Schwann cells reposition a peripheral nerve to isolate it from postembryonic remodeling of its targets

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SUMMARY
Although much is known about the initial construction of the peripheral nervous system (PNS), less well understood are the processes that maintain the position and connections of nerves during postembryonic growth. Here, we show that the posterior lateral line nerve in zebrafish initially grows in the epidermis and then rapidly transitions across the epidermal basement membrane into the subepidermal space. Our experiments indicate that Schwann cells, which myelinate axons in the PNS, are required to reposition the nerve. In mutants lacking Schwann cells, the nerve is mislocalized and the axons remain in the epidermis. Transplanting wild-type Schwann cells into these mutants rescues the position of the nerve. Analysis of chimeric embryos suggests that the process of nerve relocalization involves two discrete steps – the degradation and recreation of the epidermal basement membrane. Although the outgrowth of axons is normal in mutants lacking Schwann cells, the nerve becomes severely disorganized at later stages. In wild-type embryos, exclusion of the nerve from the epidermis isolates axons from migration of their targets (sensory neuromasts) within the epidermis. Without Schwann cells, axons remain within the epidermis and are dragged along with the migrating neuromasts. Our analysis of the posterior lateral line system defines a new process in which Schwann cells relocate a nerve beneath the epidermal basement membrane to insulate axons from the postembryonic remodeling of their targets.

KEY WORDS: Schwann cell, Zebrafish, Posterior lateral line nerve, Basement membrane invasion, Postembryonic remodeling

INTRODUCTION
Many studies have investigated the mechanisms of axonal pathfinding that connect neurons to their targets in the developing embryo (Bashaw and Klein, 2010). Axonal growth cones migrate in response to attractive and repulsive cues, such as netrins, semaphorins, ephrins and slits, to reach targets that are often significant distances from the neuronal cell body (Bashaw and Klein, 2010). Once the initial architecture of the nerve is established, it must be able to respond to changes that occur during postembryonic development, including extensive growth of the organism and changes in tissue morphology. Little is known about growth and remodeling of nerves and their targets after the initial outgrowth of axons. In addition, many cell types are present in mature peripheral nerves, including endothelial cells, fibroblasts, cells of the immune system and Schwann cells (Gamble and Goldby, 1961; Jessen and Mirsky, 2005), and the roles of these non-neuronal cells that associate with axons in postembryonic remodeling are unclear.

Schwann cells are a significant component of peripheral nerves and play well established roles in ensheathing and myelinating axons (Jessen and Mirsky, 2005). Additionally, Schwann cells provide trophic support for axons and present cues that cluster axonal sodium channels at the node of Ranvier (Jessen and Mirsky, 2005; Poliak and Peles, 2003). Recently, new roles for Schwann cells have emerged, including elimination of ectopic sodium channels from the internode (Voas et al., 2009), preventing the premature differentiation of sensory organs (Grant et al., 2005), fascilitating the nerve (Gilmour et al., 2002) and preventing oligodendrocytes from exiting the spinal cord (Kucenas et al., 2009).

The posterior lateral line nerve (PLLn) is a prominent peripheral nerve in zebrafish that innervates sensory organs called neuromasts, which detect changes in water currents (Ghysen and Dambly-Chaudiere, 2007). The components of the posterior lateral line arise from an ectodermal placode, the posterior portion of which forms a primordium that migrates towards the posterior end of the embryo and deposits clusters of cells that will give rise to the neuromasts. The anterior half of the placode becomes neurons in the ganglion of the PLLn, which will innervate the neuromasts of the lateral line. An interesting variation of axonal pathfinding occurs in the PLLn, where the precursors of the targets (i.e. the primordium) and the associated axons grow out together (Gilmour et al., 2004). Although Schwann cells are intimately associated with the axons from an early stage, they do not appear to play a role in axonal pathfinding (Gilmour et al., 2002).

The posterior lateral line system (nerve and neuromasts) initially has a simple structure with only seven to eight neuromasts distributed in a line down the midbody of the larva, but it rapidly becomes more complicated, containing hundreds of neuromasts widely distributed over the body of the adult (Ghysen and Dambly-Chaudiere, 2007). Additionally, the fish grows considerably in size after the initial formation of the lateral line, so the PLLn must be able to compensate both for the increased size and complexity of the system. Finally, while the primordium migrates within the epidermis (Metcalfe, 1985), which is the final position of the neuromasts, the mature nerve is embedded within the subepidermal space at the horizontal myoseptum, directly below the basement membrane of the epidermis (Voas et al., 2009; Winklbauer, 1989). How these complex changes in tissue architecture occur is not well understood, including how the PLLn grows within the epidermis but is ultimately excluded from it.
To learn more about the initial formation and remodeling of the lateral line, we have investigated the development of the lateral line system in embryonic and early larval stages. Ultrastructural studies reveal that the entire nerve, including axons and Schwann cells, is initially localized within the epidermis, superficial to the epidermal basement membrane. Shortly after axon outgrowth, the epidermal basement membrane is degraded and then reformed on the opposite side of the nerve, so that the nerve is repositioned to its mature location in the subepidermal space. The analysis of mutant and chimeric embryos shows that Schwann cells are required for this process of basement membrane degradation and regeneration. In mutants lacking Schwann cells, nerves become progressively disorganized as development proceeds, suggesting that the process of basement membrane invasion is essential to maintain the functional integrity of the nerve during postembryonic remodeling. These results define a new role for Schwann cells in remodeling tissues in the vicinity of nerves to ensure proper organization during postembryonic growth.

MATERIALS AND METHODS

Fish strains
Zebrafish embryos were raised at 28.5°C and were staged as described (Kimmel et al., 1995). The erbb2<sup>st61</sup>, erbb3<sup>st48</sup> and sox10<sup>clst3</sup> mutants and FoxD3:GFP(17) transgenic fish have been previously described (Gilmour et al., 2002; Kelsh and Eisen, 2000; Lyons et al., 2005; Pogoda et al., 2006).

Genotyping
The erbb2<sup>st61</sup> and erbb3<sup>st48</sup> mutations were genotyped as described (Lyons et al., 2005). sox10<sup>clst3</sup> embryos were scored for lack of pigment and then genotyped for the presence of the t3 insertion [using primers 5′ TGAAGTCGACGAGGAAGAT 3′ (forward) and 5′ CACAGCTTC- CCCAGTGTAT 3′ (reverse)] that do not amplify a fragment in the mutants.

Transmission electron microscopy
Sample preparation for electron microscopy was performed as described previously (Lyons et al., 2008) for 28 hours postfertilization (hpf), 3 days postfertilization (dpf) and 5 dpf embryos; a minimum of three nerves from three separate embryos were examined for each condition. Images were collected on a Jeol TEM1230 and pseudocolored using Adobe Photoshop software.

Immunohistochemistry and live imaging
Antibody staining for acetylated tubulin was performed following standard methods as described previously (Lyons et al., 2005). FoxD3:GFP(17) wild-type embryos were soaked in 1-phenyl-2-thiourea at a final concentration of 0.2 mM following gastrulation to prevent melanization of the pigmentation. For live imaging, embryos were anesthetized with 0.016% Tricaine (w/v) and were mounted in 1.5% low melting agarose. Fluorescent images were collected on a Zeiss Pascal LSM5 confocal microscope.

Generation of genetic chimeras
Wild-type embryos carrying the FoxD3:GFP transgene were injected with 1% Texas Red dextran. Labeled cells were transplanted at the blastula stage into sox10<sup>clst3</sup> mutant hosts. Embryos were scored live at 3 dpf and 5 dpf for GFP-positive Schwann cells along the PLLn and were imaged live as described above. Embryos were then removed from the agarose and processed for transmission electron microscopy (TEM).

RESULTS
The PLLn invades the subepidermal space
At 3 days postfertilization (dpf), the zebrafish PLLn is normally localized just beneath the basement membrane that separates the epidermis from the muscle (Voas et al., 2009). To determine the localization of the PLLn during its initial outgrowth, we performed transmission electron microscopy. At 28 hours postfertilization (hpf), the axons and Schwann cells in the posterior segment of the nerve, which are close to the migrating primordium, are located within the epidermis (Fig. 1A,C). At more anterior locations farther from the primordium, the nerve can be found in a transitional state, with basement membrane both above and below the nerve (Fig. 1A,D). At later developmental stages, the nerve is located completely below the basement membrane of the epidermis (Fig. 1E). Thus, the PLLn transitions across the basement membrane from the epidermis into the subepidermal space during its development (Fig. 1B). Additionally, this transition occurs in an anterior to posterior fashion, with more anterior segments of the nerve moving across the basement membrane prior to more posterior segments (Fig. 1A,B).
Schwann cells are required for the PLLn to relocalize across the basement membrane

To further investigate whether Schwann cells are required to shift the PLLn from the epidermis into the subepidermal space, we performed transplantation experiments to generate genetic chimeras in which Schwann cell-deficient mutants contained wild-type cells, including some Schwann cells. Cells from wild-type embryos carrying the FoxD3:GFP(17) transgene, which expresses green fluorescent protein (GFP) in Schwann cells and other neural crest derivatives (Gilmour et al., 2002), were transplanted into sox10/clst3 mutant hosts, which lack all Schwann cells in the PLLn. Chimeric embryos were screened for the presence of GFP-expressing (wild-type) Schwann cells in the PLLn and were then processed for electron microscopy to determine the localization of the nerve.

We analyzed 12 chimeras in which sox10/clst3 mutant embryos had wild-type Schwann cells covering the entire length of the PLLn (Fig. 4B). In these 12 chimeras, there were eight cases of complete rescue of the nerve position (Fig. 4C), such that the chimeric nerve occupied the mature wild-type position beneath the basement membrane. There were also four cases of partial rescue, three of which had nerves in the proper location deep to the basement membrane, but with some residual basement membrane remaining on the deep (i.e. muscle) side of the nerve (data not shown, similar to Fig. 5C). In the fourth case of partial rescue, basement membrane surrounded the nerve on both sides, suggesting that the transplanted Schwann cells were unable to degrade the original basement membrane deep to the nerve, but were nonetheless able to initiate the formation of a new superficial membrane (Fig. 4D). These results indicate that Schwann cells are required to correctly position the PLLn within the subepidermal space.

We also analyzed four other chimeras in which sox10/clst3 mutant embryos had smaller clones of wild-type Schwann cells that covered only the anterior segment of the PLLn (Fig. 5B). In the anterior segments of the PLLn, all of these had partial rescue of the nerve position, either with some residual deep basement membrane (Fig. 5C; n=1), equal basement membrane on both sides (data not shown, similar to Fig. 4D; n=2), or location in the epidermis but surrounded by a circumferential basement membrane (Fig. 5E,F; n=1). The partial rescue of nerve position suggests that Schwann cells were not present in sufficient numbers or at the correct stage to degrade the original basement membrane completely in these chimeras. We did not observe any evidence of rescue in the posterior segments of these nerves, in which axons were not associated with Schwann cells (Fig. 5D), indicating that Schwann cells can only rescue the region of the nerve with which they are associated. Finally, in all 16 chimeras with full or partial Schwann cell coverage, myelination of the PLLn was rescued, indicating that sox10 acts cell autonomously in Schwann cells to regulate myelination (see insets in Figs 4, 5).

Movement across the epidermal basement membrane protects the integrity of the PLL nerve

The foregoing results raise questions about the possible functions of nerve relocalization. It has previously been reported that, by 3 dpf, the neuromasts of the PLLn begin to migrate ventrally within the epidermis (Grant et al., 2005). Because the PLLn remains in the epidermis with the neuromasts in mutants lacking Schwann cells, we hypothesized that ventral migration of the neuromasts might drag the mislocalized lateral line nerve and cause the disorganization of the nerve that is observed in these mutants (Gilmour et al., 2002; Lyons et al., 2005; Pogoda et al.,...
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ERBB3ST48 transitions out of the epidermis, below the epidermal basement membrane. Our evidence suggests that repositioning the nerve protects it from the remodeling of its targets in the epidermis and maintains its integrity during the continued growth of the fish.

Our analysis defines a new role for Schwann cells in breaching and reforming the epidermal basement membrane to properly position the lateral line nerve. We find that the wild-type PLLn, including Schwann cells and axons, initially grows within the epidermis but then crosses the epidermal basement membrane to invade the subepidermal space (Fig. 1). This transition occurs early in development, while the distal region of the nerve is still growing. Additionally, in mutants lacking Schwann cells, including *erbb2*mut, *erbb3*mut and *sox10/cls*, the nerve is aberrantly located within the epidermis, and this results in significant disorganization of the nerve (Figs 3, 6). The phenotypic similarity of these mutants suggests that the abnormal nerve localization results from the loss of Schwann cells, rather than a specific function of any of these genes in the process of basement membrane remodeling itself. Transplantation of wild-type Schwann cells into *sox10/cls*mutants restores the nerve to its normal position beneath the epidermal basement membrane (Figs 4, 5). Therefore, Schwann cells are required for the local degradation and immediate reformation of the basement membrane on the opposite side of the nerve.

Basement membrane invasion defines a mode of axon localization that is distinct from axonal growth cone pathfinding. In the PLLn, the axons grow out along with the primordia of their final targets (Gilmour et al., 2004), the neuromasts, in the epidermis and then are moved along their entire length by the Schwann cells associated with the nerve. In addition, the segments of the axon farthest from the growth cone are the first to be moved across the basement membrane. It appears that interactions between Schwann cells and their local environment regulate the transition of the PLLn across the epidermal basement membrane. This most likely occurs through short-range or contact-dependent signaling, because Schwann cells are only competent to move segments of the nerve with which they are associated (Fig. 5C,D). In addition, it appears that this mode of nerve localization is distinct from the

**DISCUSSION**

As development progresses, organs that are initially built in a small embryo must grow to the adult size. The organism must be able to adapt to these changes in size while maintaining functionality of the organs. This is certainly true of the nervous system, where connections are initially established in the embryo and then maintained during extensive growth of the organism. Here, we present a process by which the posterior lateral line nerve in zebrafish initially forms in the epidermis and then rapidly transitions out of the epidermis, below the epidermal basement membrane. Our evidence suggests that repositioning the nerve protects it from the remodeling of its targets in the epidermis and maintains its integrity during the continued growth of the fish.

To test this hypothesis, we studied a time course of wild-type and Schwann cell mutant (*erbb2*mut and *sox10/cls*; *erbb3*mut data not shown) nerves (Fig. 6). Consistent with previous studies (Gilmour et al., 2002), wild-type and mutants nerves are indistinguishable from each other at 48 hpf, a stage before the neuromasts begin to migrate (Fig. 6A,F,K). Beginning at 3 dpf, however, as neuromasts are migrating ventrally (Grant et al., 2005) (Fig. 6B), the mutant nerves began to undulate and defasciculate (Fig. 6G,L); this phenotype worsened over time (Fig. 6G-L). In the mutants, the nerves were always the most severely disorganized in the anterior segments, where neuromasts first begin to migrate ventrally (Fig. 6). Additionally, whereas the wild-type neuromasts migrate ventrally away from the PLLn, which remains anchored at the horizontal myoseptum, mutant neuromasts are often found close to the disorganized nerve (arrowheads, Fig. 6). Finally, when mutant nerves at 5 dpf were studied using transmission electron microscopy, they often remained closely associated with neuromasts (10/19 mutant nerves examined, data not shown, Fig. 3B,C), whereas wild-type nerves were well separated from the neuromasts by this stage (Fig. 6; data not shown). These results support the possibility that moving the PLLn from the epidermis into the subepidermal space is crucial for protecting the nerve from being pulled ventrally along with the migrating neuromasts in the larva (Fig. 6A-E).

**Fig. 3. The PLLn is mislocalized in mutants lacking Schwann cells.** (A) At 5 dpf, the PLLn of wild-type siblings has migrated from the epidermis (ep), across the basement membrane (arrowheads) into the muscle (m) (n=28). (B,C) By contrast, at 5 dpf in the Schwann cell-deficient mutants *erbb2*mut (B) and *sox10/cls* (C), the PLLn remains in the epidermis, outside of the basement membrane (arrowheads, n=7 in B and n=6 in C). Defasciculation of the mutant nerves is also observed (asterisk, C). (A’,B’,C’) Higher magnification of boxed regions in A,B,C, respectively. Scale bars: 2 μm in A,B,C; 1 μm in A’,B’,C’. Schwann cells are pseudocolored green and axons red. Abbreviations: ep, epidermis; m, muscle; p, pigment; n, neuromast cell.

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Schwann cells reposition nerves

**Fig. 4. Full-length clones of transplanted wild-type Schwann cells can rescue nerve position in sox10/clst3 mutant larvae.** (A) Wild-type 3 dpf embryo carrying the FoxD3:GFP(17) transgene, labeling the PLLn, motor nerves and some pigment cells. (B) FoxD3:GFP(17) cells have been transplanted into a sox10/clst3 mutant host, creating a clone of wild-type Schwann cells that traverse the entire length of the PLLn (3 dpf). (C) Transplanted wild-type Schwann cells can reposition the mutant nerve in the subepidermal space, below the basement membrane (arrowheads). (D) Transplanted wild-type Schwann cells partially rescue the mutant nerve, positioning it within the epidermal basement membrane (arrowheads). Insets show the presence of myelinated axons in C,D. Scale bars: 200 μm for A,B; 2 μm for C,D. Schwann cells are pseudocolored green and axons red in C,D.

Abbreviations: ep, epidermis; m, muscle.

**Fig. 5. Shorter length clones of transplanted wild-type Schwann cells can partially rescue the position of sox10/clst3 mutant nerves.** (A) Wild-type 5 dpf embryo carrying the FoxD3:GFP(17) transgene, labeling the PLLn [dorsal (d) and midbody (mb, tracts), motor nerves and some pigment cells. (B) A clone of wild-type, FoxD3:GFP(17) cells that are present on the dorsal and anterior regions of the PLLn. (C,D) Partial rescue of the PLLn, Schwann cells, which are nerve crest derivatives, are present in the epidermis and move with the axons into the subepidermal space.

Our results demonstrate that localization of the lateral line nerve occurs in two key phases. First, axons grow with precursors of their target neuromasts, ensuring proper connectivity and function at early stages (Gilmour et al., 2004). Next, associated Schwann cells reposition the axons while Schwann cells traverse the epidermal basement membrane. The postembryonic remodeling of the lateral line sensory organs begins with migration of neuromasts and interneuromast cells ventrally within the epidermis by 72 hpf (Grant et al., 2005) (Fig. 6). By this time in wild type, the nerve has crossed the basement membrane and is therefore compartmentalized from neuromast migration. In mutants lacking Schwann cells, however, the nerve remains in the epidermis and is degraded by matrix metalloproteases (MMPs) (Ferguson and Muir, 2000; La Fleur et al., 1996; Lehmann et al., 2009; Mantuano et al., 2008), which cleave extracellular matrix components (Page-McCaw et al., 2007), and can secrete a basement membrane that surrounds the nerve.
mature nerves (Prockop and Kivirikko, 1995), the simplest possibility is that Schwann cells directly secrete a new basement membrane superficial to the PLLn while degrading the deep basement membrane.

MMPs mediate basement membrane invasion in many contexts, including leukocyte invasion and anchor cell invasion in C. elegans (Madsen and Sahai, 2010; Page-McCaw et al., 2007; Sherwood et al., 2005; Yadav et al., 2003). Additionally, Schwann cells activate MMP expression following nerve injury, and MMPs have been implicated in Schwann cell myelination (Ferguson and Muir, 2000; La Fleur et al., 1996; Lehmann et al., 2009; Mantuano et al., 2008). Intriguingly, MMP2 was recently shown to have a role in degrading the basement membrane to allow dendrite remodeling in Drosophila sensory neurons (Yasunaga et al., 2010). Future experiments will determine whether MMPs or other enzymes are involved in basement membrane remodeling.

The process of localizing a nerve below a basement membrane to protect it from the remodeling of its targets is likely to be a common feature of sensory epithelia. In many sensory structures, including the tongue, epidermis, nose, vestibular organ and lateral line, innervated receptive organs or free nerve endings are located within the epithelium, whereas the main nerve plexus is located below the epithelial basement membrane (Boulat and Misery, 2008; Fernandez et al., 1990; Nedelec et al., 2005; Northcutt, 2004; Oakley and Witt, 2004; Purcell and Perachio, 1997; Si et al., 2003; Winklbauer, 1989). These are tissues, with the exception of the inner ear hair cells in mammals, where there is frequent turnover of the epithelium or the sensory cells themselves. For example, taste receptors and olfactory neurons are constantly reborn and replaced throughout life, and the epidermis is continually being sloughed off and replaced (Fuchs and Horsley, 2008; Nedelec et al., 2005; Oakley and Witt, 2004). Perhaps separating innervating axons from their targets protects the nerve from the turnover of its targets (or, in the case of the nasal epithelium, the turnover of individual neurons). Thus, as we show here in the lateral line nerve, Schwann cells may be important for positioning nerves in other sensory organs.

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Competing interests statement
The authors declare no competing financial interests.

References

Fig. 6. Nerves become disorganized during development in mutants lacking Schwann cells. (A-O) Anti-acetylated tubulin antibody staining shows the position of the PLLn. (A-E) Wild-type nerves remain anchored at the horizontal myoseptum and run straight along the length of the embryo at all times examined (48 hpf, 3-6 dpf), but the neuromasts migrate ventrally over time (arrowheads). Some axons branch from the main bundle to innervate individual neuromasts. (F-O) In embryos lacking Schwann cells (F-O). Asterisks indicate the presence of pigment cells. Scale bar: 50 μm.
**Schwann cells reposition nerves**


