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A G Protein-Coupled Receptor is Essential for Schwann Cells to Initiate Myelination

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Abstract

The myelin sheath allows axons to rapidly conduct action potentials in the vertebrate nervous system. Incompletely understood axonal signals activate specific transcription factors, including Oct6 and Krox20, that initiate myelination in Schwann cells. Elevation of cAMP can mimic axonal contact in vitro, but the mechanisms that regulate cAMP levels in vivo are unknown. Using mutational analysis in zebrafish, we report that Gpr126 is required autonomously in Schwann cells for myelination. In *gpr126* mutants, Schwann cells failed to express *oct6* and *krox20*, and were arrested at the promyelinating stage. Elevation of cAMP in *gpr126* mutants, but not *krox20* mutants, could restore myelination. We propose that Gpr126 drives the differentiation of promyelinating Schwann cells by elevating cAMP levels, thereby triggering Oct6 expression and myelination.

During peripheral nervous system (PNS) development, promyelinating Schwann cells associate with one segment of an axon and differentiate into myelinating Schwann cells that iteratively wrap their membrane around an axonal segment to form the myelin sheath (1). Axonal signals transiently activate the expression of the transcription factor Oct6 in Schwann cells that will form myelin, and cAMP can mimic axonal contact in vitro (2,3). Oct6 regulates Krox20 expression (4), and both transcription factors are required for Schwann cells to initiate myelination (5–7). Neuregulin signals and their ErbB receptors are involved in regulation of *Oct6* and *Krox20* (8), but the signaling pathways in Schwann cells that regulate myelination are not well understood.

In a genetic screen for zebrafish mutants with abnormalities in myelinated axons, we previously identified two allelic mutations, *st49* and *st63*, in which Myelin basic protein (Mbp) expression was not observed in peripheral nerves (9). Central nervous system (CNS) Mbp expression and PNS axonal marker expression were unaffected (9;Fig. S1). Except for an enlargement of the ear that was evident at 5 days post fertilization (dpf), *st49* homozygous mutant larvae were morphologically indistinguishable from wild-type and heterozygous siblings (Fig. S2). High-resolution mapping experiments placed the *st49* mutation in a region of Linkage Group 20 (LG20) that contains *g-protein coupled receptor 126* (*gpr126)*, which encodes a member of the adhesion G-protein coupled receptor (GPCR)

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Supporting Online Material
Materials and Methods
Figs. S1 to S12

family (10,11). In addition to a type II seven transmembrane domain (7TM), Gpr126 contains CUB (Complement, Uegf, Bmp1), Pentraxin (PTX), and GPCR proteolytic site (GPS) domains (12; Fig. 1A). By sequencing, we identified lesions in *gpr126* in both mutations: *st49* introduces a premature stop codon before the GPS domain, and *st63* changes a highly conserved cysteine to a tyrosine in the 7TM domain (Fig. 1A). All mutants tested were homozygous for their respective lesions (*st49*, *n*=105; *st63*, *n*=122). In addition, injection of wild-type embryos with a morpholino that blocks splicing of *gpr126* pre-mRNA phenocopied *gpr126* mutants (Fig. 1B, C; Fig. S3). These results provide strong evidence that *gpr126* is the gene disrupted by the *st49* and *st63* mutations.

By in situ hybridization from 30 hours post fertilization (hpf) to 4 dpf, we observed expression of *gpr126* mRNA in Schwann cells of the posterior lateral line nerve (PLLn), a prominent sensory nerve in zebrafish (Fig. 1D-F), and in other sites (Fig. S4). Additionally, we detected *gpr126* expression in adult PLLn by RT-PCR (Fig. S4). Thus, *gpr126* is expressed in Schwann cells in the embryo, and this expression persists into adulthood.

To test whether Gpr126 was required autonomously in Schwann cells, we created genetic chimeras by transplanting fluorescently labeled wild-type cells into *gpr126*^{st49} mutant animals at blastula stages. In sensory and motor nerves of eleven such chimeras, Schwann cells expressed Mbp; in all of these cases, all Mbp-expressing Schwann cells were wild-type (Fig. 1G). In five of the eleven chimeras, we observed wild-type Schwann cells, but no wild-type neurons, associated with rescued nerves. This indicates that wild-type Schwann cells can activate Mbp expression when associated with *gpr126*^{st49} mutant neurons. In addition, we observed >40 cases in which wild-type neurons alone failed to rescue Mbp expression in *gpr126*^{st49} mutant Schwann cells (Fig. S6). In accord with the expression of *gpr126* in Schwann cells, the analysis of chimeras indicates that Gpr126 functions autonomously in Schwann cells.

To define the function of Gpr126 in myelination, we analyzed markers for different stages of Schwann cell development in *gpr126*^{st49} mutants. Expression of s*ox10*, which marks neural crest-derived cells, including Schwann cells, was not disrupted at any stage (Fig. 2A, B; Fig. S7). The earliest defect we noted was at 42–48 hpf, when PLLn Schwann cells begin to express *oct6* in wild-type larvae. We did not observe *oct6* expression in Schwann cells in *gpr126*^{st49} mutants at any stage (Fig. 2C, D; Fig. S7). Additionally, *krox20* expression was first detected in PLLn Schwann cells in wild-type larvae at 54–60 hpf, but was not observed in Schwann cells of *gpr126*^{st49} mutants (Fig. 2E, F; Fig. S7). This analysis indicates that *gpr126* is required for differentiating Schwann cells to initiate expression of *oct6* and *krox20*.

Neuregulin1 (Nrg) signaling through ErbB2 and ErbB3 receptors is required for many steps of Schwann cell development, including myelination (8;13–17). We therefore examined *erbb3* and *nrg1 type III* expression in *gpr126*^{st49} mutants and found that this expression was not disrupted (Fig. S8). Additionally, *gpr126* expression was not disrupted in the ganglia of *erbb2*^{st61} zebrafish mutants, in which Schwann cells reach the ganglia but do not co-migrate with growing axons of the PLLn (15; Fig. S8). These data show that Gpr126 does not regulate *erbb3* or *nrg1 type III* expression and that Nrg1/ErbB signals do not regulate *gpr126* expression; these signals may therefore function independently to regulate myelination.

Our marker studies indicated that early steps in Schwann cell development were normal in *gpr126*^{st49} mutants and suggested that Schwann cells are arrested at the promyelinating stage. To test this hypothesis, we examined the ultrastructure of *gpr126*^{st49} mutant PLL nerves by transmission electron microscopy (TEM). At 5 dpf in wild-type and *gpr126*^{st49}/+

heterozygotes, many PLLn axons were surrounded by several wraps of myelin (Fig. 3A, C). In contrast, no myelin was observed in the PLLn of *gpr126*^{st49} mutants (Fig. 3B, D). In the mutants, Schwann cells associated with axons, but myelination did not progress beyond 1.5 wraps of uncompacted Schwann cell cytoplasm. This phenotype was also evident in motor nerves (Fig. S9). Ultrastructural studies confirmed that early stages of Schwann cell development appeared normal in *gpr126*^{st49} mutants (Fig. S10). Additionally, total axon number and the number of large-caliber axons ensheathed by promyelinating Schwann cells were normal at all stages in *gpr126*^{st49} mutants (Fig. 3B; Fig. S10). Thus, *gpr126* is essential for promyelinating Schwann cells to initiate myelination in both sensory and motor nerves.

Homozygous *gpr126*^{st49} mutants survive to adulthood and are viable and fertile. To determine if a myelin defect persisted in adult *gpr126*^{st49} mutants, we examined PLL nerves at four months and six months of age. At both stages, dissected PLL nerves of *gpr126*^{st49} mutants were thinner and less opaque than PLL nerves from wild-type and *gpr126*^{st49}/+ zebrafish (Fig. S9). By TEM analysis, we found that axons in wild-type animals were surrounded by compact myelin at six months of age (Fig. 3E, G), but we found no evidence of compact myelin in *gpr126*^{st49} adult zebrafish (Fig. 3F, H) at the same stage. The absence of myelin at six months of age in *gpr126*^{st49} mutant PLL nerves indicates that the requirement for *gpr126* in Schwann cell myelination persists into adulthood.

In cultured Schwann cells, intracellular increase of cAMP mimics axonal contact and drives differentiation (18). However, the mechanism by which cAMP is elevated in Schwann cells in vivo is unknown. Many GPCRs signal through cAMP to activate downstream targets; we therefore treated *gpr126*^{st49} mutants with forskolin to elevate cAMP. Treatment with forskolin restored *oct6*, *krox20*, and Mbp expression (Fig. 4AL) and ultrastructurally normal myelin (Fig. S11) in *gpr126*^{st49} mutants, suggesting that Gpr126 functions to drive myelination by elevating cAMP levels in Schwann cells. Because *Krox20* is activated downstream of cAMP elevation, we would not expect forskolin treatment to rescue *Krox20* mutants. As a control, we therefore generated zebrafish *krox20* mutants by TILLING (19). Like murine *Krox20* mutants, Schwann cells in *krox20*^{fh227} mutants are arrested at the promyelinating stage (7; Fig. S9); as expected, forskolin treatment did not rescue Mbp expression in *krox20*^{fh227} mutants (Fig. 4M–P). These data support the hypothesis that Gpr126 functions in Schwann cells to elevate levels of cAMP, thereby activating *oct6* and *krox20* expression to initiate myelination.

We demonstrate that Gpr126 is essential for Schwann cells to initiate myelination. Like most adhesion GPCRs, Gpr126 is an orphan receptor that has not been shown to interact with G-proteins. Previously, a biochemical study raised the possibility that Gpr126 functions as a diffusible signal (20). Our data, however, suggest that Gpr126 acts as a receptor in Schwann cells that signals through G-proteins to transiently elevate cAMP. In Schwann cells, cAMP has been shown to activate a cascade including PKA, NF-κB, and CREB to induce transcription of *oct6* (21; Fig. S12). Our data show that Gpr126 acts autonomously in Schwann cells, and that forskolin treatment is sufficient to restore myelination in *gpr126*^{st49} mutants. We show that *gpr126* is expressed independently of Nrg1/ErbB signals, and propose that Gpr126 elevates cAMP in Schwann cells following axonal contact to trigger myelination.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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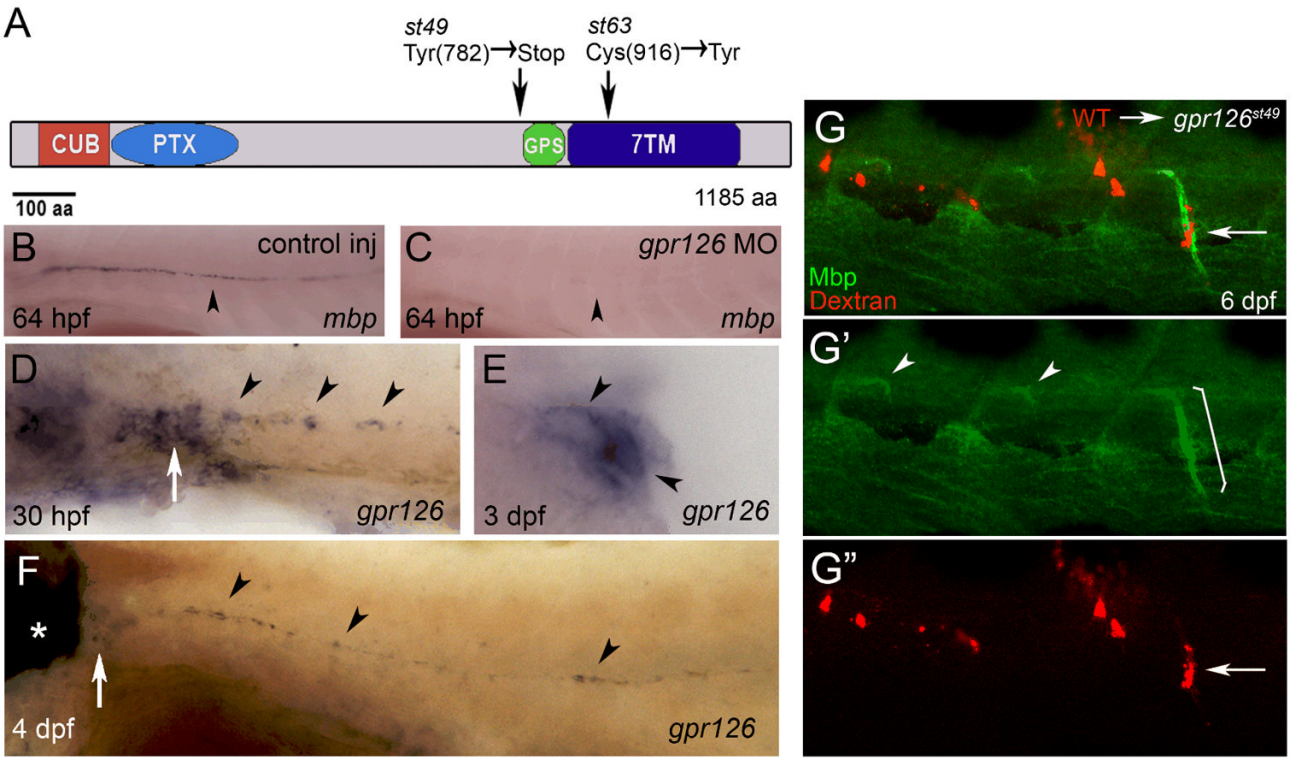

Fig. 1.

The *st49* and *st63* lesions disrupt zebrafish *gpr126*, which is required autonomously in Schwann cells for Mbp expression. (**A**) Schematic representation of Gpr126 showing functional domains and the lesions in the *st49* and *st63* mutations. (**B**) WT zebrafish embryo injected with control morpholino (MO) showing *mbp* mRNA in PLLn Schwann cells at 64 hpf (*n*=13/13). (**C**) WT zebrafish embryo injected with *gpr126* MO showing lack of *mbp* mRNA in PLLn Schwann cells at 64 hpf (*n*=19/21). (**D–F**) Expression of *gpr126* in wild-type (WT) larvae examined by whole-mount in situ hybridization. (**D**) *gpr126* mRNA in PLLn Schwann cells (arrowheads) and PLL ganglion (PLLg; arrow) at 30 hpf. (**E**) Cross-section of a WT larva at 3 dpf showing *gpr126* expression in PLLn Schwann cells (arrowheads). (**F**) *gpr126* expression in the ear (*), PLLg (arrow), and in PLLn Schwann cells (arrowheads) at 4 days postfertilization (dpf). For (**B–D** and **F**), lateral views are shown, with anterior left and dorsal top. (**G**) Lateral views at 6 dpf of a chimeric larva generated by transplantation of Texas red dextran-labeled WT cells (red) into a *gpr126*^{st49} mutant. WT Schwann cells (arrow in **G**, **G”**) express Mbp (green) when associated with mutant motor axons (bracket in **G′**). In motor nerves without transplanted WT Schwann cells, Mbp expression is only observed at motor nerve exit points as described in Fig. S1 (arrowheads, **G′**).

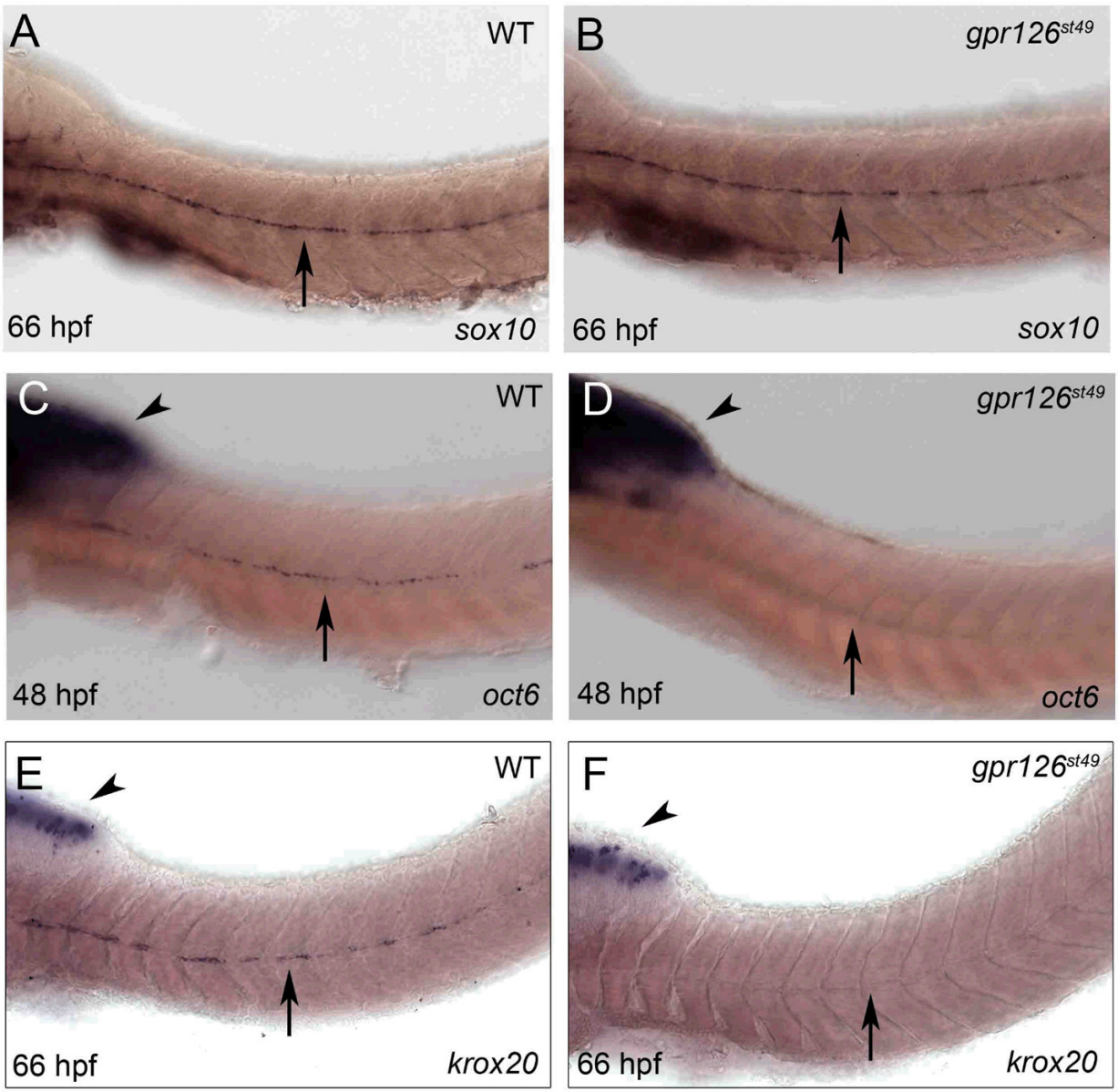

Fig. 2.

gpr126 is essential for expression of *oct6* and *krox20* in Schwann cells. Whole mount in situ hybridization of (**A**) WT and (**B**) *gpr126*^{st49} mutant zebrafish larvae at 66 hpf showing *sox10* mRNA expression in PLLn Schwann cells (arrow). (**C**) WT zebrafish embryo at 48 hpf showing *oct6* expression in the brain (arrowhead) and in PLLn Schwann cells (arrow) as previously described (22). (**D**) *gpr126*^{st49} mutant embryo at 48 hpf showing normal *oct6* expression in the brain (arrowhead) but not in PLLn Schwann cells (arrow). (**E**) WT zebrafish larva at 66 hpf showing *krox20* mRNA expression in the brain (arrowhead) and in PLLn Schwann cells (arrow). (**F**) *gpr126*^{st49} mutant larva at 66 hpf showing normal *krox20* expression in the brain (arrowhead) but not in PLLn Schwann cells (arrow). For (**A–F**), anterior is left and dorsal is up. Genotypes were determined by PCR after photography; see Fig. S7 for quantification of these experiments.

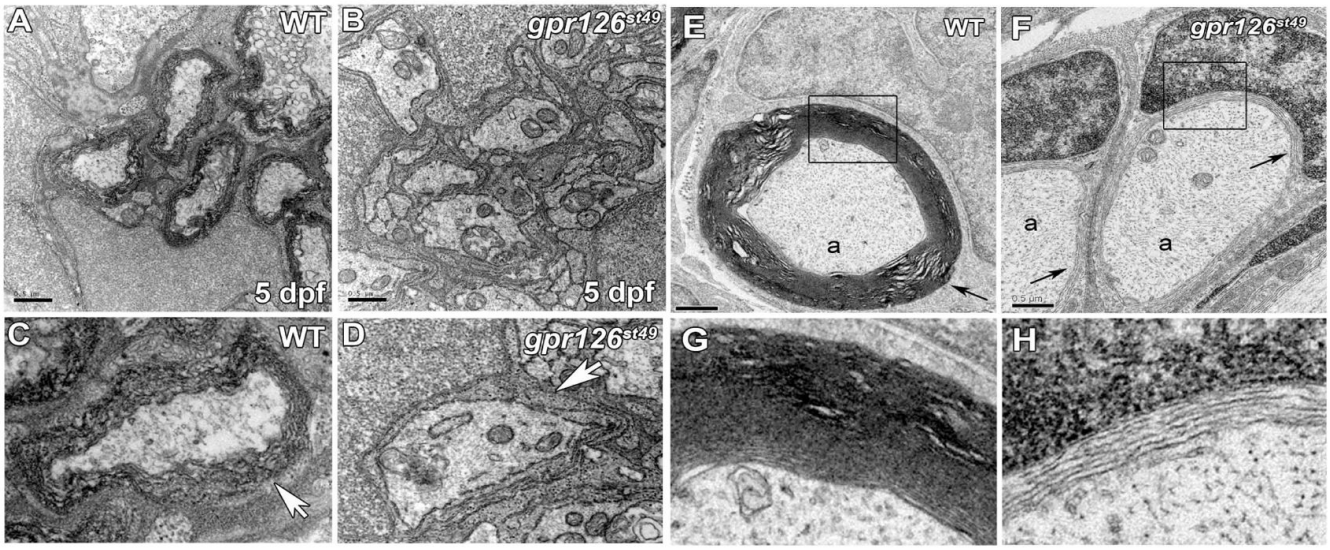

Fig. 3.

Schwann cells sort, but do not myelinate axons in *gpr126*^{st49} mutants. (**A**) Transmission electron micrograph showing cross-section through a WT PLLn at 5 dpf. (**B**) Transmission electron micrograph showing cross-section through a *gpr126*^{st49} mutant PLLn at 5 dpf. (**C**) Magnified view of a WT axon wrapped by several layers of myelin (arrow). (**D**) Magnified view of a *gpr126*^{st49} mutant axon showing that Schwann cell cytoplasm surrounds the axon (arrow), but does not turn more than 1.5 times around the axon. For (**A**, **B**), scale bar = 0.5 μm. (**E**) Transmission electron micrograph showing cross-section through a WT PLLn at 6 months of age. An axon (a) surrounded by compact myelin (arrow) is shown. (**F**) Transmission electron micrograph showing cross-section through a *gpr126*^{st49} mutant PLLn at 6 months of age. Axons (a) surrounded by a few loose wraps of Schwann cell cytoplasm (arrow) are shown. For (**E**) and (**F**), scale bar = 0.5 μm. (**G**) Magnified view of boxed region in (**E**) showing compact myelin surrounding an axon in WT PLLn. (**H**) Magnified view of boxed region in (**F**) showing loose Schwann cell cytoplasm surrounding an axon in *gpr126*^{st49} mutant PLLn. See Fig. S10 for quantification of these experiments.

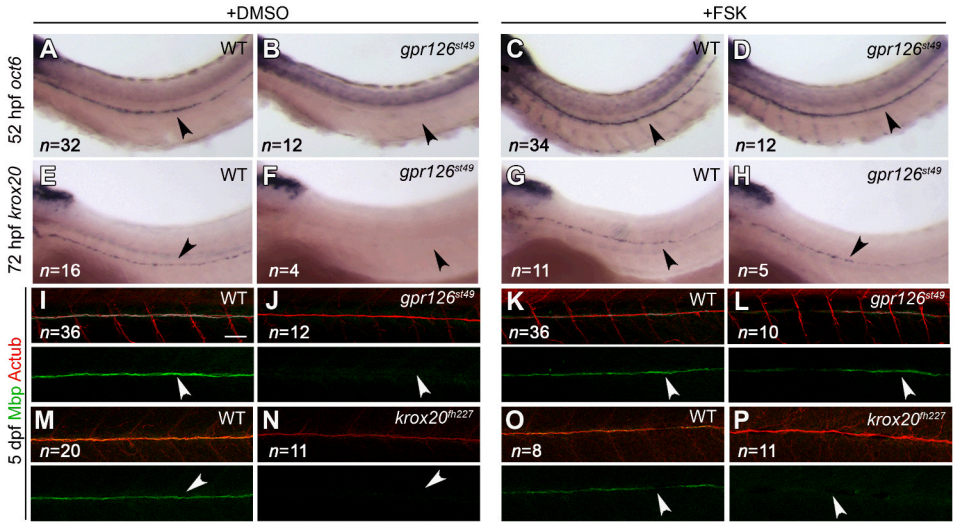

Fig. 4.

Treatment with forskolin rescues *gpr126*^{st49} mutant phenotypes. (**A–D**) *oct6* expression in 52 hpf zebrafish larvae treated with DMSO (+DMSO) or forskolin (+FSK) from 45–52 hpf. Schwann cells in *gpr126*^{st49} mutants express *oct6* after FSK treatment (**D**), but not after DMSO treatment (**B**). (**E–H**) *krox20* expression in 72 hpf zebrafish larvae treated with DMSO or FSK from 45–52 hpf. Schwann cells in *gpr126*^{st49} mutants express *krox20* after FSK treatment (**H**), but not after DMSO treatment (**F**). (**I–P**) Myelin basic protein (Mbp; green) and Acetylated Tubulin (AcTub; red) expression in 5 dpf zebrafish larvae treated with DMSO or FSK from 45–52 hpf. Schwann cells in *gpr126*^{st49} mutants express Mbp after FSK treatment (**L**), but not after DMSO treatment (**J**). Schwann cells in *krox20*^{fh227} mutants do not express Mbp after DMSO (**N**) or FSK (**P**) treatment. For (**A–P**), the PLLn is indicated with an arrowhead, and the number of larvae examined in each experiment is shown in the lower left of all panels. For (**I–P**), scale bar = 50 μm.