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swap70 Promotes neural precursor cell cycle exit and oligodendrocyte formation

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Abstract

Multipotent neural precursors produce oligodendrocyte lineage cells, which then migrate throughout the central nervous system and extend multiple, long membrane processes to wrap and myelinate axons. These dynamic cellular behaviors imply dynamic regulation of the cytoskeleton. In a previous microarray screen for new oligodendrocyte genes we identified *swap70*, which encodes a protein with domains that predict numerous signaling activities. Because mouse Swap70 can promote cell motility by functioning as a guanine nucleotide exchange factor for Rac1, we hypothesized that zebrafish Swap70 promotes oligodendrocyte progenitor cell (OPC) motility and axon wrapping. To test this we investigated Swap70 localization in OPCs and differentiating oligodendrocytes and we performed a series of gain and loss of function experiments. Our tests of gene function did not provide evidence that Swap70 regulates oligodendrocyte lineage cell behavior. Instead, we found that *swap70* deficient larvae had excess neural precursors and a deficit of OPCs. Cells associated with neural proliferative zones express *swap70*. Therefore, our data reveal a potential new role for Swap70 in regulating transition of dividing neural precursors to specified OPCs.

Keywords

Neural precursor; Oligodendrocyte; Glia; Zebrafish; Neurogenesis

Introduction

During vertebrate development, dividing neuroepithelial precursors produce first neurons and then glial cells. Diversity of neuronal and glial cell type is achieved, in part, by subdivision of the developing CNS into spatial domains of precursors that give rise to subsets of neurons and glia. In particular, pMN precursors of the spinal cord generate motor neurons and then oligodendrocyte progenitor cells (OPCs), which continue to divide as they migrate to become dispersed throughout the neural tube (Rowitch and Kriegstein, 2010). As OPCs migrate, they continuously extend and retract multiple long membrane processes, which might serve as a surveillance mechanism that influences the number of OPCs and their distribution (Kirby et al., 2006). Near the end of embryogenesis and continuing into the postnatal stage, a subset of OPCs stop dividing, wrap multiple neighboring axons with membrane and form myelin. Therefore, successful myelination requires integration of mechanisms that promote specification of OPCs from dividing, multipotent neural

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precursors, regulate OPC division to ensure formation of sufficient myelinating cells, guide OPCs to their target axons and mediate recognition and wrapping of axons by differentiating oligodendrocytes.

To search for factors that might be important for oligodendrocyte development, we performed a microarray screen that uncovered several genes expressed by oligodendrocyte lineage cells (Takada and Appel, 2010). Because of the highly dynamic nature of OPC membrane processes during migration and axon wrapping, we were particularly interested in genes that might control the cytoskeleton. One gene, *swap70*, seemed to be a good candidate because it encodes a protein that can bind the lipid second messenger phosphatidylinositol-3,4,5-triphosphate (PtdIns(3,4,5)P3) and can function as a GEF for Rac1 GTPase (Shinohara et al., 2002), which influences actin cytoskeletal rearrangements and motility (Bosco et al., 2009). To test the possibility that *swap70* gene function influences OPC behavior, we examined localization of fusion proteins and performed gain and loss-of-function experiments in zebrafish. Although we found evidence that Swap70 occupies both nuclear and cytoplasmic compartments, our data do not support the hypothesis that Swap70 regulates OPC motility or membrane extension and axon wrapping. Instead, we found that larvae lacking *swap70* function had a deficit of OPCs and myelinating oligodendrocytes. These larvae upregulated expression of *sox2*, a marker of neural precursor cells, and maintained elevated numbers of spinal cord cells in the cell cycle. Therefore, *swap70* function may be necessary for the transition of dividing precursors to specified neural cells.

Results

Neural precursors and oligodendrocyte lineage cells express swap70

To characterize *swap70* expression, we performed in situ RNA hybridization on zebrafish embryos collected at different stages of development. At 2 somite stage, which occurs about 10.5 hours post fertilization (hpf), the eye primordia prominently expressed *swap70*, whereas transcript levels appeared to be at lower levels throughout other regions of the embryo (Fig. 1A). At 20 hpf, *swap70* expression remained high within the developing eyes and was at high level in the pronephric ducts (Fig. 1B). By 50 hpf, cells occupying the ventral spinal cord expressed *swap70* (Figs. 1C,I) similar to the pattern of cells that expressed *sox10* (Figs. 1D,J), which marks newly specified OPCs. At this stage no spinal cord cells expressed *plp1a* (Fig. 1E), a marker of myelinating oligodendrocytes. At 80 hpf, cells in both ventral and dorsal spinal cords expressed *swap70* (Figs. 1F,K), consistent with the distribution of differentiating oligodendrocytes marked by *sox10* (Figs. 1G,L) and *plp1a* (Figs. 1H,M) expression. These data therefore indicate that OPCs initiate *swap70* expression prior to their differentiation as myelinating cells. We also noticed that cells lining the medial septum and central canal of the spinal cord at 50 hpf expressed *swap70* (Fig. 1I). Medial expression was maintained through 80 hpf but restricted to the ventral half of the spinal cord (Fig. 1K). The medial septum is lined initially by dividing neuroepithelial precursors and later radial glia. Similarly, *swap70* expression was evident at proliferative ventricular zones of the forebrain and hindbrain (Figs. 1N,O). These expression data indicate that at least a subset of zebrafish neural precursor cells express *swap70*. Notably, the hippocampus, subventricular zones, olfactory bulb and cerebellum of adult mouse brain appear to express *Swap70* (Lein et al., 2007) and Swap70 was purified from bovine brain lysate (Shinohara et al., 2002) raising the possibility that Swap70 has a role in neural precursor maintenance or specification.

To confirm that oligodendrocyte lineage cells express *swap70* we examined homozygous *sox10*^{tw11} mutant embryos and larvae. *sox10*^{tw11} mutants produce OPCs but the myelinating subpopulation of oligodendrocytes, which express *nkx2.2a*, die soon after initiation of axon

wrapping (Takada et al., 2010). *sox10*^{tw11} mutant larvae had a deficit of *swap70*$^{+}$ cells relative to wild type at 60 and 80 hpf (Fig. 2) consistent with the notion that myelinating oligodendrocytes express *swap70*. Notably, remaining OPCs of mutant larvae appeared to express *swap70* at normal levels. Therefore, *swap70* expression in oligodendrocyte lineage cells does not appear to be under control of the Sox10 transcription factor.

Swap70 accumulates at the plasma membrane to promote membrane ruffling (Shinohara et al., 2002) but also can move to the cell nucleus to influence B cell switching (Borggrefe et al., 1998; Borggrefe et al., 1999). To assess Swap70 localization in neural cells, we crossed fish carrying *Tg*(*UAS:cherry-swap70*), which encodes Cherry fluorescent protein fused to full-length Swap70, to *Tg*(*hsp70l:Gal4vp16*);*Tg*(*olig2:EGFP*) fish and induced expression in embryos using heat shock. This produced a mosaic pattern of expression within the spinal cord, which revealed that Cherry-Swap70 protein localized to the cytoplasm and not the nucleus of most cells (Figs. 3A,B). However, some cells usually associated with the proliferative zone of the medial spinal cord, including *olig2*:EGFP^{+} cells, had nuclear localized Cherry-Swap70 protein (Figs. 3A,B). These data raise the possibility that Swap70 can occupy different cellular compartments.

To more thoroughly investigate subcellular localization we next expressed Cherry-Swap70 in OPCs also expressing membrane-tethered GFP. To do so, we co-injected *UAS:cherry-swap70* plasmid with *sox10:Gal4vp16* plasmid into *Tg*(*nkx2.2a:mEGFP*) embryos, which express membrane-tethered EGFP in the myelinating subset of OPCs and oligodendrocytes (Kirby et al., 2006). In migrating OPCs, Cherry-Swap70 protein accumulated at the cell membrane and the tips of extending membrane processes (Fig. 4A). In myelinating oligodendrocytes, Cherry-Swap70 protein was apparent at the axon-ensheathing internode membrane (Fig. 4C). By comparison, when expressed alone Cherry fluorescent protein appeared to accumulate mostly in the soma of oligodendrocytes and, although evident in membrane processes, did not reveal ensheathing internodes as distinctly as the fusion protein (Fig. 4B). To attempt to identify Swap70 domains necessary for localization, we expressed deletion constructs. First, we deleted C-terminal sequence, which includes the Dbl-homology domain (Cherry-Swap70ΔGEF). This truncated fusion protein showed localization similar to that of full-length Cherry-Swap70 (Fig. 4D). By contrast, a fusion protein containing only the Pleckstrin Homology (PH) domain of Swap70 (Cherry-Swap70PH) was localized primarily to the cytoplasm and did not show significant internode accumulation (Fig. 4E). Therefore, Swap70 protein localizes to myelinating membrane processes of oligodendrocytes in a PH domain-dependent manner.

swap70 loss of function results in fewer OPCs

Expression of *swap70* by neural precursors and oligodendrocytes raised the possibility that it has distinct functional roles in specification or maintenance of precursors and in oligodendrocyte differentiation. However, expression of neither full-length nor deleted versions of Swap70 described above had any discernable effect on oligodendrocyte development. Therefore, we sought to further test these possibilities by reducing *swap70* function using an antisense morpholino oligonucleotide (MO) approach. We designed two different translation-blocking MOs (MO$^{swap70\text{-}UTR}$ and MO$^{swap70\text{-}ATG}$) and one splice-blocking MO (MO$^{swap70\text{-}sp}$). MO$^{swap70\text{-}sp}$ was designed to target the boundary between exon 5 and intron 5 with the prediction that it would cause exclusion of exon 5, which encodes a portion of the PH domain, from the processed mRNA (Fig. 5A). We tested this prediction using RT-PCR. Whereas primers that target sequences within exons 4 and 6 produced a band of the predicted size of 500 bp from control embryos, a smaller product of about 375 bp was produced by RT-PCR from embryos injected with MO$^{swap70\text{-}sp}$ (Fig. 5B). Sequence analysis showed that *swap70* mRNA modified by MO$^{swap70\text{-}sp}$ encodes a protein lacking the portion of the PH domain encoded by exon 5 (Fig. 5C). Because the PH domain

is necessary for PtdIns(3,4,5)P3 binding and Swap70 function (Shinohara et al., 2002), we expect that MO$^{swap70\text{-}sp}$ produces a loss-of-function effect.

At 3 dpf, larvae injected at single cell stage with up to 4 ng of any of the *swap70* MOs had no discernable morphological defects (Figs. 6A–C). To investigate the effect of *swap70* loss-of-function on oligodendrocyte development, we examined the number and distribution of oligodendrocyte lineage cells marked by EGFP encoded by a *Tg*(*olig2:EGFP*) transgene. This revealed that larvae injected with either MO$^{swap70\text{-}ATG}$ or MO$^{swap70\text{-}sp}$ had fewer dorsal spinal cord EGFP$^+$ cells then control larvae (Figs. 6A′–C″). We confirmed that injected larvae had statistically fewer oligodendrocyte lineage cells by counting Sox10$^+$ cells in the spinal cord (Figs. 6D,E). To further validate our MO approach, we tested the ability of Myc epitope-tagged Swap70 (Myc-swap70) encoded by synthetic mRNA to suppress the oligodendrocyte phenotype when co-injected with MO$^{swap70\text{-}SP}$. This partially restored the number of Sox10$^+$ cells at 3 and 4 dpf (Figs. 6F,G) indicating that the deficit of oligodendrocyte lineage cells following morpholino injection results specifically from loss of *swap70* function.

We noted that overexpression of Myc-Swap70 in the absence of MO$^{swap70\text{-}sp}$ had no effect on oligodendrocyte lineage cell number (Figs. 6F,G). To overcome the possibility that the absence of an effect resulted from the inability of the protein to persist until 3 dpf, we delayed the onset of Swap70 expression by incubating *Tg* (*UAS: Cherry-swap70*); *Tg*(*hsp70:Gal4vp16*) embryos at 39 °C for 30 min at 24 hpf and 48 hpf. At 3.5 dpf, the number of Sox10$^+$ OPCs was equivalent in Cherry-larvae, which did not overexpress Swap70, and in their sibling Cherry$^+$ larvae, which overexpressed Swap70 (Figs. 6H,I). Furthermore, Cherry-Swap70 overexpression did not change the distribution of Sox10$^+$ cells (Fig. 6I), indicating that it did not influence OPC migration. We conclude that *swap70* function is necessary for formation of the normal number of oligodendrocyte lineage cells but that overexpression is not sufficient to infiuence cell number or distribution.

Swap70 promotes exit of neural precursors from the cell cycle

The expression of *swap70* at neural proliferative zones and the reduction of OPC number following *swap70* knockdown raised the possibility that *swap70* is necessary for OPC production from neural precursors. We first examined localization of the cell polarity protein Protein kinase C, iota (Prkci) because *prkci* loss-of-function alters the numbers of spinal cord precursors and OPCs (Roberts and Appel, 2009), and Zrf-1 expression, which marks radial glia. Both Prkci and Zrf-1 labeling were indistinguishable between wild-type and MO$^{swap70\text{-}SP}$-injected larvae (Figs. 7A–D). Therefore, loss of *swap70* function does not alter the number, spatial organization and polarity of radial glia, which serve as neural precursors in late embryonic and early larval stages. We next examined expression of *sox2*, which marks neural precursors. By 2.5 dpf only a few cells bordering the spinal cord central canal of wild-type embryos expressed *sox2* (Fig. 7E) consistent with depletion of neural precursors by the end of embryogenesis. By contrast, many more medial spinal cord cells expressed *sox2* in MO$^{swap70\text{-}sp}$-injected embryos (Fig. 7F). As an additional test of the effect of *swap70* function on spinal cord precursors we investigated markers of the cell cycle. Both MO$^{swap70\text{-}ATG}$ and MO$^{swap70\text{-}sp}$-injected embryos had significantly more M-phase cells, marked by phosphohistone H3 labeling, and S-phase cells, marked by BrdU incorporation, than control embryos (Figs. 7G–K). We interpret these data to mean that in the absence of *swap70* function neural precursors fail to exit the cell cycle normally, resulting in a deficit of OPCs.

Swap70 is dispensable for oligodendrocyte migration and differentiation

OPCs continuously extend and retract multiple membrane processes as they migrate and settle among axon-rich regions of the CNS, prior to axon wrapping (Kirby et al., 2006). Because Swap70 function has been implicated in membrane motility and cell migration, we hypothesized that it would regulate OPC membrane activity necessary for axon wrapping and, consequently, myelination. To test this we reduced *swap70* function in *Tg*(*nkx2.2a:mEGFP*) embryos. However, both membrane processes of migrating OPCs and myelinating internodes of oligodendrocytes appeared normal in $MO^{swap70\text{-}sp}$ larvae (Figs. 8A–D). To examine OPC behavior more directly, we performed in vivo time-lapse imaging. No differences in direction or speed of OPC migration, membrane process extension and retraction and axon wrapping were evident between control and $MO^{swap70\text{-}sp}$ larvae (Supplementary Movies 1 and 2). We also investigated oligodendrocyte differentiation by performing in situ RNA hybridization to detect expression of *plp1a* and *mbp.* Although the numbers of cells that expressed these myelin gene markers in $MO^{swap70\text{-}sp}$ larvae were fewer than in wild type, consistent with the reduced number of oligodendrocyte lineage cells, both genes appeared to be expressed at normal levels (Figs. 8E–H) indicating that loss of *swap70* function does not alter oligodendrocyte differentiation.

Discussion

Oligodendrocyte lineage cells undergo an apparent step-wise progression from multipotent precursors to a post-mitotic, myelinating phenotype (Baumann and PhamDinh, 2001). These steps entail changes in cell cycle, motility, morphology and gene expression, implicating the action of a coordinated network of regulatory mechanisms. One of the principal mechanisms engages molecular signals transduced by receptor tyrosine kinases (RTKs). For example, Fgf receptors Fgfr1 and Fgfr2 are required for formation of OPCs in mouse embryonic forebrain (Furusho et al., 2011) and Fgf receptor signaling is necessary for development of zebrafish hindbrain oligodendrocytes (Esain et al., 2010). Once OPCs are formed, signaling mediated by the growth factor PDGF and its receptor PDGFRa promotes their proliferation (Calver et al., 1998). Fgfr signaling in oligodendrocyte formation appears to be conveyed by a Ras/MAPK pathway (Furusho et al., 2011; Kessaris, 2004) but the details of the downstream signal transduction mechanisms for RTK function in oligodendrocyte development remain largely unknown.

swap70, which we identified in a screen for genes expressed by oligodendrocyte lineage cells, encodes a protein that potentially mediates RTK signaling. The Swap70 protein has within it a pleckstrin homology domain, which binds PtdIns(3,4,5)P3 (PIP_3), a molecule produced from PIP_2 by PI3 kinase following activation by RTK signaling. Studies using cultured fibroblasts showed that mouse Swap70 can function as a PIP_3-dependent guanine nucleotide exchange factor (GEF) for Rac1 GTPase (Shinohara et al., 2002), which regulates various cell processes such as the cell cycle and motility. In fibroblasts Swap70 promoted membrane ruffling via Rac1 regulation of the actin cytoskeleton (Shinohara et al., 2002) and *Swap70* mutant B cells were less polarized, had unstable lamellipodia and inefficiently migrated into lymph nodes (Pearce et al., 2006). Therefore, we thought that Swap70 might be important for regulating OPC motility, membrane process dynamics and axon wrapping. Consistent with this possibility, we found that transgenically expressed Swap70 fusion proteins localized to OPC processes during migration and to oligodendrocyte membrane that wrapped axons to form internodes and that this localization required the pleckstrin homology domain. However, neither loss nor gain of Swap70 function affected OPC migration, membrane extension and retraction or axon wrapping. Although we did not find evidence for additional genes with similarity to *swap70* in the zebrafish genome, we cannot rule out the possibility that loss of Swap70 function is compensated in our experiments.

Nevertheless, our data do not support the hypothesis that Swap70 mediates signal transduction necessary for OPC migration and axon wrapping.

Instead, we found that loss of Swap70 function resulted in a deficit of OPCs. Our data indicate that the deficit results not from reduced OPC proliferation but from decreased production of OPCs. The *olig2*$^{+}$ precursors that give rise to OPCs remained intact in *swap70* deficient larvae, indicating that the OPC deficit did not result from failure to form or maintain neural precursors. In fact, to our surprise, *swap70*MO-injected larvae had more neural precursors than normal, as indicated by *sox2* expression, and they had elevated numbers of neural cells in the S and M phases of the cell cycle. Therefore, loss of Swap70 function results in maintenance of excessive numbers of dividing neural precursors raising the possibility that Swap70 helps promote the transition from neural precursor to specified OPC.

Loss of Fgfr function in mice and zebrafish (Furusho et al., 2011; Esain et al., 2010) and loss of Swap70 function in zebrafish result in a deficit of OPCs, raising the possibility that Swap70 mediates Fgfr signaling necessary for OPC specification. The MAPK pathway also mediates Fgfr signaling and, in culture MAPK function appears necessary for Fgfr-dependent formation of OPCs (Kessaris, 2004). Immunocytochemistry using a phospho-specific ERK antibody revealed no differences in *swap70* morpholino injected larvae (data not shown), indicating that signaling through both MAPK and PIP_3-mediated pathways downstream of Fgf receptors may be required for OPC formation.

One possible role for Swap70 in regulating neural precursor division is through regulation of Rac1, which can promote cell cycle progression via c-Jun kinase (Olson et al., 1995). However, this is difficult to reconcile with the increase of dividing neural precursors in *swap70* deficient larvae because loss of Swap70 GEF activity should result in less Rac1 stimulation of cell cycle progression. Another possibility is that Swap70 plays an unknown role in regulating the cell cycle within the nucleus. Consistent with this possibility, Swap70 contains nuclear localization sequences and we found nuclear localization of Swap70 protein in some cells, particularly in those that were associated with the proliferative ventricular zone of the spinal cord.

A nuclear function for Swap70 has been shown previously but of a very different sort than we imagine for neural precursors. Swap70 was first identified as part of a protein complex in mouse B cells that promotes heavy-chain immunoglobulin class switching by DNA recombination (Borggrefe et al., 1998). Swap70 localizes to cytoplasm of mast cells and B cells and moves to the nucleus of the latter following B cell activation (Borggrefe et al., 1999; Gross et al., 2002; Masat et al., 2000) and B cells of *Swap70* mutant mice are hypersensitive to γ-irradiation and impaired in their ability to switch to the IgE class of immunoglobulins (Borggrefe et al., 2001). The molecular mechanisms of Swap70 nuclear function remain unknown.

Apart from their altered IgE response, *Swap70* mutant mice appear phenotypically normal (Borggrefe et al., 2001), suggesting that *Swap70* function is limited to cells of the hematopoietic lineage. However, other cell and tissue types in mice, including lung and uterus (Borggrefe et al., 1999), embryonic fibroblasts (Shinohara et al., 2002) and the nervous system (Lein et al., 2007) express Swap70. Our data showing that oligodendrocyte lineage cells of zebrafish express *swap70* and that Swap70 function promotes transition of neural precursors to specified OPCs but not OPC migration or axon wrapping raises the possibility that Swap70 proteins, in addition to their role in immunoglobulin class switching and B cell motility, regulate transduction of signals that influence neural cell cycle and specification.

Experimental methods

Animals

Zebrafish embryos were raised at 28.5 °C in egg water or embryo medium (15 mM NaCl, 0.5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 0.15 mM KH_2PO_4, 0.05 mM NH_2PO_4 and 0.7 mM $NaHCO_3$). Embryos were staged to hours post fertilization (hpf) or days post fertilization (dpf) according to published criteria (Kimmel et al., 1995). To prevent pigmentation, embryos were raised in egg water containing 0.003% Phenylthiourea (PTU) from 24 hpf. The following zebrafish lines were used: *Tg*(*olig2:EGFP*)^{vu12} (Shin et al., 2003), *Tg*(*nkx2.2a:mEGFP*)^{vu17} (Kirby et al., 2006), *colorless*^{tw11} (*cls*^{tw11}) (Dutton et al., 2001) and *Tg*(*hsp70l:Gal4vp16*)^{vu22} (Inbal et al., 2007). In addition to these lines, we created *Tg*(*UAS:cherry-swap70*) fish as described below.

Plasmid construction, conditional expression experiments and generation of Tg(UAS:cherry-swap70) transgenic fish

To create an expression construct of monomeric Cherry fluorescent protein fused to Swap70 under the regulation of upstream activation sequence (UAS) sequence, we first subcloned a 5× UAS plus TATA box element in the pSKII vector (pSKII:UAS). We also cloned Cherry coding sequence without a stop codon into the pCS2+ vector (pCS2:Cherry(–stop)). Next, the sequences encoding full-length Swap70, the N-terminal sequence upstream of the PH domain (Swap70(356)) and the PH domain (Swap70(PH)) were amplified by PCR and subcloned into the 3′ end of Cherry in pCS2:Cherry(-stop). We transferred Cherry-fused Swap70 and truncated Swap70 cDNAs including SV40 poly(A) sequence downstream of UAS in pSKII:UAS. Finally, UASCcherry-Swap70, UAS:Cherry-Swap70(356) and UAS:Cherry-Swap70(PH) containing SV40 poly(A) were cloned into the pTol2 vector carrying two recognition sequences for Tol2 transposase, creating pTol2:UAS:Cherry-Swap70, pTol2:UAS:Cherry-Swap70 (356) and pTol2:UAS:Cherry-Swap70(PH). Similarly, we created pTol2:UAS:Cherry, which has a stop codon at the end of Cherry coding sequence. To create pTol2:sox10:Gal4vp16, a 7.2 kb regulatory sequence of sox10 (Dutton et al. 2001) was first subcloned into pTol2. Then, Gal4vp16 sequence fused to SV40 poly(A) was isolated from pCS2:Gal4vp16 and cloned downstream of sox10 sequence in pTol2:sox10. Fertilized eggs were injected with a solution containing 0.2 mg/ml of the plasmid and 0.3 mg/ml in vitro synthesized Tol2 mRNA.

To produce *Tg*(*UAS:Cherry-Swap70*) fish, pTol2:UAS:Cherry-Swap70 was injected into one-cell stage embryos with Tol2 mRNA as described above. To identify germ-line-transformed founders, we crossed plasmid-injected adult fish (G0 fish) to homozygous *Tg*(*hsp7l0:gal4vp16*) and heat shocked the embryos by incubating them at 39 °C for 30 min. *Tg*(*UAS:Cherry-Swap70*);*Tg*(*hsp70l: Gal4vp16*) double-transgenic embryos were identified by Cherry fluorescence. To establish stable lines we mated G0 founders to wild-type fish, raised the embryos to adulthood and screened them as before.

To induce overexpression of Cherry-Swap70, embryos were collected from mating of homozygous *Tg*(*hsp70l:Gal4vp16*) and heterozygous *Tg*(*UAS:Cherry-Swap70*) adults and raised at 28.5 °C. At appropriate stages, embryos and larvae were incubated at 39 °C for 30 min and 37 °C for 30 min.

Whole-mount in situ RNA hybridization, immunocytochemistry and BrdU labeling

Embryos and larvae were fixed in 4% paraformaldehyde (pfa) in 1X PBS overnight at 4 °C and stored in methanol at −20 °C for a minimum of 1 day. In situ hybridization was performed essentially as described previously (Hauptmann and Gerster, 2000). After processing embryos for in situ RNA hybridization embryos and larvae for sectioning were

embedded in 1.5% agar/30% sucrose and placed in 30% sucrose in water to equilibrate. The blocks were frozen in 2-methyl-butane chilled by immersion in liquid nitrogen. 12-μm sections were collected using a cryostat microtome and mounted with 75% glycerol in 1X PBS.

For immunocytochemistry, embryos and larvae were fixed in 4% AB fix (4% paraformaldehyde, 8% sucrose, and 1× PBS) overnight at 4 °C. Embryos and larvae for sectioning were embedded in agar/sucrose solution as described above. Primary antibodies used in this study were rabbit anti-Sox10 (1:1000) (Park et al., 2005), mouse anti-BrdU (G3G4) (1:1000) (Developmental Studies Hybridoma Bank), rabbit anti-phospho-Histone-H3 (1:1000) (Upstate Biotechnology), mouse anti-ZRF-1 (1:500) (University of Oregon Monoclonal Antibody Facility), rabbit anti-PKCζ (#sc-216) (1:200) (Santa Cruz Biotechnology) and mouse anti-HuC/D (16A11) (1:20) (Invitrogen). For nuclear staining, SYTOX Green and DAPI (Invitrogen) were used according to the company's instruction. For fluorescent detection of antibody labeling, we used Alexa Fluor 488 and Fluor 568 goat anti-mouse or goat anti-rabbit conjugates (1:200, Invitrogen).

For BrdU labeling, larvae were incubated in 0.5% solution of BrdU (Roche) in embryo medium. The larvae were then fixed using 4% pfa in PBS and sectioned as described earlier. Before anti-BrdU immunocytochemistry, the sections were treated for 30 min with 2 M HCl.

Imaging was conducted on a Zeiss Axiovert 200 equipped with epifluorescence and DIC optics or a Zeiss Axiovert 200 microscope equipped with a Perkin Elmer spinning-disk confocal system. Z-stack images were obtained and complied using Volocity software (Improvision). Images were exported to Photoshop (Adobe). Image adjustments were limited to contrast, levels, color-matching settings, and cropping.

Morpholino oligonucleotides injection

Antisense Morpholino oligonuceotides (MO) were purchased from Gene Tools. The sequences of MOs were followed: $MO^{swap70\text{-}UTR}$: TTTCAGAGGTAGTTTCTCCGTCTGC; $MO^{swap70\text{-}ATG}$: TGAGAAGCTCGTCCCTTAGTCCCAT; $MO^{swap70\text{-}sp}$: AGACAATGAGCTCTTGCCTCAACAC. $MO^{swap70\text{-}UTR}$ was complementary to a sequence in the 5′ UTR. $MO^{swap70\text{-}ATG}$ was complementary to a sequence spanning just before and including the translation start codon. $MO^{swap70\text{-}sp}$ was complementary to a sequence spanning exon 5–intron 5 boundary. MO were diluted in water and injected into one-cell stage embryos with 1–2 nl volume per embryo.

Live imaging

The larvae were raised in embryo medium containing PTU. Larvae were anesthetized using Tricane and immersed in 1% low-melting temperature agarose. They were then mounted in lateral orientation in glass-bottom dishes and incubated in embryo medium. Images were captured using the confocal microscope described above. The data sets were analyzed using Volocity software.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CNS	central nervous system
OPCs	oligodendrocyte progenitor cells
GEF	guanine nucleotide exchange factor
PIP_3	phosphatidylinositol-3,4,5-triphosphate

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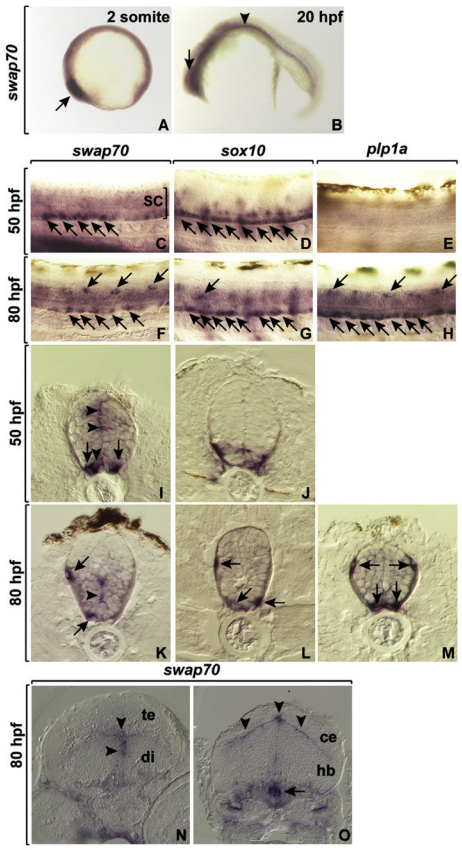

Fig. 1.

Developmental expression of *swap70*. (A,B) Lateral views of embryos hybridized with *swap70* antisense RNA probe at 2 somite (11 hpf) and 20 hpf. The eye primordia (arrows) and pronephric ducts (arrowhead) express *swap70*. (C–H) Lateral views of whole embryos focused on the trunk spinal cord, with dorsal up. The bracket marks spinal cord (sc). Expression of *swap70* (C,F) is similar to that of *sox10* (D,G), a marker of OPCs (arrows) at 50 and 80 hpf. By contrast, expression of *plp1a*, which marks differentiating oligodendrocytes, is not evident until 80 hpf (E,H). (I–M) Transverse sections through trunk spinal cord, dorsal up. *swap70* (I,K) is expressed similarly to *sox10* (J,L) in OPCs (arrows) except that cells that line the proliferative ventricular zone (arrowheads) also express *swap70* but not *sox10*. (M) *plp1a* expression marking oligodendrocytes (arrows). (N,O) Transverse sections through forebrain (N) and hindbrain (O). *swap70* expression is evident at ventricular zones of the tectum (te), diencephelon (di) and at the boundary between cerebellum (ce) and hindbrain (hb).

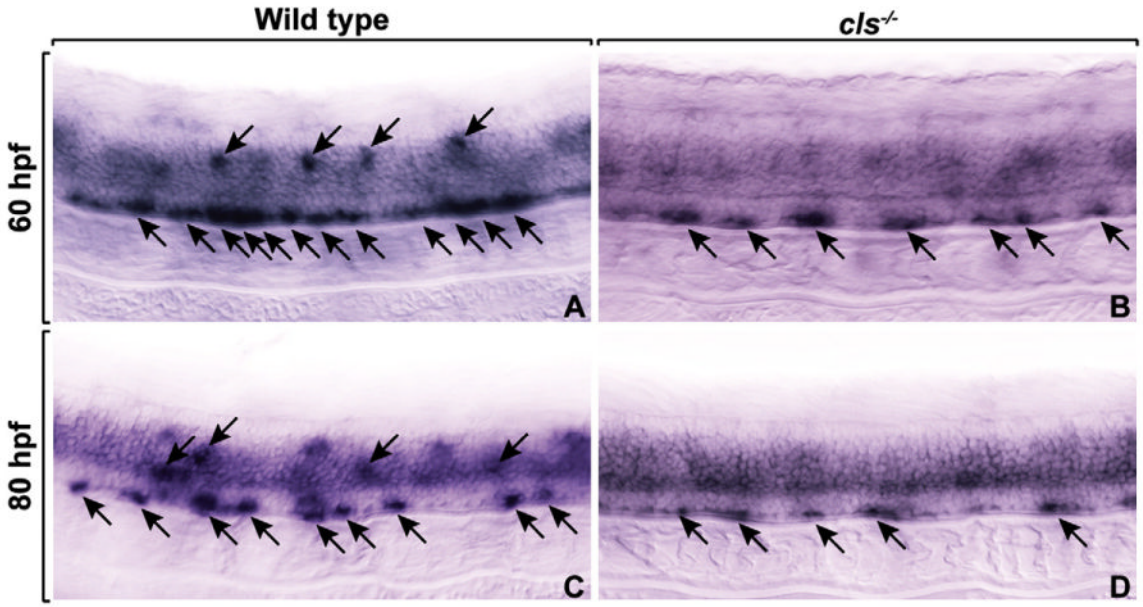

Fig. 2.

OPCs express *swap70* independently of *sox10* function. Lateral views of the spinal cords of wild-type (A, C) and *cls*$^{-/-}$ (B, D) embryos at 60 hpf (A, B) and 80 hpf (C, D). Arrows mark *sox10* expression. Whereas fewer cells express *swap70* in *cls*$^{-/-}$ embryos the level of expression appears to be similar in wild type and *cls*$^{-/-}$.

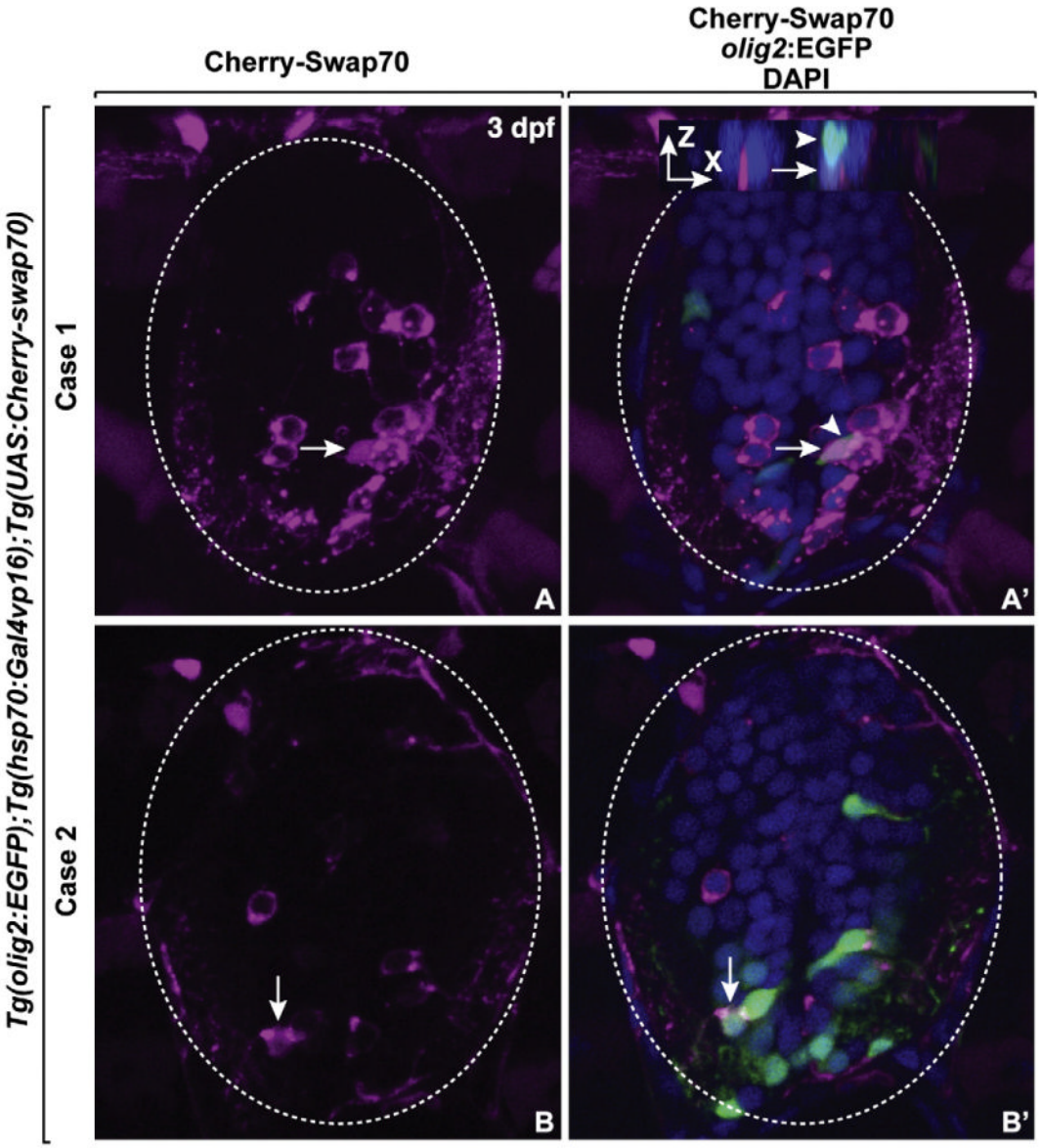

Fig. 3.

Swap70 is localized to nuclear and cytoplasmic compartments. Cherry-Swap70 was transiently expressed by crossing *Tg*(*hsp70l:Gal4vp16*);*Tg*(*olig2:EGFP*) fish to *Tg*(*UAS:Cherry-Swap70*) and performing heat shock induction. Two examples (A, A′ and B, B′) showing nuclear localization of Cherry-Swap70. (A, A′) Z-stack images were merged in single plane. An *olig2*-EGFP^{-} cell (arrow) next to *olig2*-EGFP^{+} cell (arrowhead) in anterior–posterior position had nuclear localized Cherry-Swap70. The inset is an X-Z projection of the image. (B, B′) Single plane image showing nuclear localization of Cherry-Swap70 in an *olig2*:EGFP^{+} precursor cell (arrow). Nuclei are labeled with DAPI (blue).

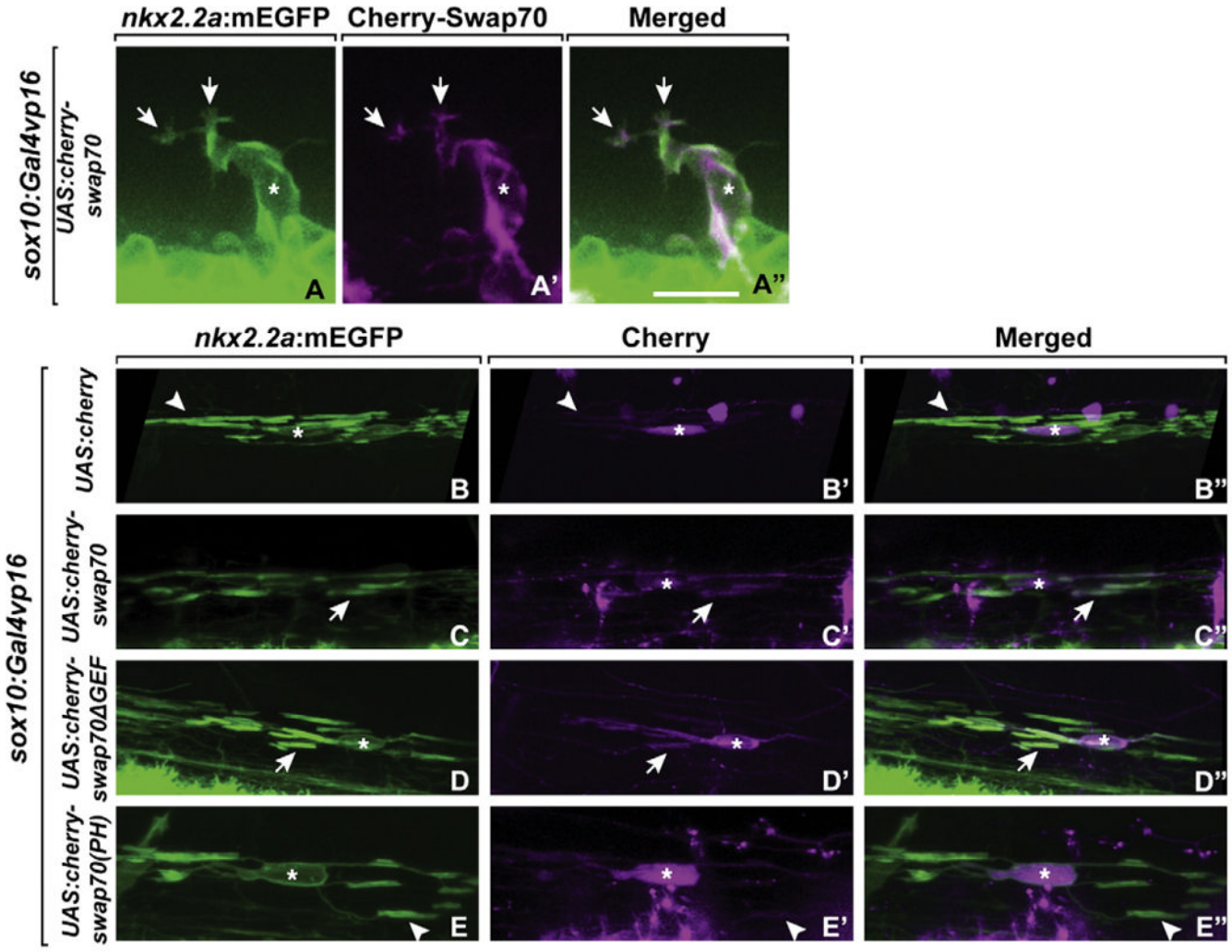

Fig. 4.

Swap70 localization in migrating OPCs and myelinating oligodendrocytes. Cherry-Swap70 was transiently expressed in OPCs by co-injecting *sox10:Gal4vp16* plasmid and the following plasmids: *UAS:Cherry-Swap70* (A–A″, C–C″), *UAS-Cherry* (B–B″), *UAS-Cherry-Swap70ΔGEF* (D–D″), *UAS-Cherry-Swap70(PH)* (E–E″). (A–A″) Cherry-Swap70 was localized at the tips of membrane processes (arrows) and the cell membrane of migrating OPCs. (B–B″) In myelinating oligodendrocytes, Cherry localized to the cytoplasm (asterisks) but not to internode membrane (arrowheads). (C–C″) Cherry-Swap70 was localized to membrane (asterisks) and the axon-wrapping internodes (arrows). (D–D′) C-terminal deleted Swap70 fused to Cherry (Cherry-Swap70ΔGEF) was also localized in the membrane and axon wrapping internodes. (E–E″) Cherry-fused PH domain of Swap70 (Cherry-Swap70(PH)) was localized to the cytoplasmic and nuclear compartments (asterisks) but not internode membrane (arrowheads). Scale bar in A, 15 μm.

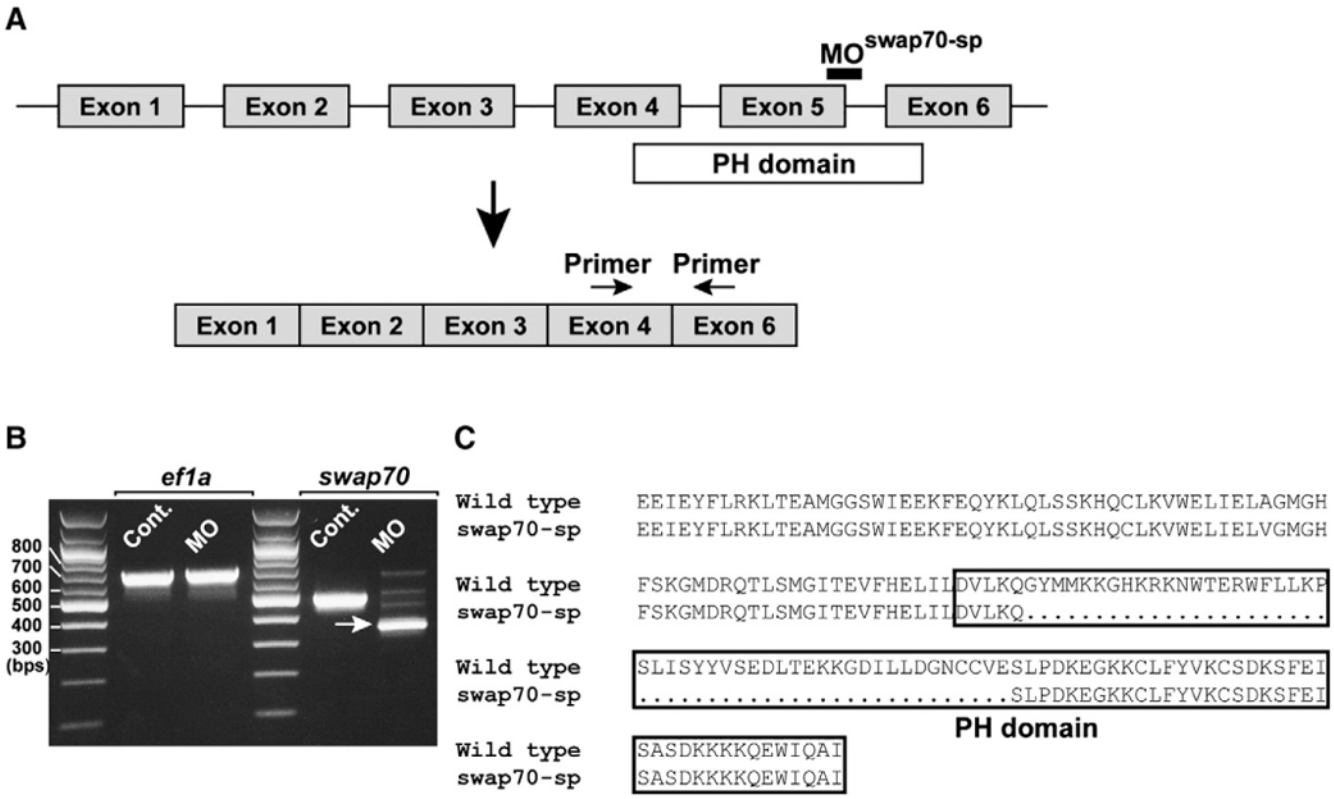

Fig. 5.

A splice-blocking MO causes deletion of a portion of the Swap70 PH domain. (A) A schematic representation of exon 1 to exon 6 of the *swap70* locus is shown. The PH domain is encoded by exons 4, 5, and 6. $MO^{swap70\text{-}sp}$ targets the exon 5/intron 5 boundary, resulting in the deletion of exon 5. Location of primer sets used for RT-PCR is shown. (B) Gel image of RT-PCR products amplified from wild-type control (cont.) and $MO^{swap70\text{-}sp}$-injected larvae at 3 dpf. Arrow indicates shorter *swap70* fragment produced by $MO^{swap70\text{-}sp}$ injection. Primers to amplify *ef1a* were used as a control. (C) The sequence of the indicated *swap70* product in (B) of $MO^{swap70\text{-}sp}$-injected larvae. The box indicates the PH domain. $MO^{swap70\text{-}sp}$-injected larvae lack 49 amino acids of the PH domain.

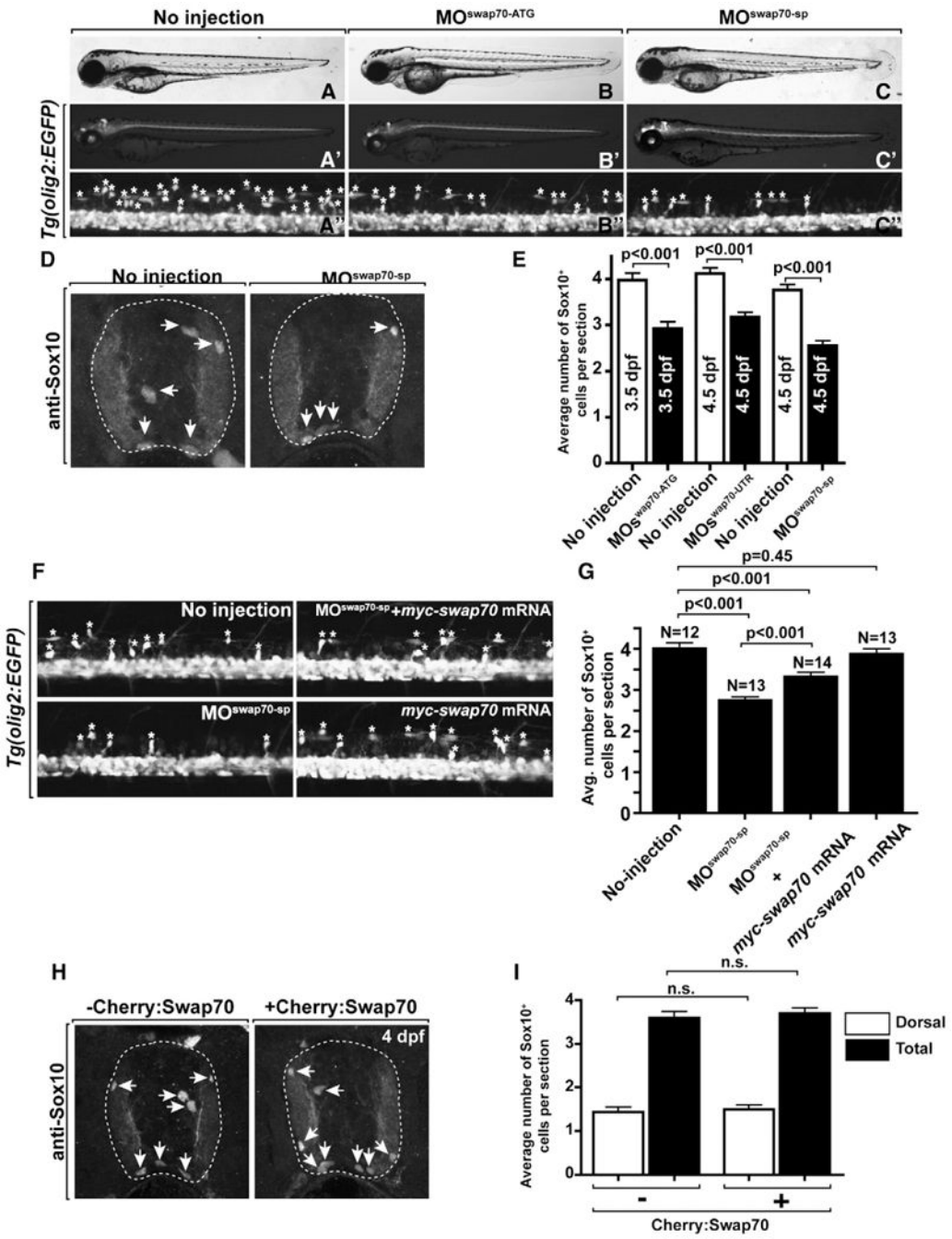

Fig. 6.

swap70 regulates OPC number. Lateral views of wild-type (A–A″), MO$^{swap70\text{-}ATG}$ (B–B″) and MO$^{swap70\text{-}sp}$ (C–C″) larvae at 3 dpf. Asterisks in A″, B″, C″ mark dorsally migrated olig2:EGFP^{+} OPCs. MO-injected larvae appear to have fewer OPCs than wild type. (D) Transverse sections showing Sox10^{+} OPCs (arrows) in the spinal cord of wild-type and MO-injected larvae at 4.5 dpf. (E) Quantification of OPCs reveals statistically fewer OPC in MO-injected larvae at 3.5 and 4.5 dpf. (F, G) swap70 overexpression by mRNA injection partially rescues the OPC deficit produced by MO knockdown. (F) Lateral view of Tg(olig2:EGFP) spinal cord at 3 dpf. Asterisks mark dorsally migrated OPCs. (G) Quantification of Sox10^{+} OPCs on spinal cord sections at 3 dpf shows that myc-swap70

overexpression reduces the OPC deficit caused by swap70 MO but does not alter OPC number in the absence of MO. (H, I) Transgenically induced Cherry-Swap70 does not alter OPC number or distribution. (H) Transverse sections through spinal cords of control and Cherry-Swap70 overexpressing larvae. Arrows point to $Sox10^{+}$ OPCs. (I) Quantification of dorsally migrated and total OPCs in control and Cherry-Swap70 overexpressing larvae reveals no differences. White bars indicate the number of dorsally migrated OPCs. Black bars indicate the total OPC number. n.s., not significant.

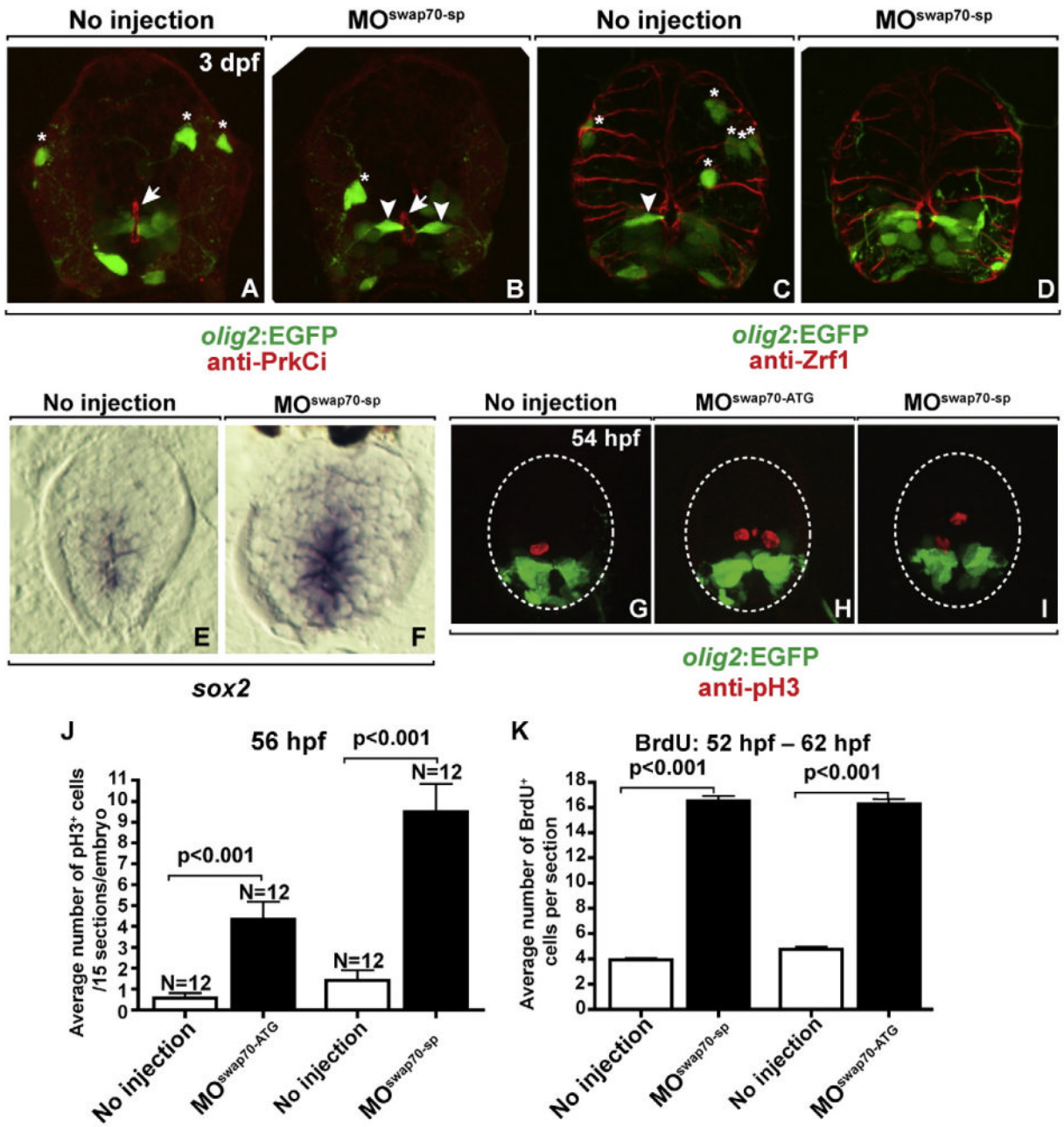

Fig. 7.

swap70 loss-of-function does not dramatically alter spinal cord development. All images show transverse sections through trunk spinal cord with dorsal up. (A, B) Anti-PrkCi immunocytochemistry performed on control and MO$^{swap70\text{-}sp}$-injected larva at 3 dpf. PrkCi labeling of apical membrane (arrows) and EGFP labeling of olig2^{+} radial glia (arrowheads) is indistinguishable between control and experimental animals whereas fewer EGFP^{+} OPCs (asterisks) are evident following MO$^{swap70\text{-}sp}$ injection. (C, D) Anti-Zrf1 immunocytochemistry to label radial glia at 3 dpf. The shape and number of Zrf1^{+} radial fibers look similar in wild type and MO$^{swap70\text{-}sp}$-injected larva. Arrowheads and asterisks mark EGFP^{+} radial glia and OPCs, respectively. (E, F) sox2 mRNA expression in the spinal cord at 3 dpf. More medial spinal cord cells express sox2 in MO$^{swap70\text{-}sp}$-injected larva. (G–

I) Anti-pH3 immunocytochemistry to label M-phase cells (red) on spinal cord sections of 54 hpf Tg(olig2:EGFP) embryos. In MO-injected embryos more spinal cord cells are positive for pH3. (J) Quantification of pH3 labeling. Y axis indicates total pH3 positive cells on 15 sections per embryo. N indicates number of embryos analyzed. (K) Quantification of BrdU incorporation to label S-phase cells. Embryos were soaked in BrdU solution from 52 hpf to 62 hpf. Y axis indicates number of BrdU positive cells number per section.

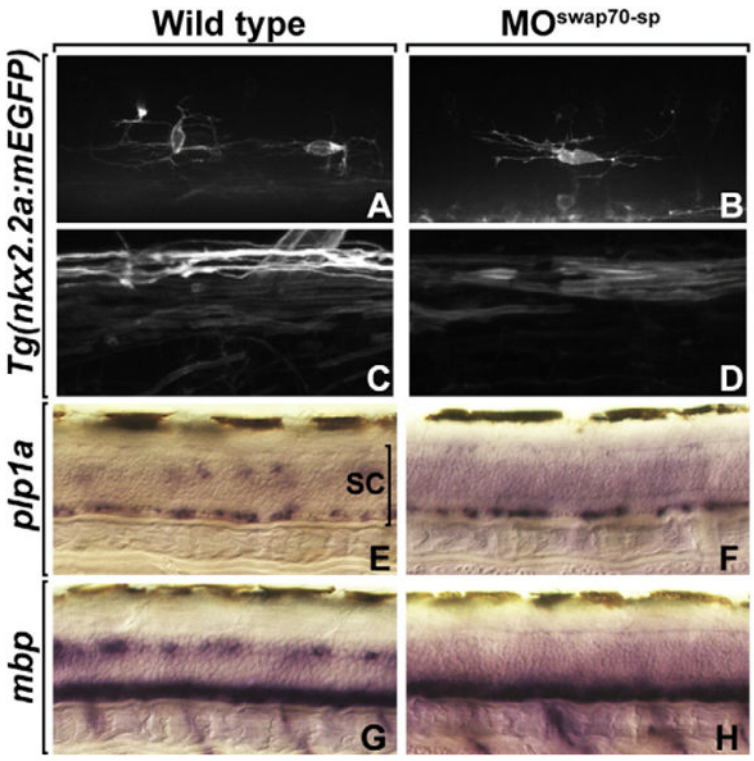

Fig. 8.

Swap70 loss-of-function does not affect OPC morphology and differentiation. Images of migrating OPCs (A, B) and wrapping oligodendrocytes (C, D) in living 3 dpf larvae visualized using a *Tg*(*nkx2.2a:mEGFP*) transgenic reporter. Membrane processes of OPCs and myelinating oligodendrocytes appear normal in $MO^{swap70\text{-}sp}$-injected larvae. (E, F) *plp1a* mRNA expression at 3 dpf. (G, H) *mbp* mRNA expression at 3 dpf. Both *plp1a* and *mbp* appear to be expressed at normal levels in $MO^{swap70\text{-}sp}$-injected larvae, although the number of cells expressing these genes in MO-injected larvae is fewer than in wild type.