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Analysis of Gpr126 function defines distinct mechanisms controlling the initiation and maturation of myelin

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SUMMARY

In peripheral nerves, Schwann cells form the myelin sheath, which allows the efficient propagation of action potentials along axons. The transcription factor Krox20 regulates the initiation of myelination in Schwann cells and is also required to maintain mature myelin. The adhesion G protein-coupled receptor (GPCR) Gpr126 is essential for Schwann cells to initiate myelination, but previous studies have not addressed the role of Gpr126 signaling in myelin maturation and maintenance. Through analysis of Gpr126 in zebrafish, we define two distinct mechanisms controlling the initiation and maturation of myelin. We show that *gpr126* mutant Schwann cells elaborate mature myelin sheaths and maintain *krox20* expression for months, provided that the early signaling defect is bypassed by transient elevation of cAMP. At the onset of myelination, Gpr126 and protein kinase A (PKA) function as a switch that allows Schwann cells to initiate *krox20* expression and myelination. After myelination is initiated, *krox20* expression is maintained and myelin maturation proceeds independently of Gpr126 signaling. Transgenic analysis indicates that the *Krox20* cis-regulatory myelinating Schwann cell element (MSE) becomes active at the onset of myelination and that this activity is dependent on Gpr126 signaling. Activity of the MSE declines after initiation, suggesting that other elements are responsible for maintaining *krox20* expression in mature nerves. We also show that elevated cAMP does not initiate myelination in the absence of functional Neuregulin 1 (Nrg1) signaling. These results indicate that the mechanisms regulating the initiation of myelination are distinct from those mediating the maturation and maintenance of myelin.

KEY WORDS: Schwann cells, Myelination, Zebrafish

INTRODUCTION

The myelin sheath insulates axons and allows rapid conduction of action potentials in vertebrates (Sherman and Brophy, 2005). Schwann cells, which form the myelin sheath in peripheral nerves, originate from neural crest progenitors and co-migrate with growing axons in the peripheral nervous system (PNS) (Jessen and Mirsky, 2005). As an immature Schwann cell progresses to the promyelinating stage, it associates with one segment of a single axon in a 1:1 ratio, and extends 1-1.5 wraps of cell membrane around that axon. When a promyelinating Schwann cell progresses to the myelinating stage, it reiteratively wraps its membrane around the axon and forms the myelin sheath.

Several transcription factors regulate the transition from promyelinating to myelinating Schwann cells, including Oct6 (Scip/Pou3f1), Brn2 (Pou3f2) and Krox20 (Egr2) (Bermingham et al., 1996; Jaegle et al., 2003; Jaegle et al., 1996; Topilko et al., 1994). Krox20 controls the initiation of myelination, and the loss of *Krox20* in mouse results in a complete arrest of Schwann cells at the promyelinating stage (Topilko et al., 1994). *Krox20* expression is maintained throughout life in myelinating Schwann cells (Ghislain et al., 2002; Zorick et al., 1996), and the elimination of *Krox20* expression in mature nerves results in Schwann cell dedifferentiation and demyelination (Decker et al., 2006). Krox20 is therefore a key regulator of both the initiation and maintenance of myelination, and mutations in *KROX20* (*EGR2*) cause peripheral neuropathy in humans (Suter and Scherer, 2003).

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The adhesion GPCR Gpr126 is essential for the initiation of myelination in both zebrafish and mammals (Monk et al., 2009; Monk et al., 2011). In *gpr126* mutant zebrafish, the early events of Schwann cell development are normal: mutant Schwann cells express *sox10*, proliferate, migrate and enter a 1:1 relationship with axons (Monk et al., 2009). *gpr126* mutant Schwann cells do not, however, express *oct6* or *krox20*, nor do they progress beyond the promyelinating stage (Monk et al., 2009). Cell transplantation studies demonstrated that Gpr126 functions in Schwann cells to initiate myelination (Monk et al., 2009). Whether Gpr126 signaling is also required to maintain *krox20* expression and the mature myelin sheath is unknown.

In this study, we show that Gpr126 signaling has a specific function in the initiation of myelination. We show that PKA is sufficient to initiate myelination downstream of the Gpr126 receptor. After myelination is initiated, Gpr126 signaling is no longer required for maintenance of *krox20* expression, Schwann cell membrane wrapping, compaction, or maintenance of the myelin sheath. The initiation of myelination is closely paralleled by expression of an EGFP reporter construct corresponding to the *Krox20* cis-regulatory MSE, previously defined in mouse (Ghislain et al., 2002). Activity of the MSE declines after the initiation of myelination, suggesting that other elements are responsible for maintenance of *krox20* in the mature nerve. These results define two distinct mechanisms controlling myelin formation in Schwann cells: initiation requires Gpr126 signaling to activate *krox20* expression and the MSE; and the maintenance of *krox20* expression is controlled independently of Gpr126 signaling and is likely to be mediated by other regulatory elements.

MATERIALS AND METHODS

Zebrafish strains

The *gpr126*^{st49} and *erbb2*^{st61} nonsense mutations have been previously described (Lyons et al., 2005; Monk et al., 2009). Transgenic fish harboring

the *UAS:hNrg1typeIII* transgene were described previously (Perlin et al., 2011). *S1101:Gal4* transgenic fish express the Gal4 transcriptional activator in all neurons (Scott et al., 2007).

Genotyping

The *gpr126*^{st49} and *erbb2*^{st61} lesions were genotyped as previously described (Lyons et al., 2005; Monk et al., 2009).

Drug treatments

Forskolin (FSK; Sigma) was dissolved in DMSO to generate 20 mM stock solutions, which were diluted 1:400 in standard zebrafish embryo medium (Westerfield, 2007) to generate 50 μM solutions used for all experiments. Embryos were treated in 50 μM FSK or DMSO as controls, for 5 hours, from 50 to 55 hours post-fertilization (hpf).

Transmission electron microscopy (TEM)

TEM was performed as described (Lyons et al., 2008). Stained grids were imaged on a JEOL JEM-1400 transmission electron microscope. The number of samples analyzed are as follows: heterozygous control 9 days post-fertilization (dpf), six posterior lateral line (PLL) nerves from three animals; mutant control 9 dpf, five PLL nerves from three animals; mutant FSK treated 9 dpf, six PLL nerves from three animals; heterozygous control 30 dpf, four PLL nerves from three animals; mutant control 30 dpf, seven PLL nerves from four animals; mutant FSK treated, 11 PLL nerves from six animals.

RNA extraction and RT-PCR

PLL nerves from five 3-month-old fish for each genotype and treatment condition were dissected and pooled together to extract RNA using the RNeasy Micro Kit (Qiagen). cDNA was generated using the Superscript First Strand Synthesis System for RT-PCR (Invitrogen). PCR was performed using the following primers: *krox20*: ATGACAGCTAAAACTTTGGAGAAAGC and GTGACGAGGATGCTGAGGATGG; *mbp*: AAGCACCTCTGGACAAAACC and CTGCAGTGCAACATCTGAGG; and *sox10*: TCTCAGTGAAGTCCGACGAGG and TGACCTCACCCTCAGAGTGG.

Quantitative PCR (qPCR)

PLL nerves were dissected from 6-month-old zebrafish and cDNA was prepared as described above, with the exception that nerves were not pooled, but were collected from four individual fish for each genotype and treatment condition shown in Fig. 2B. qPCR was performed in triplicate using the standard curve method on the BioRad CFX384 Touch Real-Time PCR Detection System. The following primer sets were used: *krox20*: GATAGCATCTATTCGGTGGACGAGC and GCAGGTGACGAGGATGCTGAGG; *mbp*: TCAAATCAGCAGGTTCTTCG and CTGAAGAAATGCACGACAGG; *sox10*: AACGCGTTCATGGTGTGG and GCTTATCCGTCTCGTTCAGC; *EF1α* (*eef1a1l1*): AGGACATCCGTCGTGGTAAT and AGAGATCTGACCAGGGTGGTT. Fold differences were calculated after normalizing to *EF1α*. *krox20* and *mbp* were additionally normalized to *sox10*, after ensuring that *sox10* levels were not different in wild-type and mutant nerves.

Generation of transgenic constructs with *huC* and *sox10* regulatory sequences

The *huC* regulatory sequence (Park et al., 2000) was amplified by PCR from plasmid DNA using the following primers: 5′-caagtaggccgtttaaactcgagcggccgcGAATTCACTAATTTGAATTTAAATGC-3′, and 5′-catgtctggatcatcatcgatTCTTGACGTACAAAGATGATATGATC-3′. The *huC* sequence was cloned upstream of EGFP into a modified Tol2 expression vector (Williams et al., 2010) by homologous recombination (CloneEZ kit, Genscript). The pBH-*huC:PKA*^{act} clone was similarly constructed with the *PKA*^{act} sequence that had been generated by PCR with the following primers: 5′-atcatctttgtacgtcaagaATGGGCAACGCCGCCGCCGCCAAGAAG-3′ and 5′-catgtctggatcatcatcgatTCTAAAACTCAGTAAACTCCTTGCCAC-3′. Upper case letters denote gene-specific primer sequence, and lower case letters represent homology arms used for cloning by homologous recombination.

The *sox10* regulatory sequence (Carney et al., 2006) was amplified by PCR from zebrafish genomic DNA using the following primers: 5′-CCTTTAAACTCGAGCGAGTGTCACTTTCTCCAAAGC-3′ and 5′-CCTTTGCGGCCGCCGGTCCACTCGTTCTGCGGCCAC-3′. *EGFP* and *PKA*^{act} were cloned downstream of the *sox10* sequence in the Tol2 expression vector as described above for *huC*.

Synthesis of mRNA for microinjection

Synthetic mRNA encoding *erbb2* was prepared as described (Lyons et al., 2005). *Tol2* mRNA was similarly transcribed with SP6 polymerase using *Not*I linearized *Tol2-pCS2+* as template.

Microinjection of zebrafish embryos

To rescue Schwann cell migration in *erbb2*$^{st61/st61}$ mutants, embryos were injected at the one-cell stage with 200 pg *erbb2* mRNA (Lyons et al., 2005). To make transient transgenic zebrafish expressing *huC:EGFP*, *sox10:EGFP*, *huC:PKA*^{act,} or *sox10:PKA*^{act}, 25 pg of the appropriate constructs were injected as supercoiled plasmid DNA into one-cell-stage zebrafish embryos. Each construct was co-injected with 25 pg of synthetic *Tol2* transposase mRNA (Kawakami, 2005).

Generation of MSE:*EGFP* transgenic fish

The MSE sequence was amplified by PCR from mouse genomic DNA using the following primers: 5′-TTAATGTCACCACAGGCACCTCTCC-3′ and 5′-GTGCACACTGCTGAGTATGTATGG-3′. The MSE was cloned into an *EGFP* expression vector containing the basal *E1b* promoter (Li et al., 2010).

MSE*:EGFP* (25 pg) was injected as supercoiled plasmid DNA, along with 25 pg of *Tol2* mRNA, into one-cell-stage zebrafish embryos. The injected fish were raised and were screened for germline transmission by crossing with wild-type fish and screening for EGFP expression in the progeny. The resulting transgenic founder was outcrossed to *gpr126*^{st49} fish to start the MSE*:EGFP* transgenic line used in this study.

Confocal microscopy and antibody staining

All fish were imaged live, with the exception of the experiments shown in Fig. 3E,F and Fig. 4G,H. Live imaging was performed by anesthetizing in 0.016% (w/v) Tricaine, and mounting in 1.5% low-melting-point agarose. For double labels with MBP (Fig. 3E; Fig. 4G,H), fish were fixed overnight at 4°C (4% paraformaldehyde, 8% sucrose in PBS), followed by 5 hours of water washes. MBP antibody staining was then performed as described (Lyons et al., 2005). All fluorescent images were captured using a Zeiss LSM 5 Pascal confocal microscope with the Axioplan 2 imaging system. The objectives used in this study were the Plan-Neofluar 10× (numerical aperture 0.30) and the Plan-Apochromat 20× (numerical aperture 0.75).

In situ hybridization

In situ hybridization was performed by standard methods (Thisse and Thisse, 2008), using probes for *mbp* (Lyons et al., 2005) and *sox10* (Dutton et al., 2001). Imaging was performed using a Zeiss Stemi SV 11 Apo stereomicroscope using the 10× objective (numerical aperture 0.45), and images were captured using the Zeiss AxioCam HRc color camera using AxioVision imaging software.

RESULTS

Gpr126 signaling activity is required specifically at the onset of myelination

In *gpr126* mutant zebrafish, treatment with forskolin (FSK), which activates adenylate cyclase to increase cAMP (Seamon et al., 1981), rescues myelination in a subset of axons of the posterior lateral line nerve (PLLn) (Monk et al., 2009). To investigate whether *gpr126* is required for later stages of myelination, we examined mutants at various stages after transient FSK treatment. Zebrafish embryos from a *gpr126*^{st49}/+ intercross were treated with either DMSO (control) or 50 μM FSK from 50 to 55 hpf. The drug was then removed, and, after a one-week recovery period following treatment, larvae were fixed at 9 dpf for TEM. Whereas DMSO treated mutants did not have any myelinated axons in the PLLn at

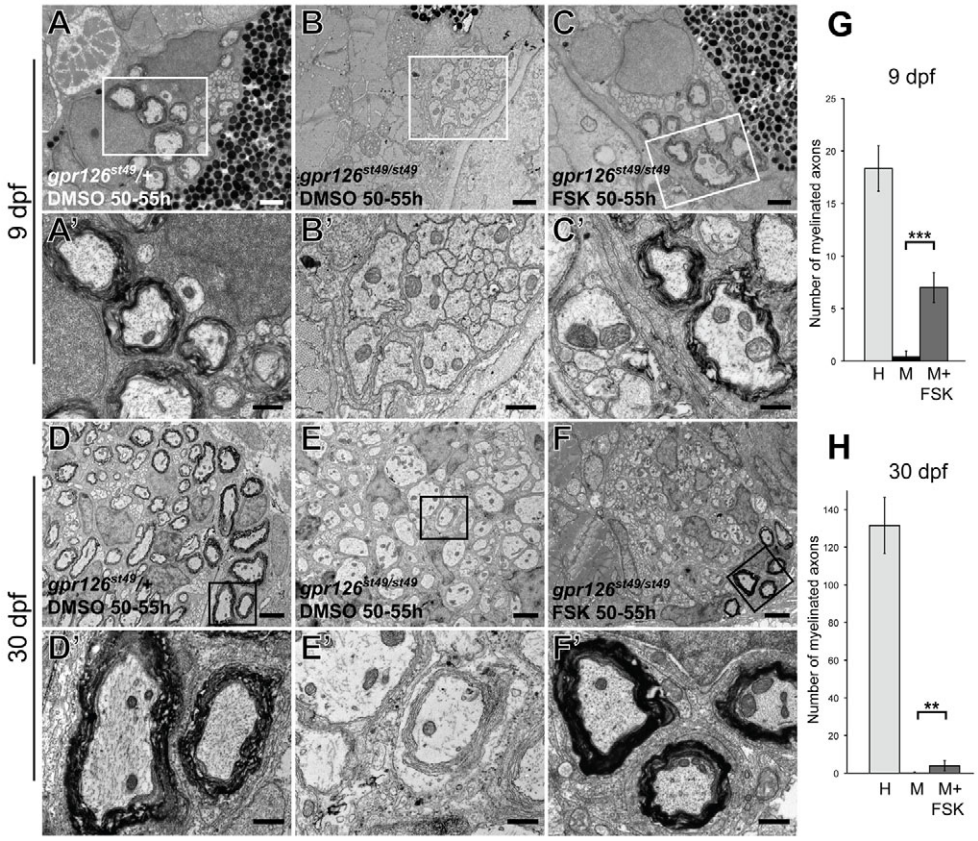

Fig. 1. Schwann cell membrane wrapping, compaction, and the maturation of the myelin sheath proceed in *gpr126* mutant nerves after transient elevation of cAMP. (**A-C′**) TEM images of transverse sections through zebrafish larvae at 9 dpf showing the ultrastructure of the PLLn one week following a pulse treatment with either DMSO (A,B) or 50 µM FSK (C). (A) *gpr126*^{st49}/+ control DMSO-treated fish have normal myelination in the PLLn. (B) The PLLn of *gpr126*$^{st49/st49}$ mutant larvae has no myelinated axons at 9 dpf. (C) The PLLn of *gpr126*$^{st49/st49}$ mutant larvae that were treated with FSK from 50 to 55 hpf contains some rescued myelinated axons. A′-C′ show higher magnification TEM images of the boxed regions in A-C, respectively. (**D-F′**) TEM images of transverse sections through juvenile zebrafish at 30 dpf showing the ultrastructure of the PLLn four weeks following a pulse treatment with either DMSO (D,E) or 50 µM FSK (F). (D) *gpr126*^{st49}/+ control DMSO-treated fish have normal myelination in the PLLn at 30 dpf. (E) The PLLn of *gpr126*$^{st49/st49}$ mutant fish has no myelinated axons at 30 dpf. (F) The PLLn of *gpr126*$^{st49/st49}$ mutant fish that were treated with FSK from 50 to 55 hpf contains a subset of rescued myelinated axons. D′-F′ show higher magnification TEM images of the boxed regions in D-F, respectively. (**G**,**H**) Quantification of the number of myelinated axons present per PLL nerve in heterozygous (H), mutant (M) and FSK-treated mutant (M+FSK) nerves at 9 dpf (G) and 30 dpf (H). Error bars represent s.d. Significance with two-tailed Student's *t*-test: ***P<0.001, **P<0.01. Scale bars: 1 µm in A-C; 0.5 µm in A′-C′,D′-F′; 2 µm in D-F.

9 dpf (Fig. 1B), mutants treated with FSK from 50 to 55 hpf contained an average of seven myelinated axons in the PLLn (Fig. 1C,G). Control heterozygous siblings had an average of 18 myelinated axons per PLLn (Fig. 1A,G). No additional axons were myelinated after the drug treatment, so that FSK-treated mutants never had as many myelinated axons as heterozygous siblings at 9 dpf. These results suggest that the myelinated axons observed in the FSK-treated mutants represent the subset of Schwann cells that were competent to respond to cAMP during the 50-55 hpf treatment period. Although the FSK-treated *gpr126* mutants had fewer total myelinated axons than heterozygous siblings, the myelin that was present in the mutants was indistinguishable from wild type (compare Fig. 1A′ and 1C′). These results indicate that myelination is able to proceed normally in *gpr126* mutants, provided that the block at the promyelinating stage is overcome with a pulse of cAMP. Our results from the FSK pulse-chase experiment show that in the week following drug treatment, similar numbers of myelin wraps were added in FSK-treated *gpr126* mutant fish compared with their heterozygous siblings.

We next sought to determine whether the rescued myelin in FSK-treated *gpr126* mutants is able to mature and persist in the absence of Gpr126 signaling. This experiment was possible because *gpr126*$^{st49/st49}$ mutant zebrafish are viable despite the lack of peripheral myelin (Monk et al., 2009). We treated embryos with FSK from 50 to 55 hpf and allowed the fish to recover for 4 weeks before fixing at 30 dpf for TEM analysis. Whereas control heterozygous fish contained many myelinated axons in the PLLn at 30 dpf (Fig. 1D,H), the PLLn of mutants treated with DMSO remained unmyelinated at 30 dpf (Fig. 1E). In these unmyelinated nerves, there were many large caliber axons that had been successfully sorted into a 1:1 relationship with Schwann cells, but all of these Schwann cells arrested at the promyelinating stage (Fig. 1E′). By contrast, *gpr126* mutants that received a FSK pulse from 50 to 55 hpf had PLL nerves that contained a small number of myelinated axons, which were typically located at the periphery of the nerve (Fig. 1F). The number of myelinated axons observed in FSK-treated mutants at 30 dpf (mean=4) was slightly less than that observed at 9 dpf (Fig. 1G,H). The PLLn undergoes dramatic

postembryonic remodeling (Ghysen and Dambly-Chaudière, 2007; Raphael et al., 2010), with many axons branching off to innervate sensory neuromasts. Therefore, it is likely that the difference between seven and four myelinated axons at 9 dpf and 30 dpf, respectively, reflects the changing anatomy of the nerve during that period rather than a loss of myelinated axons. Strikingly, the myelin observed in FSK-treated mutants at 30 dpf was thick and compact, and was indistinguishable from wild-type myelin (compare Fig. 1D′ and 1F′). Furthermore, the rescued myelin in FSK-treated *gpr126* mutants at 30 dpf was thicker and more compact than that observed in FSK-treated mutants at 9 dpf (compare Fig. 1F′ and 1C′). These results indicate that Gpr126 signaling is specifically required at the onset of myelination: if the initiation block in *gpr126* mutants is overcome with a transient increase in cAMP, Gpr126 signaling is not required for the maturation or maintenance of compact myelin for at least one month.

krox20 expression is maintained independently of Gpr126 signaling

Although *krox20* is not expressed in *gpr126* mutants (Monk et al., 2009), our results described above suggested that *krox20* might be expressed and maintained after rescue of *gpr126* mutant fish with cAMP. Embryos from a *gpr126*^{st49}/+ intercross were treated with DMSO or 50 μM FSK from 50 to 55 hpf, and raised in the absence of the drug. At time points 3 and 6 months later, PLL nerves were dissected from mutants and heterozygous siblings, RNA was extracted to make cDNA and RT-PCR analysis was performed. Control-treated and FSK-treated heterozygous siblings showed robust expression of *krox20* and *myelin basic protein* (*mbp*) in their PLLn, whereas nerves from control-treated *gpr126* mutant fish showed nearly undetectable levels of *krox20* and *mbp* (Fig. 2A,B). By contrast, nerves from FSK-treated *gpr126* mutants showed significant levels of both *krox20* and *mbp* expression (Fig. 2A,B). The expression of both of these markers was much lower than in heterozygous siblings, as expected because myelination was rescued in only a subset of axons in these fish (Fig. 1F). *sox10* was expressed at similar levels in Schwann cells of both wild type and *gpr126* mutants, as expected from previous studies (Monk et al., 2009). These results indicate that although *krox20* is not normally expressed in *gpr126* mutant nerves, a pulse of cAMP from 50 to 55 hpf is capable of not only rescuing *krox20* expression in larval myelinating Schwann cells, but also maintaining this expression in *gpr126* mutant nerves, even six months following the drug treatment.

Activity of the MSE parallels the initiation, but not the maintenance, of myelination

The MSE is a 1.3-kb enhancer located ~35 kb downstream of the transcriptional start site of the mouse *Krox20* gene (Ghislain et al., 2002). Reporter construct analyses have shown that the MSE drives expression in myelinating Schwann cells, similar to *Krox20* expression (Ghislain et al., 2002). Using the MSE from mouse, we generated a transgenic line of zebrafish carrying an MSE:*EGFP* expression construct. Schwann cells expressed the MSE:*EGFP* transgene, indicating conserved regulatory function (Fig. 3). EGFP was also expressed in muscle pioneer cells, the heart, the autonomic nervous system, some neuromasts, and neural progenitors in the spinal cord (not shown). The expression in these other cell types could be due to leaky activity of the MSE, or to background from the expression vector as described in other studies (Clarke et al., 2012). EGFP expression was first detected in Schwann cells of the PLLn at 60 hpf (Fig. 3A), when *krox20* mRNA is first detected in Schwann

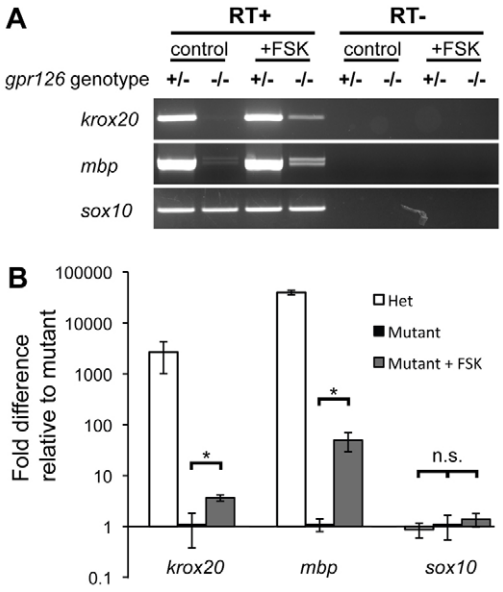

Fig. 2. The maintenance of *krox20* and *mbp* expression does not require Gpr126 signaling. (**A**) RT-PCR from dissected PLL nerves from 3-month-old *gpr126*^{st49}/+ and *gpr126*$^{st49/st49}$ mutant fish showing expression of *krox20*, *mbp* and *sox10*. Fish received either a control pulse of DMSO or 50 μM FSK from 50 to 55 hpf. Mutants that received the FSK treatment show continued expression of *krox20* and *mbp* at 3 months of age, whereas control treated mutants show nearly undetectable levels of both genes. *sox10* was expressed at the same level in all samples. (**B**) qPCR analysis of the mRNA expression levels of *krox20*, *mbp* and *sox10* in dissected PLL nerves from 6-month-old zebrafish that received treatments as described in A. Fold differences are reported relative to the mutants. Note that the scale is logarithmic. The fold difference between FSK-treated mutants and control mutants for *krox20* and *mbp* was 3.7 and 49.7, respectively. Significance with two-tailed Student's *t*-test: **P*<0.05; n.s., not significant. Error bars represent s.e.m.

cells (Monk et al., 2009), and increased over the next 30 days of development (Fig. 3A-C). In contrast to the robust expression during the initiation of myelination, EGFP was barely detectable in the PLL nerve at 6 and 10 months, although the nerve was heavily myelinated (Fig. 3D-F). New axons join the PLLn at different stages spread over several months (Ghysen and Dambly-Chaudière, 2007), resulting in a staggered pattern of myelination (supplementary material Fig. S1). The pattern of MSE:*EGFP* expression suggests that the reporter marks Schwann cells in the initiation phase, and declines thereafter, so that there are nearly undetectable levels of EGFP in the PLLn by 6 months (Fig. 3D,E). If MSE activity corresponds to the initiation of myelination, one would expect nerves that are myelinated later in development to show MSE:*EGFP* expression at those later stages. Accordingly, we observed EGFP expression in Schwann cells of nerves in the pectoral fin beginning at 30 dpf, as well as along axons innervating lateral line stitches (supplementary material Fig. S2), which do not begin to form until the juvenile stage (Ghysen and Dambly-Chaudière, 2007). We conclude that MSE activity is high during the initiation of myelination, but much lower during myelin maintenance.

The initiation of MSE activity requires Gpr126 signaling

Because the MSE marks Schwann cells that are initiating myelination, we reasoned that Gpr126 signaling would be required

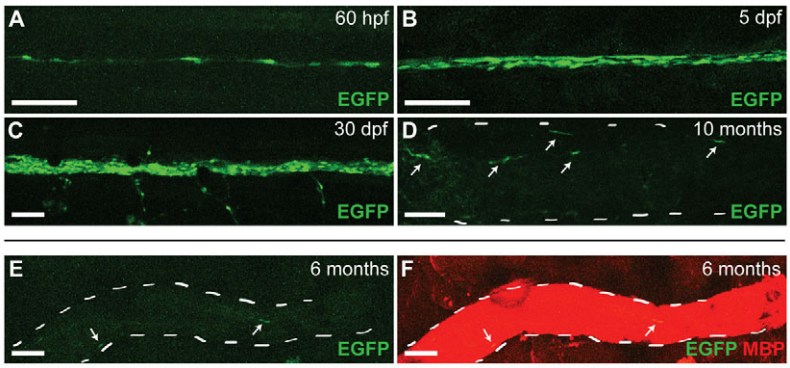

Fig. 3. The MSE is highly active in Schwann cells during the initiation of myelination, but activity is downregulated in the mature nerve. (**A-D**) Lateral views (anterior to the left, dorsal to the top) of wild-type zebrafish imaged live at the indicated stage carrying the MSE:*EGFP* transgene. EGFP expression is first detected in the PLL nerve at 60 hpf (A), and increases by 5 dpf (B) and 30 dpf (C). At 10 months, only a few Schwann cells express EGFP (D). (**E**,**F**) Lateral view of the PLLn from a wild-type fish fixed and stained for MBP expression at 6 months. EGFP expression is nearly undetectable (E), although the nerve is heavily myelinated at this time as indicated by MBP staining (F). The white dashed lines in D-F outline the nerve, and the white arrows mark EGFP-positive Schwann cells. *n*=at least five transgenic fish at each stage shown. Scale bars: 50 µm.

to activate the MSE. At 6 dpf, when heterozygous siblings showed EGFP expression in the PLL and motor nerves (Fig. 4A), transgenic *gpr126* mutant larvae showed no EGFP expression in Schwann cells along the PLL or motor nerves (Fig. 4B), suggesting that Gpr126 signaling activates *krox20* expression through the MSE, probably via the intermediary action of *oct6* (Ghislain and Charnay, 2006). Upon FSK treatment, however, *gpr126* mutant larvae expressed EGFP in Schwann cells along the PLLn (Fig. 4D). Schwann cells along motor nerves did not express detectable EGFP. The drug treatment period of 50-55 hpf corresponds to the time when the very first myelin is observed in the PLLn in wild type, but not yet in motor nerves, suggesting that Schwann cells along motor nerves are not yet competent to respond to cAMP at 50-55 hpf. Heterozygous larvae showed no change in MSE activity after treatment with FSK (Fig. 4C). Although FSK-treated mutants continued to express EGFP in Schwann cells of the PLLn at 11 dpf (Fig. 4F), expression was not detected by 20 dpf (Fig. 4G). This provides further evidence that MSE activity marks the initiation, but not the maintenance phases of myelination, and also suggests that MSE activity is markedly reduced in Schwann cells ~2-3 weeks after initiation.

Activated PKA rescues *myelin basic protein* expression in *gpr126* mutants

Because the effects of FSK are global, cAMP levels are increased in a wide variety of cell types in zebrafish embryos during treatment with the drug. PKA is activated by cAMP in many GPCR signaling cascades (Rosenbaum et al., 2009), and has been implicated in Schwann cell myelination (Howe and McCarthy, 2000; Yoon et al., 2008). In order to determine which cell type is responding to this increase in cAMP, we used the expression of a constitutively active form of PKA (PKA^{act}) (Orellana and McKnight, 1992) under the control of cell type-specific regulatory sequences. We generated expression constructs using the *sox10* promoter, which is expressed in Schwann cells and neural crest (Carney et al., 2006), and the *huC*

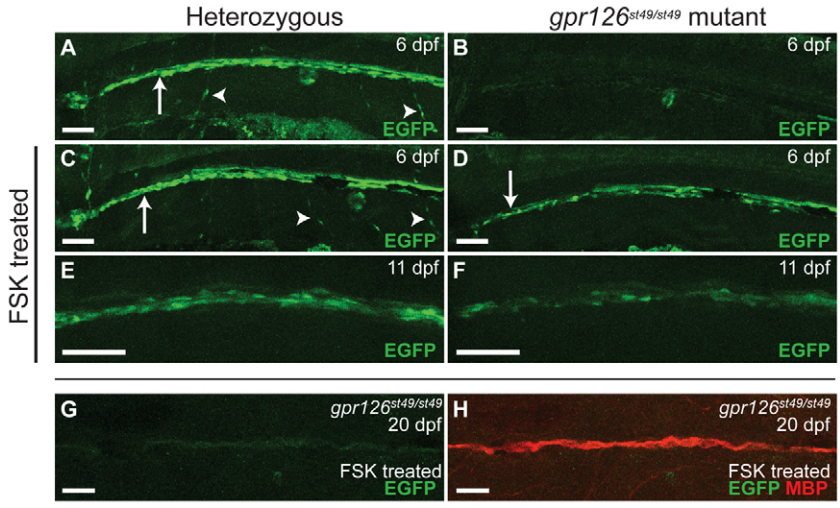

Fig. 4. The MSE is not active in Schwann cells of *gpr126* mutants, but activity is restored during the initiation of myelination after elevating cAMP. (**A-F**) Lateral views (anterior to the left, dorsal to the top) of zebrafish larvae imaged live at the indicated stage carrying the MSE:*EGFP* transgene. Heterozygous fish are in the left column and mutants in the right column. White arrows in A, C and D indicate Schwann cells along the PLLn. Arrowheads in A and C indicate Schwann cells along motor nerves. (A) EGFP-positive Schwann cells are present in $gpr126^{st49}$/+ fish (*n*=10/10 transgenic fish). (B) Mutant larvae do not express EGFP in Schwann cells (*n*=0/6 transgenic fish). (C) Elevating cAMP with a pulse of FSK from 50 to 55 hpf has no effect on MSE:*EGFP* expression in $gpr126^{st49}$/+ fish (*n*=5/5 transgenic fish). (D) Elevating cAMP rescues the expression of MSE:*EGFP* in Schwann cells along the PLLn in mutant larvae treated with a FSK pulse from 50 to 55 hpf (*n*=5/5 transgenic fish). (E) Schwann cells along the PLLn continue to express EGFP at 11 dpf in $gpr126^{st49}$/+ control larvae (*n*=3/3 transgenic fish). (F) Schwann cells along the PLLn continue to express EGFP at 11 dpf in mutant larvae that were transiently treated with FSK from 50 to 55 hpf (*n*=7/7 transgenic fish). (**G**,**H**) Lateral view of the PLLn from a mutant larva that was treated with FSK from 50 to 55 hpf and then fixed and stained for MBP expression at 20 dpf. EGFP expression is nearly undetectable (G) but MBP expression is strong in rescued Schwann cells (H) (*n*=10). Scale bars: 50 µm.

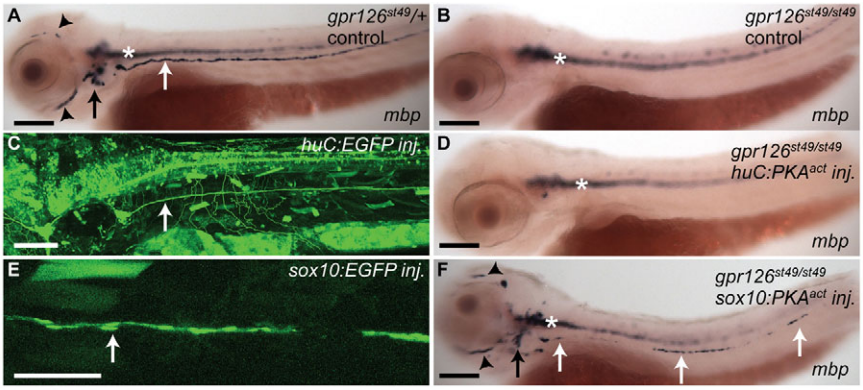

Fig. 5. Activated PKA expression in Schwann cells rescues *mbp* expression in *gpr126* mutants. (A,B,D,F) Lateral views of 3 dpf zebrafish embryos showing expression of *mbp* mRNA. (C,E) Confocal microscope images showing expression of the indicated *EGFP* reporter constructs. Anterior is to the left and dorsal to the top in all images. (**A**) *mbp* is expressed in the PNS of a control *gpr126*^{st49}/+ embryo, including the PLLn (white arrow), the anterior lateral line (black arrowheads) and cranial ganglia (black arrow) (*n*=20). Expression is also observed in the CNS (white asterisk). (**B**) *mbp* is expressed in the CNS (white asterisk) but not the PNS in a control *gpr126*$^{st49/st49}$ mutant embryo (compare expression in A with that in B) (*n*=10). (**C**) Zebrafish embryo (3 dpf) injected with the *huC:EGFP* reporter construct, which drives expression in the nervous system. The white arrow points to axons of the PLLn. Expression was observed in the PLLn of all embryos analyzed (*n*=8). (**D**) *mbp* expression in a *gpr126*$^{st49/st49}$ mutant embryo injected with the *huC:PKA*^{act} transgene. Expression is identical to that seen in control mutant embryos (compare D with B). No *mbp* expression was observed in the PNS (*n*=70). (**E**) 2 dpf zebrafish embryo injected with the *sox10:EGFP* reporter construct. White arrow points to Schwann cells along the PLLn. Expression in Schwann cells was observed in 13% (*n*=3/24) of injected embryos. (**F**) *mbp* expression in a *gpr126*$^{st49/st49}$ embryo injected with the *sox10:PKA*^{act} transgene. Rescued *mbp* expression is observed in clones of Schwann cells along the PLLn (white arrows), the anterior lateral line (black arrowheads) and cranial ganglia (black arrow). *mbp* expression was observed in the PNS in 15% (*n*=8/52) of injected embryos. Scale bars: 100 μm.

promoter, which is expressed in neurons (Park et al., 2000). Importantly, *sox10* is expressed in Schwann cells in *gpr126* mutants (Monk et al., 2009). Analysis of transient transgenic zebrafish injected with a *sox10:EGFP* reporter construct confirmed that the *sox10* promoter drives expression in Schwann cells (Fig. 5E). Transient transgenic analysis with the *huC:EGFP* construct confirmed that *huC* drives strong expression in neurons, including those of the lateral line system (Fig. 5C). We next performed transient transgenic rescue experiments using the expression constructs *sox10:PKA*^{act} and *huC:PKA*^{act}. When injected into *gpr126* mutant embryos, *sox10:PKA*^{act} was able to partially rescue *mbp* expression in peripheral nerves (Fig. 5F). The rescue was partial because the injection of DNA constructs results in mosaic expression in zebrafish embryos, such that a minority of Schwann cells will express the transgene. By contrast, mutant embryos injected with *huC:PKA*^{act} did not exhibit any *mbp* expression in the PNS (Fig. 5D). These results indicate that PKA^{act} is sufficient to rescue *mbp* expression in *gpr126* mutants when expressed in Schwann cells. Additionally, this result suggests that PKA is an important effector of Gpr126 signaling that functions downstream of the receptor in Schwann cells.

cAMP does not induce myelination in the absence of ErbB2 function

In order to test the ability of elevated cAMP to induce myelination in the absence of axonal Nrg1 signaling *in vivo*, we designed an experiment to test whether FSK treatment is sufficient to rescue myelination in *erbb2* mutants. *erbb2*$^{st61/st61}$ mutant zebrafish lack

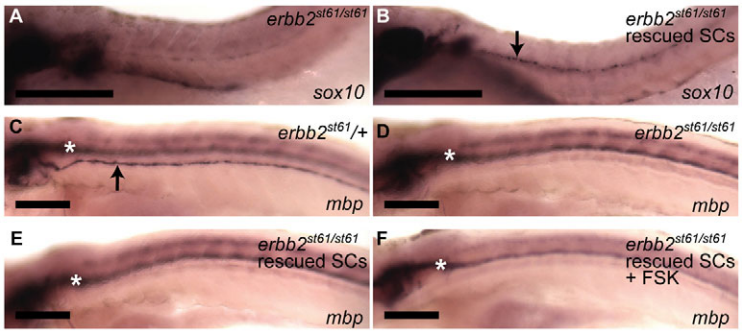

Fig. 6. cAMP does not rescue myelination in *erbb2* mutants. (**A**,**B**) Lateral views of zebrafish embryos at 2 dpf. Anterior is to the left and dorsal to the top in all panels. Schwann cells (marked by *sox10*, arrow in B) are not present along the PLLn in *erbb2*$^{st61/st61}$ mutants (A, *n*=15), but migration is rescued upon injection of *erbb2* mRNA (B, *n*=38). (**C-F**) Lateral views of zebrafish larvae at 5 dpf showing expression of *mbp* in (C) control *erbb2*^{st61}/+ (arrow indicates PLLn), (D) *erbb2*$^{st61/st61}$ mutant, (E) *erbb2*$^{st61/st61}$ mutant with rescued Schwann cell migration, and (F) *erbb2*$^{st61/st61}$ mutant with rescued Schwann cell migration that was also treated with a FSK pulse from 50 to 55 hpf. *mbp* expression was not observed in the PLLn of *erbb2*$^{st61/st61}$ mutants, even when Schwann cell migration was rescued with *erbb2* mRNA injection and cAMP was elevated by FSK treatment. White asterisks mark *mbp* expression in the CNS. The number of fish analyzed in C-F is: C, *n*=60; D, *n*=8; E, *n*=10; F, *n*=12. Scale bars: 100 μm.

peripheral myelin, and have defects in Schwann cell migration (Lyons et al., 2005). Schwann cells in *erbb2*$^{st61/st61}$ mutant embryos fail to migrate from the PLL ganglion, which precludes an analysis of myelination, but migration is rescued upon injection of *erbb2* mRNA (Lyons et al., 2005) (Fig. 6B). Although Schwann cell migration is rescued in these mutants, *mbp* expression is not (Fig. 6E), presumably because the *erbb2* mRNA has degraded before myelination begins. Embryos from an *erbb2*^{st61}/+ intercross were injected with *erbb2* mRNA to rescue Schwann cell migration, and were then treated with FSK from 50 to 55 hpf. Analysis of *mbp* expression in these mutants demonstrated that FSK is not sufficient to rescue *mbp* expression in Schwann cells in the absence of ErbB2 (Fig. 6F). Thus, elevated levels of cAMP cannot bypass the requirement for Nrg1 signaling through the ErbB2 receptor to initiate myelination *in vivo*.

Overexpression of NRG1 Type III does not rescue myelination in *gpr126* mutants

To determine whether activation of the Nrg1 signaling pathway is sufficient to rescue peripheral myelination in *gpr126* mutants, we analyzed fish containing a human *NRG1* type III transgene under control of the *UAS* promoter sequence (Perlin et al., 2011) in a *gpr126*$^{st49/st49}$ background. A previous study shows that this transgene causes ectopic Schwann cell migration as well as an expansion of *mbp* expression in zebrafish (Perlin et al., 2011). *gpr126*^{st49}/+*;UAS:hNrg1 type III* fish were crossed with transgenic fish harboring a *Gal4* transgene expressed in all neurons (Scott et al., 2007) in a *gpr126* mutant background. Progeny from this cross include *gpr126* mutant fish that also express human NRG1 type III in all neurons. Overexpression of human NRG1 type III was not sufficient to rescue the expression of *mbp* in the PLLn of *gpr126* mutants at 72 hpf (Fig. 7D). Furthermore, wild-type fish that overexpress NRG1 type III in neurons show *mbp* expression along motor nerves at 72 hpf (Fig. 7F), whereas non-transgenic fish do not (Fig. 7E). This precocious *mbp* expression in motor nerves in response to NRG1 also requires Gpr126, because *gpr126* mutants do not show *mbp* expression along motor nerves at 72 hpf (Fig. 7H). This indicates that strong Nrg1 signals can elicit the early expression of *mbp* in certain populations of Schwann cells, but only if these Schwann cells also have Gpr126 signaling function. In conjunction with the experiments shown in Fig. 6, this suggests that Nrg1 and Gpr126 act in two parallel signaling pathways that converge to control the expression of myelin genes, and that inputs from both pathways are required for the initiation of myelination *in vivo*.

DISCUSSION

Our results demonstrate that *gpr126* mutant Schwann cells are able to elaborate mature myelin sheaths over the course of one month, provided that the early signaling defect is bypassed by transiently elevating cAMP (Fig. 1). This indicates that Gpr126 functions during a specific temporal window at the onset of myelination and provides evidence that distinct signals control the initiation and maturation of myelin. Although *krox20* expression is not initiated in Schwann cells of *gpr126* mutants, its expression is maintained for months in Schwann cells lacking *gpr126*, provided they have been subjected to a transient increase in cAMP in the embryo (Fig. 2). Our analysis demonstrates that Gpr126 signaling through cAMP is specifically required at the onset of myelination to initiate *krox20* expression. The action of Gpr126 on *krox20* is likely to be indirect, because previous work in mouse has shown that Oct6 binding sites are present in the MSE, and mutation of these binding sites reduces expression of an MSE reporter (Ghislain and Charnay, 2006). The

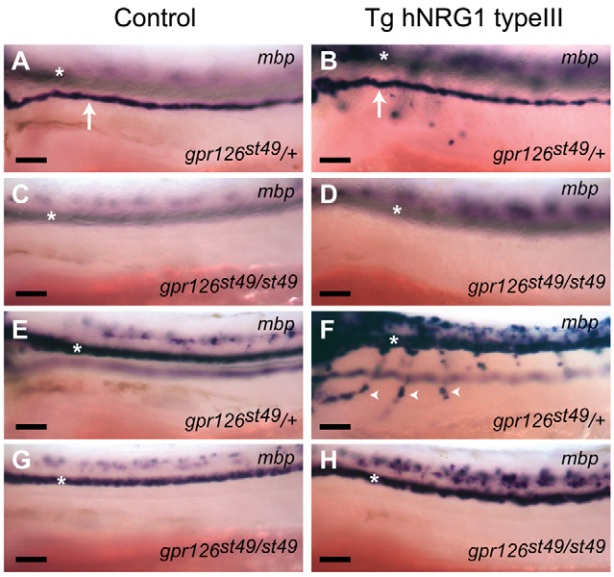

Fig. 7. Overexpression of NRG1 Type III does not rescue myelination in peripheral nerves of *gpr126* mutants. (**A-H**) Lateral views (anterior to the left, dorsal to the top) of *mbp* expression in control (A,C,E,G) or hNRG1 type III transgenic (B,D,F,H) embryos at 3 dpf. The white arrows in A and B mark *mbp*-expressing Schwann cells of the PLLn, and the white arrowheads in F mark Schwann cells along motor nerves. Control heterozygous embryos at 3 dpf express *mbp* in the PLLn (A) but not motor nerves (E) (*n*=35 fish), but transgenic embryos overexpressing hNRG1 type III show precocious *mbp* expression in motor nerves (F), in addition to expression in the PLLn (B) (*n*=89 fish). No expression in the PLLn or motor nerves was observed in *gpr126*$^{st49/st49}$ mutant embryos in either control (C,G; *n*=6 fish) or hNRG1 type III transgenic fish (D,H; *n*=29 fish), although *mbp* staining in the spinal cord was evident. White asterisks mark CNS expression in all panels. Scale bars: 50 μm.

failure to initiate *oct6* expression is the first known defect in *gpr126* mutant fish (Monk et al., 2009), and regulatory elements controlling *Oct6* expression in mouse Schwann cells are reported to contain cAMP response elements (Jagalur et al., 2011). The signals that activate Gpr126 and trigger myelination are not known, but recent work shows that other adhesion GPCRs interact with multiple ligands (Bjarnadóttir et al., 2007; Paavola and Hall, 2012), so it is possible that Gpr126 integrates multiple signals, which may be presented by the axon or other locations such as the extracellular matrix.

Distinct signaling pathways control the initiation and maintenance of *krox20* expression in myelinating Schwann cells

In addition to its important role in the initiation of myelination, *Krox20* is essential for myelin maintenance (Decker et al., 2006; Ghislain et al., 2002; Zorick et al., 1996). Our experiments, however, have demonstrated that Gpr126 signaling is not required for myelin maturation or for the maintenance of *krox20* expression. Thus, distinct mechanisms control the initiation versus the maintenance of *krox20* expression. Previous studies have identified the cis-regulatory MSE as an important regulator of *Krox20* expression in developing nerves in mice (Ghislain et al., 2002; Ghislain and Charnay, 2006). Similarly, we have found that the MSE drives expression in myelinating Schwann cells in zebrafish peripheral nerves (Fig. 3). In *gpr126* mutants, expression of an MSE:*EGFP* transgene is not activated, but expression is rescued

upon elevation of cAMP (Fig. 4). We found that the activity of the MSE marks Schwann cells during the initiation of myelination, but not myelin maintenance, because expression of the MSE:*EGFP* transgene declines several weeks after myelination is initiated. In mouse, an MSE:*lacZ* transgene is expressed in Schwann cells of the sciatic nerve at postnatal day (P)30, ~2-3 weeks after most Schwann cells have initiated myelination (Ghislain and Charnay, 2006; Ghislain et al., 2002). This is consistent with our data showing that high levels of MSE activity occur during the initiation of myelination and remain detectable for 2-3 weeks. Although we did not detect significant levels of EGFP expression in mature nerves, we cannot exclude the possibility that the MSE is active at very low levels.

The high levels of MSE activity we observe during the initiation of myelination are consistent with observations that the MSE contains multiple binding sites for the transiently expressed transcription factors Oct6 and Brn2 (Ghislain and Charnay, 2006), both of which are known to regulate the initiation of myelination (Bermingham et al., 1996; Jaegle et al., 2003; Jaegle et al., 1996). These data suggest that the long-term maintenance of *krox20* might involve other regulatory elements. One intriguing possibility is that *krox20* in Schwann cells could exhibit autoregulation, as has been reported for *Krox20* expression in the hindbrain (Ghislain et al., 2003). Our results define two phases of myelination. The initiation of myelination requires Gpr126 signaling activity and is associated with high activity of the MSE. The maintenance of myelination does not require Gpr126 signaling, and is associated with low levels of MSE activity.

During peripheral nerve development, many Schwann cell membrane wraps are added to the growing myelin sheath as the nerve matures. In the mammalian PNS, Nrg1/ErbB signaling is required for Schwann cells to produce a mature sheath of appropriate thickness (Garratt et al., 2000; Michailov et al., 2004), although Nrg1/ErbB signaling appears to be dispensable for maintenance of the mature myelin sheath in the absence of injury (Atanasoski et al., 2006; Fricker et al., 2011). We show that Schwann cell myelin maturation, compaction and maintenance occur independently of Gpr126 signaling. Interestingly, parallels between this uncoupling of myelin initiation and maturation have been reported in the mammalian central nervous system: inhibiting integrin signaling delays the initiation of myelination, but has no effect on the formation of the myelin sheath at subsequent stages (Câmara et al., 2009).

Gpr126 and the interpretation of axonal Nrg1 signals

GPCRs are known to trigger many downstream effects, including activation of the second messenger cAMP, which in turn activates PKA (Rosenbaum et al., 2009). Our experiments suggest that Gpr126 functions to activate PKA in Schwann cells (Fig. 5). One likely substrate of PKA is CREB, which is postulated to activate Oct6 expression in Schwann cells (Jagalur et al., 2011). ErbB receptors activate many downstream effectors, including PI3K and Akt (Nave and Salzer, 2006). Whether the downstream effectors of the Gpr126 and Nrg1 signaling pathways interact directly, or independently converge on common targets is unclear, but our experiments indicate that the over-activation of one pathway is not sufficient to initiate myelination in the absence of the other.

Several studies have suggested that different levels of axonal Nrg1 may direct different Schwann cell behaviors in a concentration-dependent manner, with lower levels supporting Schwann cell proliferation and migration, higher levels promoting radial sorting, and even higher levels initiating myelination (Michailov et al., 2004; Nave and Salzer, 2006; Taveggia et al., 2005). The level of axonal Nrg1 also determines the thickness of the myelin sheath (Michailov et al., 2004). It is not clear, however, whether different threshold responses underlie all of the distinct activities of Nrg1, and it has been suggested that cAMP may modulate the response of a Schwann cell to Nrg1 (Arthur-Farraj et al., 2011). One possibility is that cAMP signaling serves to amplify Schwann cell responses to Nrg1. cAMP has been shown to enhance the Nrg1-mediated activation of ERK and Akt in cultured Schwann cells (Monje et al., 2006), and PKA was shown to phosphorylate the ErbB2 receptor (Monje et al., 2008), which provides a possible mechanism for cross talk between the cAMP and Nrg1 signaling pathways. If cAMP functions *in vivo* to amplify the signaling downstream of ErbB2 in Schwann cells, one might predict that high levels of Nrg1 could bypass the requirement for cAMP and induce myelination in *gpr126* mutants. However, our results show that Schwann cells in *gpr126* mutant zebrafish do not express *mbp* even when axonal Nrg1 is overexpressed at high levels (Fig. 7). Thus, rather than simply amplifying the Nrg1 signal, Gpr126 might function as a switch that qualitatively changes the response of Schwann cells to Nrg1, thereby triggering the initiation of myelination at the appropriate developmental stage.

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Competing interests statement
The authors declare no competing financial interests.

Author contributions
T.D.G. performed all experiments. T.D.G. and W.S.T. analyzed the data and wrote the paper.

Supplementary material
Supplementary material available online at
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References

Arthur-Farraj, P., Wanek, K., Hantke, J., Davis, C. M., Jayakar, A., Parkinson, D. B., Mirsky, R. and Jessen, K. R. (2011). Mouse schwann cells need both NRG1 and cyclic AMP to myelinate. *Glia* **59**, 720-733.

Atanasoski, S., Scherer, S. S., Sirkowski, E., Leone, D., Garratt, A. N., Birchmeier, C. and Suter, U. (2006). ErbB2 signaling in Schwann cells is mostly dispensable for maintenance of myelinated peripheral nerves and proliferation of adult Schwann cells after injury. *J. Neurosci.* **26**, 2124-2131.

Bermingham, J. R., Jr, Scherer, S. S., O'Connell, S., Arroyo, E., Kalla, K. A., Powell, F. L. and Rosenfeld, M. G. (1996). Tst-1/Oct-6/SCIP regulates a unique step in peripheral myelination and is required for normal respiration. *Genes Dev.* **10**, 1751-1762.

Bjarnadóttir, T. K., Fredriksson, R. and Schiöth, H. B. (2007). The adhesion GPCRs: a unique family of G protein-coupled receptors with important roles in both central and peripheral tissues. *Cell. Mol. Life Sci.* **64**, 2104-2119.

Câmara, J., Wang, Z., Nunes-Fonseca, C., Friedman, H. C., Grove, M., Sherman, D. L., Komiyama, N. H., Grant, S. G., Brophy, P. J., Peterson, A. et al. (2009). Integrin-mediated axoglial interactions initiate myelination in the central nervous system. *J. Cell Biol.* **185**, 699-712.

Carney, T. J., Dutton, K. A., Greenhill, E., Delfino-Machín, M., Dufourcq, P., Blader, P. and Kelsh, R. N. (2006). A direct role for Sox10 in specification of neural crest-derived sensory neurons. *Development* **133**, 4619-4630.

Clarke, S. L., VanderMeer, J. E., Wenger, A. M., Schaar, B. T., Ahituv, N. and Bejerano, G. (2012). Human developmental enhancers conserved between deuterostomes and protostomes. *PLoS Genet.* **8**, e1002852.

Decker, L., Desmarquet-Trin-Dinh, C., Taillebourg, E., Ghislain, J., Vallat, J. M. and Charnay, P. (2006). Peripheral myelin maintenance is a dynamic process requiring constant Krox20 expression. *J. Neurosci.* **26**, 9771-9779.
Dutton, K. A., Pauliny, A., Lopes, S. S., Elworthy, S., Carney, T. J., Rauch, J., Geisler, R., Haffter, P. and Kelsh, R. N. (2001). Zebrafish colourless encodes sox10 and specifies non-ectomesenchymal neural crest fates. *Development* **128**, 4113-4125.
Fricker, F. R., Lago, N., Balarajah, S., Tsantoulas, C., Tanna, S., Zhu, N., Fageiry, S. K., Jenkins, M., Garratt, A. N., Birchmeier, C. et al. (2011). Axonally derived neuregulin-1 is required for remyelination and regeneration after nerve injury in adulthood. *J. Neurosci.* **31**, 3225-3233.
Garratt, A. N., Voiculescu, O., Topilko, P., Charnay, P. and Birchmeier, C. (2000). A dual role of erbB2 in myelination and in expansion of the schwann cell precursor pool. *J. Cell Biol.* **148**, 1035-1046.
Ghislain, J. and Charnay, P. (2006). Control of myelination in Schwann cells: a Krox20 cis-regulatory element integrates Oct6, Brn2 and Sox10 activities. *EMBO Rep.* **7**, 52-58.
Ghislain, J., Desmarquet-Trin-Dinh, C., Jaegle, M., Meijer, D., Charnay, P. and Frain, M. (2002). Characterisation of cis-acting sequences reveals a biphasic, axon-dependent regulation of Krox20 during Schwann cell development. *Development* **129**, 155-166.
Ghislain, J., Desmarquet-Trin-Dinh, C., Gilardi-Hebenstreit, P., Charnay, P. and Frain, M. (2003). Neural crest patterning: autoregulatory and crest-specific elements co-operate for Krox20 transcriptional control. *Development* **130**, 941-953.
Ghysen, A. and Dambly-Chaudière, C. (2007). The lateral line microcosmos. *Genes Dev.* **21**, 2118-2130.
Howe, D. G. and McCarthy, K. D. (2000). Retroviral inhibition of cAMP-dependent protein kinase inhibits myelination but not Schwann cell mitosis stimulated by interaction with neurons. *J. Neurosci.* **20**, 3513-3521.
Jaegle, M., Mandemakers, W., Broos, L., Zwart, R., Karis, A., Visser, P., Grosveld, F. and Meijer, D. (1996). The POU factor Oct-6 and Schwann cell differentiation. *Science* **273**, 507-510.
Jaegle, M., Ghazvini, M., Mandemakers, W., Piirsoo, M., Driegen, S., Levavasseur, F., Raghoenath, S., Grosveld, F. and Meijer, D. (2003). The POU proteins Brn-2 and Oct-6 share important functions in Schwann cell development. *Genes Dev.* **17**, 1380-1391.
Jagalur, N. B., Ghazvini, M., Mandemakers, W., Driegen, S., Maas, A., Jones, E. A., Jaegle, M., Grosveld, F., Svaren, J. and Meijer, D. (2011). Functional dissection of the Oct6 Schwann cell enhancer reveals an essential role for dimeric Sox10 binding. *J. Neurosci.* **31**, 8585-8594.
Jessen, K. R. and Mirsky, R. (2005). The origin and development of glial cells in peripheral nerves. *Nat. Rev. Neurosci.* **6**, 671-682.
Kawakami, K. (2005). Transposon tools and methods in zebrafish. *Dev. Dyn.* **234**, 244-254.
Li, Q., Ritter, D., Yang, N., Dong, Z., Li, H., Chuang, J. H. and Guo, S. (2010). A systematic approach to identify functional motifs within vertebrate developmental enhancers. *Dev. Biol.* **337**, 484-495.
Lyons, D. A., Pogoda, H. M., Voas, M. G., Woods, I. G., Diamond, B., Nix, R., Arana, N., Jacobs, J. and Talbot, W. S. (2005). erbb3 and erbb2 are essential for schwann cell migration and myelination in zebrafish. *Curr. Biol.* **15**, 513-524.
Lyons, D. A., Naylor, S. G., Mercurio, S., Dominguez, C. and Talbot, W. S. (2008). KBP is essential for axonal structure, outgrowth and maintenance in zebrafish, providing insight into the cellular basis of Goldberg-Shprintzen syndrome. *Development* **135**, 599-608.
Michailov, G. V., Sereda, M. W., Brinkmann, B. G., Fischer, T. M., Haug, B., Birchmeier, C., Role, L., Lai, C., Schwab, M. H. and Nave, K. A. (2004). Axonal neuregulin-1 regulates myelin sheath thickness. *Science* **304**, 700-703.
Monje, P. V., Bartlett Bunge, M. and Wood, P. M. (2006). Cyclic AMP synergistically enhances neuregulin-dependent ERK and Akt activation and cell cycle progression in Schwann cells. *Glia* **53**, 649-659.
Monje, P. V., Athauda, G. and Wood, P. M. (2008). Protein kinase A-mediated gating of neuregulin-dependent ErbB2-ErbB3 activation underlies the synergistic action of cAMP on Schwann cell proliferation. *J. Biol. Chem.* **283**, 34087-34100.
Monk, K. R., Naylor, S. G., Glenn, T. D., Mercurio, S., Perlin, J. R., Dominguez, C., Moens, C. B. and Talbot, W. S. (2009). A G protein-coupled receptor is essential for Schwann cells to initiate myelination. *Science* **325**, 1402-1405.
Monk, K. R., Oshima, K., Jörs, S., Heller, S. and Talbot, W. S. (2011). Gpr126 is essential for peripheral nerve development and myelination in mammals. *Development* **138**, 2673-2680.
Nave, K. A. and Salzer, J. L. (2006). Axonal regulation of myelination by neuregulin 1. *Curr. Opin. Neurobiol.* **16**, 492-500.
Orellana, S. A. and McKnight, G. S. (1992). Mutations in the catalytic subunit of cAMP-dependent protein kinase result in unregulated biological activity. *Proc. Natl. Acad. Sci. USA* **89**, 4726-4730.
Paavola, K. J. and Hall, R. A. (2012). Adhesion G protein-coupled receptors: signaling, pharmacology, and mechanisms of activation. *Mol. Pharmacol.* **82**, 777-783.
Park, H. C., Kim, C. H., Bae, Y. K., Yeo, S. Y., Kim, S. H., Hong, S. K., Shin, J., Yoo, K. W., Hibi, M., Hirano, T. et al. (2000). Analysis of upstream elements in the HuC promoter leads to the establishment of transgenic zebrafish with fluorescent neurons. *Dev. Biol.* **227**, 279-293.
Perlin, J. R., Lush, M. E., Stephens, W. Z., Piotrowski, T. and Talbot, W. S. (2011). Neuronal Neuregulin 1 type III directs Schwann cell migration. *Development* **138**, 4639-4648.
Raphael, A. R., Perlin, J. R. and Talbot, W. S. (2010). Schwann cells reposition a peripheral nerve to isolate it from postembryonic remodeling of its targets. *Development* **137**, 3643-3649.
Rosenbaum, D. M., Rasmussen, S. G. and Kobilka, B. K. (2009). The structure and function of G-protein-coupled receptors. *Nature* **459**, 356-363.
Scott, E. K., Mason, L., Arrenberg, A. B., Ziv, L., Gosse, N. J., Xiao, T., Chi, N. C., Asakawa, K., Kawakami, K. and Baier, H. (2007). Targeting neural circuitry in zebrafish using GAL4 enhancer trapping. *Nat. Methods* **4**, 323-326.
Seamon, K. B., Padgett, W. and Daly, J. W. (1981). Forskolin: unique diterpene activator of adenylate cyclase in membranes and in intact cells. *Proc. Natl. Acad. Sci. USA* **78**, 3363-3367.
Sherman, D. L. and Brophy, P. J. (2005). Mechanisms of axon ensheathment and myelin growth. *Nat. Rev. Neurosci.* **6**, 683-690.
Suter, U. and Scherer, S. S. (2003). Disease mechanisms in inherited neuropathies. *Nat. Rev. Neurosci.* **4**, 714-726.
Taveggia, C., Zanazzi, G., Petrylak, A., Yano, H., Rosenbluth, J., Einheber, S., Xu, X., Esper, R. M., Loeb, J. A., Shrager, P. et al. (2005). Neuregulin-1 type III determines the ensheathment fate of axons. *Neuron* **47**, 681-694.
Thisse, C. and Thisse, B. (2008). High-resolution in situ hybridization to whole-mount zebrafish embryos. *Nat. Protoc.* **3**, 59-69.
Topilko, P., Schneider-Maunoury, S., Levi, G., Baron-Van Evercooren, A., Chennoufi, A. B., Seitanidou, T., Babinet, C. and Charnay, P. (1994). Krox-20 controls myelination in the peripheral nervous system. *Nature* **371**, 796-799.
Westerfield, M. (2007) *The Zebrafish Book: A Guide for the Laboratory Use of Zebrafish (Danio rerio), 5th Edition*
Williams, P. R., Suzuki, S. C., Yoshimatsu, T., Lawrence, O. T., Waldron, S. J., Parsons, M. J., Nonet, M. L. and Wong, R. O. (2010). In vivo development of outer retinal synapses in the absence of glial contact. *J. Neurosci.* **30**, 11951-11961.
Yoon, C., Korade, Z. and Carter, B. D. (2008). Protein kinase A-induced phosphorylation of the p65 subunit of nuclear factor-kappaB promotes Schwann cell differentiation into a myelinating phenotype. *J. Neurosci.* **28**, 3738-3746.
Zorick, T. S., Syroid, D. E., Arroyo, E., Scherer, S. S. and Lemke, G. (1996). The transcription factors SCIP and Krox-20 mark distinct stages and cell fates in Schwann cell differentiation. *Mol. Cell. Neurosci.* **8**, 129-145.

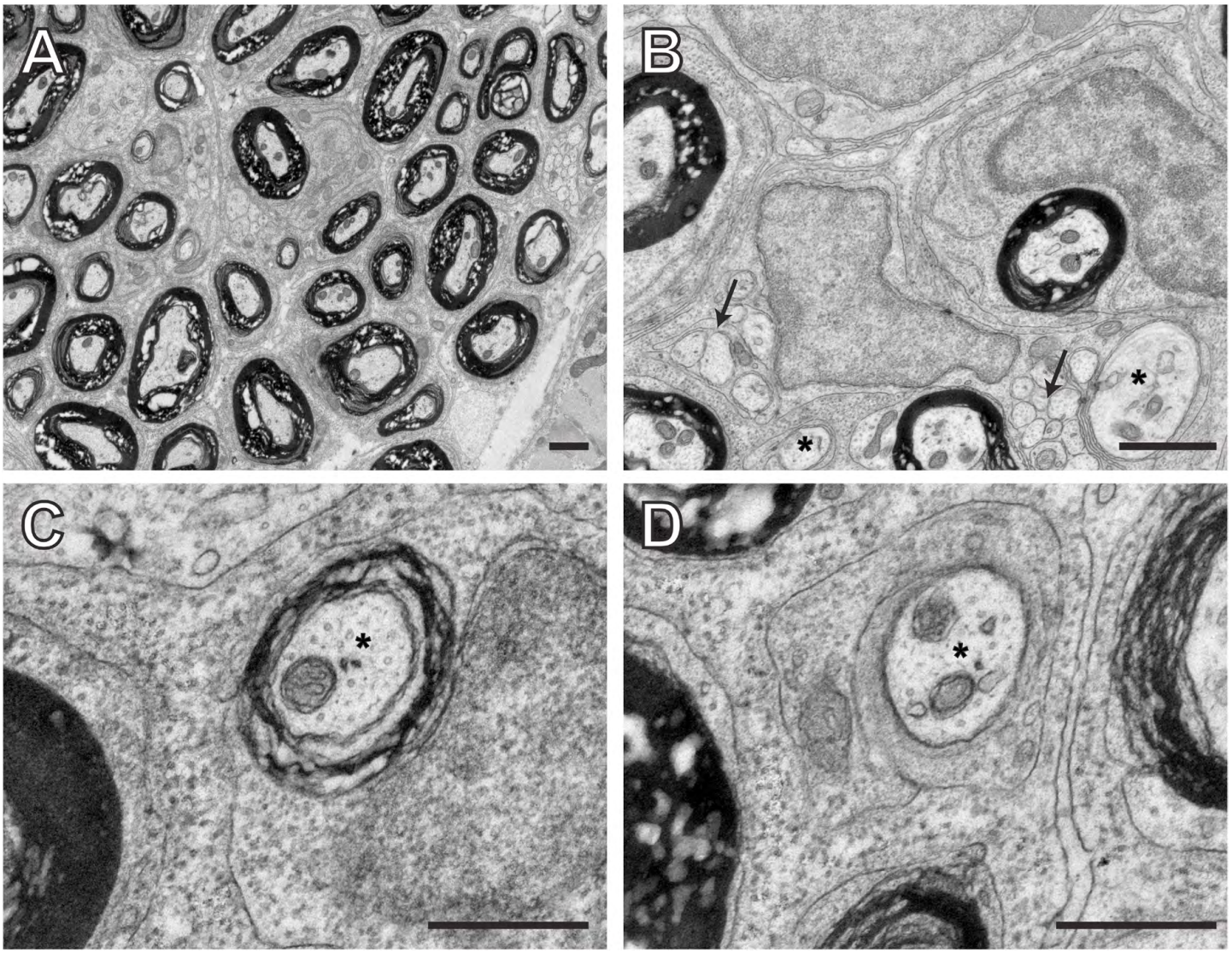

Fig. S1. Schwann cells at different developmental stages in the posterior lateral line nerve at 50 dpf. (**A-D**) TEM images of transverse sections through adult wild-type zebrafish at 50 dpf showing the ultrastructure of the PLL nerve. (A) Low magnification view of the PLLn showing many myelinated axons. (B-D) Higher magnification views of the PLLn show Schwann cells at multiple stages of development. The arrows in B mark groups of unsorted axons that are ensheathed by immature Schwann cells, and the asterisks mark sorted axons. Multiple myelinated axons are also visible in the same field. (C) A myelinating Schwann cell has elaborated several wraps around an axon (marked with asterisk) although the level of myelination is similar to that observed at earlier stages (compare with Fig. 1). (D) A Schwann cell has established a 1:1 relationship with an axon (marked with asterisk), but has not yet initiated myelination. Scale bars: 1 μM in A,B; 0.5 μM in C,D.

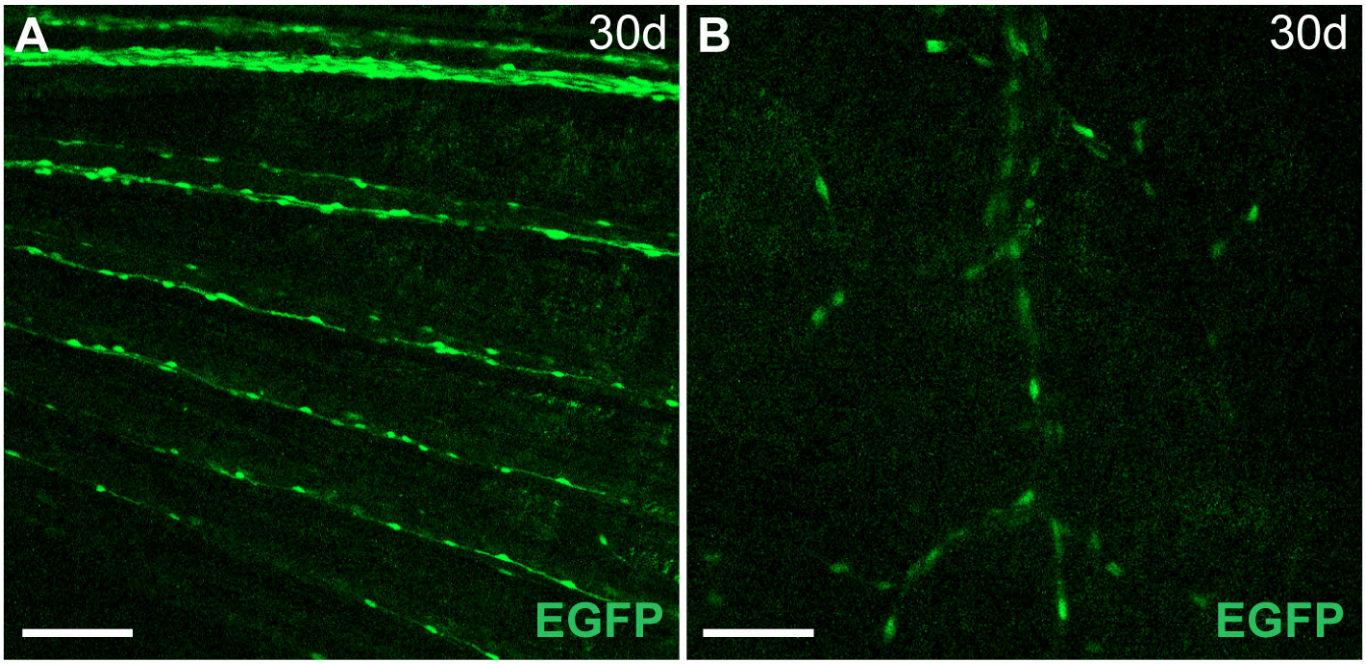

Fig. S2. Peripheral nerves that initiate myelination later in development exhibit MSE activity at the appropriate stages. (**A**) Schwann cells along nerves in the pectoral fin express MSE:*EGFP* at 30 dpf. (**B**) Schwann cells along axons innervating lateral line stitches, which do not form until the juvenile stage, express EGFP at 30 dpf. Anterior is to the left, and dorsal to the top in both images. Scale bars: 100 μM in A; 50 μM in B.