structure (Marcelle et al., 1997). First, we ablated the neural crest cell population in half of the neural tube by electroporating the diphtheria toxin fragment A (DTA) under the control of a neural crest-specific promoter [U2-DTA; as previously demonstrated, this efficiently and specifically eliminates the neural crest population (Rios et al., 2011)]. To detect electroporated cells, we co-electroporated the DTA vector with a second vector containing EGFP under the control of a strong ubiquitous chick beta-actin promoter (GAGGS-GFP). On the electroporated side, the massive depletion of neural crest cells (as demonstrated by the loss of HNK1 staining) led to a significant decrease of *WNT11* expression in the DML, compared with the control side (Fig. 2A-C; n=5/6). In parallel, a significant decrease of the WNT response was observed in the neighboring somite (supplementary material Fig. S1E-H) in comparison to a control (supplementary material Fig. S1A-D).

Second, we blocked the migration of neural crest cells using Dishevelled protein deleted of its DEP domain (DshΔDEP). The PCP pathway is required for the migration of neural crest cells in *Xenopus* (De Calisto et al., 2005) and DshΔDEP is a specific inhibitor of the PCP pathway (Rothbacher et al., 2000; Wallingford et al., 2000). We

Development (2014) 141, 2057-2063 doi:10.1242/dev.107656

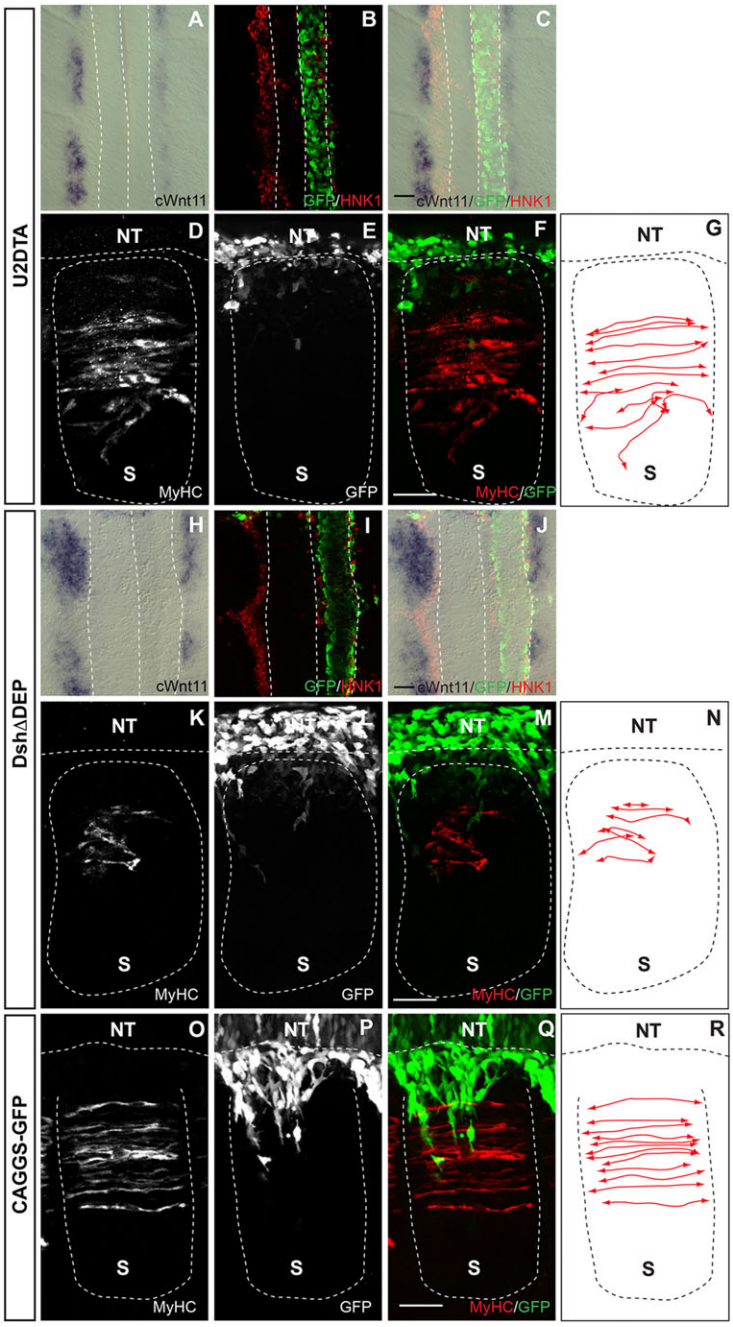

Fig. 2. Neural crest migration is required for the activation of *WNT11* expression at the DML and for myotome organization. (A-C,H-J) Dorsal views of HH16 chick embryos electroporated in the neural tube with U2-DTA (A-C) or CAGGS-DshΔDEP (H-J). A CAGGS-GFP plasmid (green) was co-electroporated to detect the transfected cells. Twenty-four hours after electroporation, *WNT11 in situ* hybridizations (A,C,H,J) followed by immunostaining for GFP and HNK1 (B,C,I,J) show a decrease of *WNT11* expression in the electroporated side of the embryo compared with the control side. (D-G,K-R) Lateral views of HH16 embryos electroporated in the neural tube with U2-DTA (D-G), CAGGS-DshΔDEP (K-N) or only CAGGS-GFP (O-R). In E-F and L-M a CAGGS-GFP plasmid (green) was co-electroporated to detect the transfected cells. Twenty-four hours after electroporation an immunostaining for MyHC was performed to observe the orientation of myocytes within the myotome. (G,N,R) Schematics illustrating the orientation of myocytes shown in D,F and K,M. NT, neural tube; S, somite. Scale bars: 50 µm.

have previously shown that DshΔDEP expression in chick neural crest cells inhibits their migration without affecting their induction (Rios et al., 2011). Indeed, the electroporation of DshΔDEP-expressing vector into the dorsal neural tube, from which neural crest originates, led to a decrease in the number of migrating HNK1-expressing cells, and resulted in the strong reduction of *WNT11* expression in the DML of the electroporated side compared with the control side (Fig. 2H-J; n=4/4). Since the expression of *WNT1* and of *WNT3a* in the dorsal neural tube (which induce *WNT11* expression) are not affected by either the electroporation of U2-DTA or that of DshΔDEP in this structure (see figure 13 in the supplementary material of Rios et al., 2011) this demonstrates that neural crest migration is essential for the activation of *WNT11* in the DML.

A direct outcome of the decrease in *WNT11* expression in the DML should be the disorganization of the early myotome (Gros et al., 2009). Indeed, we observed that the ablation of the neural crest cell population (Fig. 2D-G) or the arrest of its migration (Fig. 2K-N) affected the orientated elongation of MyHC-expressing myocytes in the adjacent somites, compared with control somites (Fig. 2O-R).

Altogether, our data show that neural crest plays an essential role in the β-catenin-dependent WNT response in somites and the resulting expression of *WNT11*, and that this has a direct consequence for the spatial organization of the early myotome.

GPC4 in neural crest is necessary for WNT11 expression in the DML and for myotome organization

Although neural crest cells are decorated by WNT at their cell surface, it is unclear how they deliver the ligand to the receiving cells of the somite. Frizzled (Fz) WNT receptors certainly play a role in this process: they are expressed by DML cells (Linker et al., 2005)

Development (2014) 141, 2057-2063 doi:10.1242/dev.107656

and functional testing suggests that FZ7 is the likely candidate for mediating WNT1 and WNT3a activities in this tissue (Gros et al., 2009). In addition, HSPG family members Dally and Dlp are known to bind WNT proteins and enhance the response of receiving cells to WNT (Yan and Lin, 2009). We investigated whether their vertebrate homologs are expressed at a time and place compatible with a role in transporting WNTs. Glypican 3 and glypican 5 precursors (*GPC3* and *GPC5*) are the vertebrate homologs of Dally, whereas *GPC1*, *GPC2*, *GPC4* [also known as *knypek* in zebrafish (Topczewski et al., 2001)] and *GPC6* are the vertebrate homologs of Dlp. We analyzed the expression patterns of five out of the six chicken GPC genes (*GPC2* was not tested) during embryonic development. Of these, only *GPC4* displayed an expression pattern compatible with a role in neural crest in chick embryos. At stage HH16, its expression was restricted to the roof plate of the neural tube and to the early migrating neural crest cell population emanating from the neural tube. In addition, *GPC4* was observed in the medial portion of the dermomyotome, including the DML (Fig. 3A-F).

We first examined whether *GPC4* expressed by migrating neural crest cells plays a role on *WNT11* expression in somites. To abrogate GPC4 function, we electroporated small interfering RNAs (siRNAs) directed against chick *GPC4* into one half of the neural tube. This efficiently decreased the levels of endogenous *GPC4* transcripts on the electroporated side of the roof plate and in the migrating neural crest cells (supplementary material Fig. S2A-C), whereas a control siRNA directed against the *Luciferase* gene had no effect on *GPC4* expression (supplementary material Fig. S2D-E). The loss of *GPC4* expression in migrating neural crest cells induced

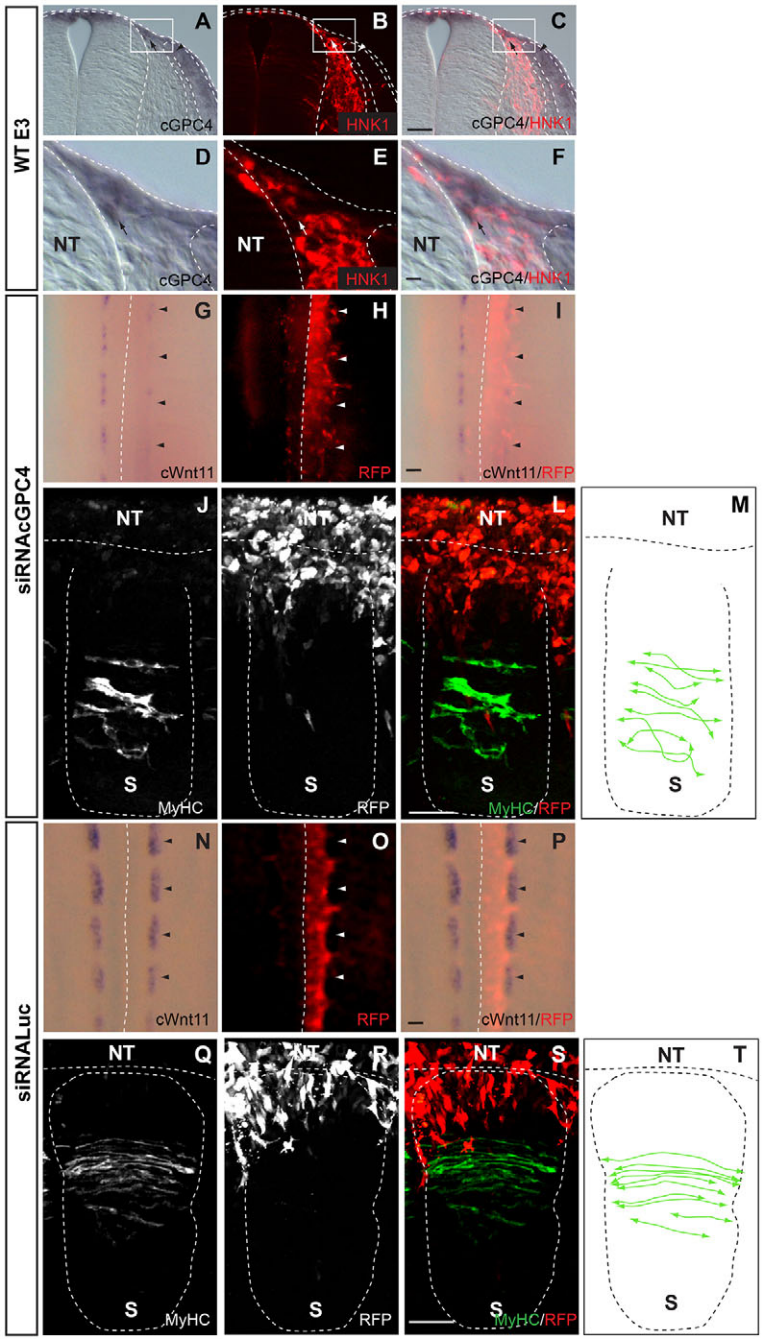

Fig. 3. GPC4 in neural crest cells regulates *WNT11* expression and myotome organization. (A-F) Transverse sections of a 3-day-old chicken embryo after *in situ* hybridization for *GPC4* followed by HNK1 immunostaining. GPC4 expression is detected in the roof plate of the dorsal neural tube, the early migrating neural crest cells as recognized by HNK1 (arrows in A-F) and the medial dermomyotome, including the DML (arrowheads in A-C). D-F are magnifications of the boxed areas in A-C, showing cells stained for HNK1 and *GPC4* mRNA. (G-I,N-P) Embryos were electroporated with a plasmid that encodes an siRNA against *GPC4* (G-I) or a control siRNA against *Luciferase* (N-P), and also for RFP to allow detection of the electroporated cells (red). Twenty-four hours after electroporation, *WNT11 in situ* hybridization demonstrates a decrease in *WNT11* expression in the electroporated side of the embryo after downregulation of *GPC4* (G-I) as compared with the control side or with the control siRNA against *Luciferase* (N-P). (J-M,Q-T) Lateral views of embryos electroporated with siRNA against *GPC4* (J-M) or *Luciferase* (Q-T) and stained for MyHC indicates a disorganization in the orientation of myocytes, which is not seen in the controls. (M,T) Schematics illustrating the orientation of myocytes shown in J and Q, respectively. Scale bars: 20 µm in C; 50 µm in I,L,P,S; 5 µm in F.

Development (2014) 141, 2057-2063 doi:10.1242/dev.107656

a significant decrease of *WNT11* expression in the DML of somites on the electroporated side of the embryos (Fig. 3G-I, arrowheads) (n=6/8). We verified that this was not due to a decrease of *WNT1* and *WNT3a* expression in the dorsal neural tube, to a decrease of WNT1 immunostaining in neural crest, or to their abnormal migration (supplementary material Fig. S2G-O). The electroporation of the control siRNA directed against *Luciferase* did not affect *WNT11* expression (Fig. 3N-P, arrowheads; n=4/5).

The functional outcome of the downregulation of *WNT11* after *GPC4* knockdown should be a disorganization of the myotome. To verify this, we electroporated the *GPC4* siRNA into the neural tube and examined the orientation of myocytes in adjacent somites. We observed a disorganization of the MyHC-expressing myocytes within the myotome in electroporated embryos (Fig. 3J-M), whereas control siRNAs had no effect on its organization (Fig. 3Q-T).

Altogether, these results show that *GPC4* expressed by the neural crest plays a role in the signaling activity of WNT1 (and WNT3a) on DML cells, which results in *WNT11* expression in this structure. In turn, this has a direct consequence for the orientation of myocytes in the myotome. Owing to the experimental protocol that we utilized (electroporation targets only a proportion of the neural crest and the siRNA technology leads to a decrease in expression and not to a complete knockout), we cannot determine whether *GPC4* is necessary for, or merely enhances, WNT activity on the DML. In *Drosophila*, Dally and Dlp are not essential for *wingless* signal, but rather boost or sustain its activity (Franch-Marro et al., 2005), and it is therefore possible that, by analogy, GPC4 in the chick neural crest enhances WNT1/3a signaling on the DML.

GPC4 is required in donor but not receiving cells for WNT11 activation

Interestingly, *GPC4* is also expressed in the DML. This observation gave us the opportunity to test whether GPC4 also plays a role in the receiving, responding cells of the DML. We electroporated the medial somites of E2.5 embryos with the *GPC4* siRNA. As observed in the dorsal neural tube (see above), this led to a significant decrease in the level of *GPC4* transcripts in the DML (Fig. 4G-I; n=5/5). However, this did not affect *WNT11* mRNA expression in this structure (Fig. 4J-L; n=6/7), as compared with a *Luciferase* siRNA that did not alter *GPC4* expression (Fig. 4A-C) nor *WNT11* expression in the DML (Fig. 4D-F) (n=4/5). Altogether, this indicates that GPC4 does not play any significant role in the DML to regulate WNT11 expression. A similar function of Dlp restricted to the donor, and not in the receiving cells, has been described in the *Drosophila* embryo (Franch-Marro et al., 2005). In this organism, it was suggested that Dally and Dlp have complementary, yet distinct, functions in Wingless signaling: Dally plays the role of co-receptor in the donor cell, whereas Dlp plays a role in transmitting the signal to the neighboring cell (Franch-Marro et al., 2005).

Although the experiments described above suggest a role for GPC4 in the transmission of WNTs, they did not address whether it has a role in cis. Previous studies have shown that WNT/β-catenin signaling plays an instructive role in the sensory neuronal fate of early migrating neural crest cells in mouse (Lee et al., 2004). To determine whether GPC4 may be implicated in WNT/β-catenin signaling in migrating neural crest as well, we electroporated a WNT reporter [TopFlash12X-d2GFP, which contains 12 TCF/LEF binding sites upstream of a destabilized EGFP (Rios et al., 2010)] in the dorsal neural tube and compared its activity after electroporation of the *GPC4* siRNA or the control *Luciferase* siRNA. We observed a dramatic reduction in the number of GFP-expressing neural crest cells after electroporation of the *GPC4* siRNA, indicating a decrease in canonical WNT signaling in the neural crest (Fig. 4P-R) as compared with controls (Fig. 4M-O). This indicates that, in addition to its role in the transmission of the WNT signal from the neural crest to the DML, GPC4 is also playing a role in mediating the β-catenin-dependent

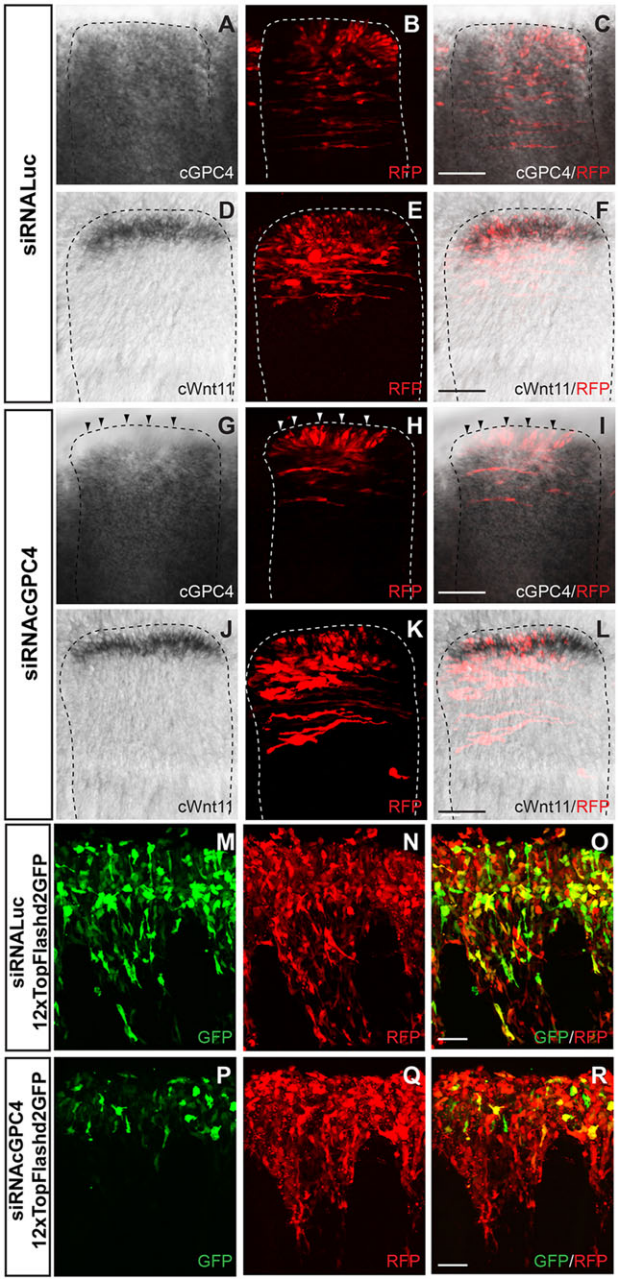

Fig. 4. GPC4 is required in donor but not receiving cells for *WNT11* activation. (A-L) siRNA against *Luciferase* (A-F) or chicken *GPC4* (G-L) were electroporated into the DML of E2.5 chick embryos. One day later, embryos were sequentially processed by *in situ* hybridization for *GPC4* (A-C,G-I) or *WNT11* (D-F,J-L) and by immunostaining for RFP (red) to detect electroporated cells. Arrowheads (G-I) show the downregulation of *GPC4* transcripts specifically in the DML, whereas *WNT11* expression remains normal (J-L). (M-R) GPC4 plays a role in the WNT/β-catenin-dependent signaling of migrating neural crest. One day after co-electroporation with the WNT/β-catenin-dependent reporter (12XTopFlashd2GFP) together with control *Luciferase* siRNA (M-O) or siRNA against *GPC4* (P-R), embryos were stained for GFP to detect the activity of the reporter, and for RFP to detect electroporated cells. Scale bars: 50 µm.

Development (2014) 141, 2057-2063 doi:10.1242/dev.107656

activity of WNT1/3a in the neural crest cell population, presumably to regulate their neuronal fate.

DISCUSSION

Altogether, our results uncover a novel mechanism of long-range WNT signaling, whereby WNT ligands are loaded onto migrating neural crest cells that deliver their message at a distance. To improve the delivery process, neural crest cells express the Dally-like protein GPC4 that acts in trans to deliver the WNT ligand to the receiving cells in somites. This mode of WNT signaling is compatible with its known biochemical properties that impede long-distance signaling unless WNT is transported by ad hoc cellular or molecular mechanisms. There are higher levels of WNT1 protein on early emigrating neural crest cells, which decrease as they migrate away from the neural tube. This creates a gradient of WNT activity that, as in other signaling gradients, relies on the rate of synthesis, diffusion and degradation of the signal (Kicheva et al., 2007; Rogers and Schier, 2011). In *Drosophila*, the rate of degradation of WNT is driven by its endocytosis and targeting to lysosomes (Dubois et al., 2001) and this is the likely mechanism in the neural crest and DML. The rate of WNT diffusion from the neural tube to somites relies on the speed of migration of the neural crest cells away from the neural tube. This is a unique mode of dispersion since, in most systems studied, gradients of morphogens are established by the transfer of secreted signals from cell to cell within epithelia. Given the universality of cell migration and tissue movements during embryogenesis, it is possible that this mode of signal transduction represents a common mechanism for regulating differentiation or patterning programs within distant stem cell pools.

Our data reveal a novel role played by neural crest cells in the patterning of the dermomyotome and the direct impact that this has on the spatial organization of the myotome. For years, a widely accepted view was that neural crest comprises cells that only passively respond to signals from their environment. Recently, it was demonstrated that trunk neural crest cells play an active role in triggering myogenesis in selected epithelial progenitors in the DML and in maintaining the pool of PAX7-positive resident muscle progenitor cells during fetal life through expression of the ligands DELTA1 and neuregulin 1, respectively (Ho et al., 2011; Rios et al., 2011). Together with earlier work showing that cephalic neural crest cells promote the formation of head muscles (Tzahor et al., 2003), this calls for a complete reassessment of the role of the neural crest in the patterning of their environment in general and of muscles in particular. From an evolutionary perspective, it is important to note that WNT reporters (this work) and NOTCH reporters (our unpublished observations) are also active in early migrating neural crest cells. Although their specific role in this cell population is unknown, this suggests that, during evolution, somites have 'highjacked' signals utilized for neural crest for their own purpose. Whether these signals are necessary for skeletal muscle differentiation and organization or are only utilized for the fine-tuning of these processes remains to be determined. It is however truly remarkable that, during evolution, two independent morphogenic movements in two distinct tissues have become so perfectly coordinated to generate such sophisticated signaling mechanisms.

MATERIALS AND METHODS

Chicken electroporation

Electroporations were performed as described (Rios et al., 2012). Plasmids were electroporated at 1 µg/µl final concentration in the electroporation mix. Neural tube electroporations were performed in 2-day-old embryos (HH12) and by positioning the electrodes to target the dorsal neural tube. Embryos were reincubated at 38°C for 24 h. Only embryos that displayed efficient electroporation with no visible developmental defects were analyzed. *GPC4* siRNA target sequences are cloned in pRFP-RNAic as described (Das et al., 2006; Rios et al., 2012). Target sequences CTCAAGAAATGGAAGAGA-GATA and TGGCAACATTTGCAGTGATGAA, which were designed using the GeneScript target finder (http://www.genscript.com/siRNA_target_finder.html), were tested for their efficiency in downregulating chick *GPC4* mRNA independently by *in situ* hybridization and were combined for increased efficiency. The vectors U2-DTA and CAGGS-DshΔDEP were co-electroporated with pCAGGS-GFP to detect the electroporated cells as described (Rios et al., 2011). Double electroporations were performed by a first electroporation in the neural tube, followed 6 h later by electroporation of the DML of the somite. We constructed a TOPFLASH reporter (12TOPFLASH-DsRed-DR) in which 12 TCF/LEF binding sites (Rios et al., 2010) were placed upstream of a destabilized RFP variant (DsRed-Express-DR, Clontech) and a CAGGS-BFP by placing a CAGGS promoter upstream of the mTagBFP blue fluorescent protein (Evrogen).

Sectioning, immunochemistry and confocal analysis

Electroporated embryos were selected under a fluorescence stereomicroscope, dissected and fixed for 30 min in 4% formaldehyde. Embryos were embedded in 15% sucrose/7.5% gelatin/PBS solution and sectioned on a cryostat at 20 µm. The following antibodies were used: anti-GFP chicken polyclonal (ab13970, Abcam; 1/500), anti-RFP rabbit polyclonal (ab6234, Abcam; 1/500), anti-WNT1 rabbit polyclonal (ab15251, Abcam; 1/100), anti-SOX10 goat polyclonal (AF2864, R&D Systems; 1/200), anti-acetylated tubulin IgG2b mouse monoclonal (T6793, Sigma; 1/500), anti-HNK1 IgGM mouse monoclonal (3H5, Developmental Studies Hybridoma Bank; 1/10), anti-myosin heavy chain (MyHC) IgG2b mouse monoclonal (MF20, Hybridoma Bank; 1/10). WNT1 signal was amplified with the TSA Plus kit (PerkinElmer). Sections were mounted in Fluoromount DAPI solution (Southern Biotech). Stained sections were examined using a Leica SP5 confocal microscope with 40× oil-immersion lens and images were analyzed with an Imaris software suite (Bitplane). Whole-mounts in Figs 2-4 are projections of confocal image series (typically 20-40 images, 1 µm apart) of somites of fixed embryos immunostained with the indicated antibodies. *In situ* hybridizations were performed as described (Henrique et al., 1995) with the following probes: two 400 bp cDNA clones encoding fragments of chick *WNT1* and *WNT3a* and a 1 kb chick *WNT11* probe (Marcelle et al., 1997), and an 800 bp EST probe (from BBSRC ChickEST database) for chicken *GPC4* (ChEST111j21).

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Competing interests

The authors declare no competing financial interests

Author contributions

O.S. and C.M. designed the experiments and wrote the paper. O.S. performed the experiments.

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Supplementary material

Supplementary material available online at
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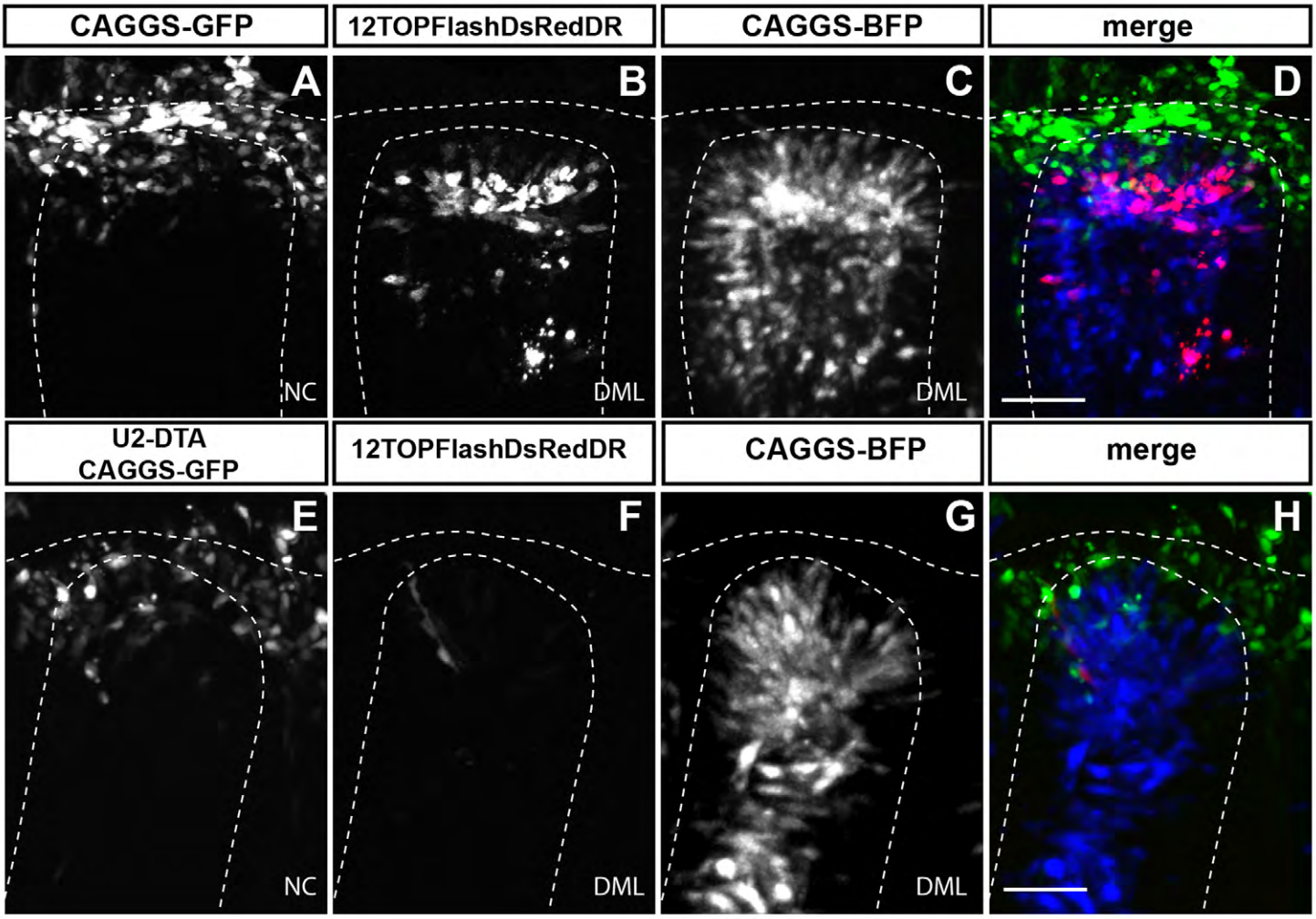
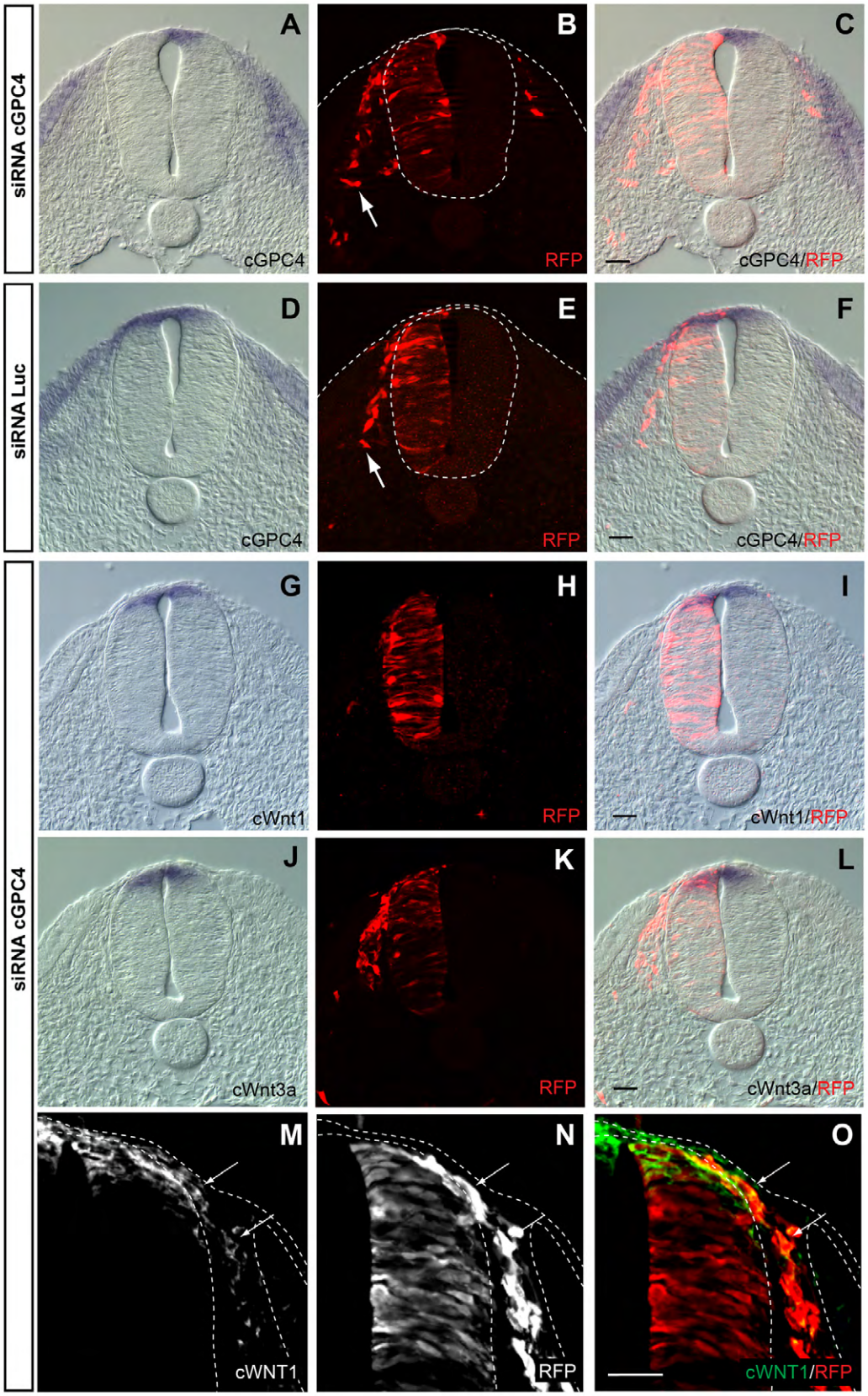


Supplementary Figure 1. The neural crest is required for β-catenin dependent WNT signaling in somites. A vector coding for U2 DTA was co-electroporated with CAGGS-GFP in the dorsal neural tube of E2 chick embryos (E-H). A few hours later, the neighbouring somites were co-electroporated with a combination of a TOPFLASH reporter (12TOPFLASH-DsRed-DR) and a CAGGS-BFP. As control, embryos were electroporated in the neural tube only with CAGGS-GFP (A-D). A-H are dorsal views of somites processed one day later for expression of GFP (A,E and green in D,H), RFP (B,F and red in D,H) and BFP (C,G and blue in D,H). A strong reduction of TOPFLASH activity is observed when neural crest cells are absent (F,H), compared to controls (B,D).



Supplementary Figure 2. *GPC4* siRNA down-regulates the endogenous *cGPC4* transcript in the dorsal neural tube and migrating neural crest cells, but not *WNT1* or *WNT3a*. siRNAs against the chick *GPC4* (A-C, G-L) or Luciferase (D-F) were electroporated in one half of the neural tube of E2.5 chick embryos. One day later, embryos were processed for *in situ* hybridization for *cGPC4* (A,F), *cWNT1* (G-I) or *cWNT3a* (J-L), sectioned, and immunostained for RFP to detect the electroporated cells. Arrows in (B,E) show electroporated migrating neural crest cells. (M-O) immunostaining for cWNT1 and RFP after neural tube electroporation of GPC4 siRNA showing a persistence of the binding of cWNT1 at the surface of the neural crest cells (Arrows). Bars (C,F,I,L,O): 25µm.