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) (Birmingham 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).

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<sup>g675</sup> and erb2<sup>367</sup> 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<sup>stop</sup> and erb2<sup>stop</sup> 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 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: ATGACAGCTA AAAACTTGGGAGAAGC and GTGACGAGGAGTGAGGAGTGG; mbp: AAGCACCTCTGGACAAAACC and CTGACTGCAACATGTGAAG; and sox10: TCTCAATGTTAGTGGCAAGG and TGACCTCACCCTCGTACAGG.

**Quantitative PCR (qPCR)**
P LL 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: GATAGACGCTATTGGGAGAAGG and GCGAGGATGCTGAGG; mbp: TCAATACGACAGTCTCTCG and CTGAAAGATGACAGCAGACG; sox10: AAGCGCGTCGTAGGCTTG; and EFla: AAGCACCTCTGGACAAAACC and CTGACTGCAACATGTGAAG.

**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'-caatagacagacaacctgggacgctgtctggatcatcatcgatTCTTGACGTACAAAGATGATATGATC-3' and 5'-CCCTTGGGCGCGGGTGCACTGTTCTCGGCGCCAC-3'. The MSE sequence was amplified by PCR from mouse genomic DNA using the following primers: 5'-GTCCTGAGGATGG; and 5'-GTGCCGACGAGG and TGACCTCACCCTCGTACAGG.

**Synthesis of mRNA for microinjection**
Synthetic mRNA encoding erb2 was prepared as described (Lyons et al., 2005). To generate transgenic zebrafish expressing huC:EGFP, sox10:EGFP, huC:PKA<sup>act</sup> or sox10:PKA<sup>act</sup>, 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).

**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 Axiosplan 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).

**Results**
**Gpr126 signaling activity is required specifically at the onset of myelination**
In gpr126 mutant zebrafish, treatment with forskolin (FSK), which activates adenylyl cyclase to increase cAMP (Seammon 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<sup>stop/+</sup> 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
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 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 gpr126st49/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<sup>49/+</sup> 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 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...
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 (PKAact) (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. 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 gpr126st61/+ 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) but not the PNS in a control gpr126st61/+ 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 all embryos analyzed (n=8). (D) mbp expression in a gpr126st61/+ mutant embryo injected with the huC:PKAact 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 gpr126st61/+ embryo injected with the sox10:PKAact 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.

Fig. 6. cAMP does not rescue 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 mutant zebrafish lack

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:PKAact and huC:PKAact. When injected into gpr126 mutant embryos, sox10:PKAact 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:PKAact did not exhibit any mbp expression in the PNS (Fig. 5D). These results indicate that PKAact 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.
Role of Gpr126 in myelination

Peripheral myelin, and have defects in Schwann cell migration (Lyons et al., 2005). Schwann cells in erbb2-2081a61 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 erbb2-2081/+ 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 gpr126st49/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). gpr126st49/+;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 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 GPCR 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 Bm2 (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; Michaelov 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 (Cámara 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 (Jagaur 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 (Michaelov et al., 2004; Nave and Salzer, 2006; Taveggia et al., 2005). The level of axonal Nrg1 also determines the thickness of the myelin sheath (Michaelov 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 Schwann cell responses to Nrg1. cAMP may 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 http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.093401/-/DC1

**References**


**Development**


