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Tcf7l1 is required for spinal cord progenitor maintenance

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Abstract

Neural progenitor cells must be maintained during development in order to produce the full complement of neuronal and glial derivatives. While molecular pathways have been identified that inhibit progenitor differentiation, it is unclear whether the progenitor state itself is actively maintained. In this study we have investigated the role of Tcf7l1 (formerly named Tcf3) in maintaining spinal progenitor characteristics and allowing the continued production of neurons and glia following primary neurogenesis. We find that spinal cord progenitor markers are progressively lost in embryos lacking Tcf7l1, and that the number of proliferative progenitors decreases accordingly. Furthermore, we show that the production of both neuronal and glial secondary derivatives of the pMN progenitor pool requires Tcf7l1. Together, these results indicate that Tcf7l1 plays an important role in spinal cord progenitor maintenance, indicating that this core function is conserved throughout multiple epithelial cell populations.

Keywords

zebrafish; Tcf7l1; spinal cord; neurogenesis

Introduction

During embryonic development, the rate of neurogenesis is tightly regulated by extrinsic and intrinsic factors. In this process, a balance must be achieved between the maintenance of proliferative progenitors and neuronal or glial differentiation. The vertebrate spinal cord has been well established as a model for CNS neurogenesis, due to its relatively simple anatomy and orderly progression of neuronal and glial differentiation. In the spinal cord, distinct dorso-ventral subregions of progenitors are established by extrinsic signals such as Shh, Bmp, Notch and Wnt (Artavanis-Tsakonas et al., 1999; Jessell, 2000; Briscoe and Ericson, 2001). Over time these specified progenitors undergo differentiation, first producing distinct populations of neurons, then producing glia. Previous work has shown that Notch signaling is required to prevent spinal progenitors from differentiating at the same time, and thus regulates cell diversity (Peng et al., 2007; Shin et al., 2007; Kim et al., 2008; Kimura et al., 2008). However, while Notch signaling primarily acts to block differentiation by repressing proneural gene expression (Holmberg et al., 2008), it is still unclear whether other factors actively maintain the progenitor pool. Furthermore, it is unknown how different aspects of the progenitor state, such as cell cycle progression, progenitor gene expression, and morphology, are coordinated.

One candidate regulator of the CNS progenitor state is the transcription factor Tcf7l1 (formerly known as Tcf3). The vertebrate Tcf/Lef family of molecules mediate canonical Wnt signaling by regulating downstream target gene expression (Behrens et al., 1996;

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Molenaar et al., 1996; Brannon et al., 1997; Lee et al., 2006). Vertebrates have four Tcf/Lef family members, Lef1, Tcf7, Tcf7l1 and Tcf7l2, which all contain highly conserved β-catenin binding domains and HMG DNA binding domains. These transcription factors function alternately as activators or repressors, depending on the presence or absence, respectively, of β-catenin (Gradl et al., 2002; Houston et al., 2002; Standley et al., 2006). Among the known roles of Tcf/Lef factors, Tcf7l1 is unique in that it acts to maintain progenitor cells in the absence of Wnt signaling. Tcf7l1 is expressed in multipotent embryonic epidermal progenitors and is down-regulated as these cells differentiate during development (Nguyen et al., 2006). In addition, Tcf7l1 expression is maintained in stem cells of the epidermal hair follicle bulge, which contribute to the normal hair cycle as well as wound repair (DasGupta and Fuchs, 1999). Identified transcriptional targets of Tcf7l1 match the gene signature of stem or progenitor cells (Nguyen et al., 2009), and loss of function analyses suggest that Tcf7l1 acts to maintain progenitor multipotency (Nguyen et al., 2009). Finally, Tcf7l1 is an integral component of the core regulatory circuitry of embryonic stem cells acting to influence the balance between pluripotency and differentiation (Cole et al., 2008; Yi et al., 2008).

Wnt signaling, mediated by Tcf/Lef factors, plays well characterized roles in early neural tube proliferation and patterning. The role of Wnt as a mitogen, directly regulating cell cycle control genes, has been confirmed in the CNS using multiple experimental systems (Megason and McMahon, 2002; Zechner et al., 2003; Chesnutt et al., 2004), leading to models in which the Wnt pathway acts to expand the neural progenitor population. Activation of targets that positively specify dorsal fates in concert with BMP signaling, and negatively influence ventral signals such as Hedgehog, support an important function for Wnt signaling in dorsal specification throughout the CNS (Zechner et al., 2007; Alvarez-Medina et al., 2008; Yu et al., 2008). Our previous work showed that Tcf7l1 plays an additional role as a repressor to inhibit precocious progenitor differentiation in the zebrafish spinal cord (Gribble et al., 2009). In zebrafish, Tcf7l1 activity is encoded by two orthologous genes, *tcf7l1a* and *tcf7l1b*. While both maternal and zygotic function of *tcf7l1a* is required for normal embryonic patterning(Kim et al., 2000), zygotic function of the tow genes is redundant in the embryo, as single homozygous mutants for each gene are viable and fertile as adults, and have no developmental phenotypes (Dorsky et al., 2003; Gribble et al., 2009). Interestingly, among *tcf/lef* genes, only the expression of these family members correlates with the presence of neural progenitors (Gribble et al., 2009). Earlier studies have demonstrated that Tcf7l1 functions primarily as a transcriptional repressor in the absence of Wnt signaling based on both its expression and biochemical activity (DasGupta and Fuchs, 1999; Dorsky et al., 2003; Gribble et al., 2009), as well as loss-of-function phenotypes (Dorsky et al., 2003; Merrill et al., 2004). We found that *sox4a*, a gene required for neuronal differentiation (Bergsland et al., 2006), is directly repressed by Tcf7l1 (Gribble et al., 2009). However, the full role of Tcf7l1 in spinal progenitors remains unclear. Specifically, it is unknown whether Tcf7l1 functions to generally maintain the progenitor state, in addition to specifically repressing genes associated with differentiation.

Here, we use a combination of loss-of-function techniques to examine the role of Tcf7l1 in spinal progenitor maintenance. By analyzing the expression of multiple spinal progenitor markers, we demonstrate that cells lacking Tcf7l1 lose their progenitor identity while continuing to proliferate. We next characterize the production of later-generated fates known to arise from a single progenitor pool, and find that both secondary motoneuron and oligodendrocyte precursor production requires Tcf7l1 function. Together, these data support a model in which the spinal progenitor population is actively maintained by Tcf7l1, and in its absence this population is gradually depleted, leading to a decreased ability to generate later derivatives..

Results

tcf7l1 genes are expressed in the zebrafish beyond primary neurogenesis

Zebrafish have two *tcf7l1* genes, *tcf7l1a* and *tcf7l1b* (Dorsky et al., 2003). In previous studies, we showed that the zygotic function of these two genes is redundant during embryonic development and early spinal cord neurogenesis (Dorsky et al., 2003; Gribble et al., 2009). To determine whether these genes may play a continuing role in spinal progenitor maintenance, we examined their expression at later stages. At 24 hours post-fertilization (hpf), *tcf7l1a* and *tcf7l1b* are mainly expressed in the developing spinal cord, however *tcf7l1a* also exhibits strong expression in the last developing somites at the tail tip (Fig. 1A,D). By 36 hpf, both *tcf7l1a* and *tcf7l1b* are expressed in the spinal cord, notochord, urogenic duct and blood vessels (Fig. 1B,E). Cross-section analysis reveals that both genes are specifically expressed in spinal cord progenitors at 36 hpf (Fig. 1C,F). The two patterns are not completely identical, as *tcf7l1a* is expressed throughout the ventral spinal cord while *tcf7l1b* is restricted from the most ventral region. Nevertheless, these expression patterns suggest that expression of *tcf7l1a* and *tcf7l1b* overlaps significantly and the two genes may be functionally redundant through 36 hpf in the spinal cord.

Tcf7l1 is required for maintenance of progenitor markers in the developing spinal cord

Our previous work showed that Tcf7l1 inhibits precocious neurogenesis in the developing spinal progenitors, and specific neuronal subpopulations, such as *isl1*-positive motoneurons, were reduced in Tcf7l1-deficient embryos (Gribble et al., 2009). To address whether these phenotypes were related to progenitor maintenance defects, we performed immunohistochemistry for GFP on *Tg(gfap:GFP)*^{mi2001} transgenic zebrafish. This transgene has been previously shown to label proliferating spinal radial glial progenitors, which endogenously express GFAP (Bernardos and Raymond, 2006). We performed BrdU labeling for 20 minutes in wild-type and *tcf7l1* splice-blocking morpholino-injected embryos, followed by fixation and cryosectioning at 26 hpf. The overall expression level of *gfap:gfp* was drastically reduced in *tcf7l1* morphant spinal cords compared to uninjected controls (Fig. 2A–F). The number of GFP+ cells was decreased at 26 hpf to 20.78±3.10 per section in *tcf7l1* morphants, from 29.56±2.34 per section in wild-type embryos (±SD, n=9 sections, $p<0.05$). Strikingly, in *tcf7l1* morphants many BrdU+ cells located in the medial spinal cord failed to express *gfap:gfp*, while nearly all BrdU+ cells in controls were GFP+ (Fig. 2A,D,G,H). At 26 hpf, secondary motoneurons marked by Isl1/2 continue to be produced from *gfap:gfp*-expressing progenitors. In control embryos, these cells are labeled by *gfap:gfp* and Isl1/2 co-expression likely due to GFP perdurance (Fig. 2B). In *tcf7l1* morphants, we observed a significant decrease in *gfap:gfp*/Isl double-positive cells (Fig. 2E,H), suggesting that the motoneuron progenitor pool may be decreased at this timepoint. Consistent with this possibility, the total number of ventral Isl+ cells was decreased to 2.00±0.79 per section in *tcf7l1* morphants, from 3.33±0.79 per section in wild-type embryos (±SD, n=9 sections, $p<0.05$). Together, these results indicate that Tcf7l1 may be required for maintenance of the progenitor state, and that the majority of Isl+ cells produced in morphants may be primary motoneurons.

It is possible that Tcf7l1 specifically regulates the expression of *gfap:gfp*, rather than progenitor identity in general. We therefore examined the expression of Sox3, which also marks spinal progenitors and may play a role in the regulation of progenitor identity (Bylund et al., 2003; Harrington et al., 2010). As before, control and *tcf7l1* morphant *gfap:gfp* embryos were labeled with BrdU for 20 minutes. We found that the overall expression level of Sox3 protein was reduced in *tcf7l1a* morphants at both 26 and 36 hpf (Fig. 3A–D). In addition, the number of Sox3+ cells was decreased at 26 hpf to 22.78±1.50 per section in *tcf7l1* morphants, from 29.11±1.17 per section in wild-type embryos (±SD, n=9 sections,

p<0.005). By 36 hpf fewer BrdU+ cells in morphants expressed Sox3 than in wild-type controls (Fig. 3C–E). These results indicate that Tcf7l1 is required for the continued expression of multiple spinal progenitor markers. As we reported previously (Gribble et al., 2009), some BrdU+ cells also ectopically expressed HuC/D, which marks differentiating neurons (not shown).

Tcf7l1 is necessary for generation of secondary motoneurons in the ventral spinal cord

Because morphants exhibited decreased Isl+ motoneuron generation from *gfap:gfp*-expressing cells at 26 hpf (Fig. 2), we hypothesized that secondary motoneurons may be affected by the loss of Tcf7l1 function. We analyzed secondary motoneuron formation using the Neurolin (zn8) antibody, which specifically marks these cells (Kim et al., 2008). To allow phenotypic analysis beyond 36 hpf, we examined *tcf7l1a/b* double mutant embryos (Gribble et al., 2009) instead of morphants for these experiments. At both 36 and 48 hpf, some Neurolin-positive cells were double-labeled with BrdU in control embryos (Fig. 4A–D), suggesting that secondary motoneurons were still being produced from ventral progenitors. However, as with Isl1/2 labeling, Neurolin/BrdU double-positive cells were significantly reduced in *tcf7l1* mutants compared to wild-type embryos (Fig. 4A–D,G). Single mutants for *tcf7l1a* and *tcf7l1b* had no significant phenotype (not shown), suggesting that the two genes act redundantly in the spinal cord at this stage of development.

Interestingly, the zone of Neurolin expression in wild-type embryos normally coincides with low expression of the Wnt activity reporter *topd:gfp* (Dorsky et al., 2002). At 36 hpf, *top:dgfp* is normally expressed at low levels in intermediate spinal progenitors, and excluded from the ventral Neurolin-positive zone (Fig. 4E). However in *tcf7l1* morphants, *top:dgfp* expression is increased overall and expanded into the most ventral region of the spinal cord, concomitant with decreased Neurolin expression (Fig. 4F). These results indicate that Tcf7l1-mediated repression of Wnt target genes may function to maintain the progenitor population long enough to allow secondary motoneuron formation.

To test whether Tcf7l1 function is specifically required for late-born secondary motoneuron formation rather than for all ventral neurogenesis, we examined the expression of GABA, which marks earlier-born ventral interneurons (Bernhardt et al., 1992). We found that unlike the decrease in Neurolin-positive secondary motoneurons, ventral GABA-positive cells were present in a normal distribution at 36 hpf (Supplementary Fig. 1A–D). Finally, to rule out the possibility that Neurolin expression was reduced by general developmental delay, we performed Neurolin whole mount immunostaining on wild-type and *tcf7l1a/b* mutants at 72 hpf. The number of Neurolin-positive cells located in the ventral spinal cord was reduced in the mutant embryos even at this later stage (Fig. 4H–L), indicating that Tcf7l1 function is required for the continued generation of secondary motoneurons by spinal progenitors.

Tcf7l1 is required for normal oligodendrocyte precursor cell development

In addition to secondary motoneurons, oligodendrocytes are also derived from *olig2*-positive progenitor cells in the pMN domain of the developing zebrafish spinal cord following primary neurogenesis (Park et al., 2007). Our previous data showed that *olig2* expression was not affected in *tcf7l1* morphants at early embryonic stages (Gribble et al., 2009). However since *tcf7l1* genes are strongly expressed throughout the ventral spinal cord through at least 36 hpf, and secondary motoneuron production from the pMN domain is dependent on Tcf7l1, we hypothesized that oligodendrocyte formation might be similarly affected. To test whether Tcf7l1 is required for *olig2*-expressing progenitor proliferation, we performed short-pulse BrdU labeling in *tcf7l1* MO-injected *Tg(olig2:EGFP)*^{vu12} embryos (Park et al., 2007), at 36 and 48 hpf. The number of GFP and BrdU double-positive cells were reduced in *tcf7l1* morphants compared to controls at both stages (Fig. 5A–E).

We then examined whether the decrease in proliferation among *olig2:gfp* expressing cells correlated with a decreased number of oligodendrocyte precursor cells (OPCs). At 72 hpf, dorsally-migrating GFP-positive OPCs were greatly decreased in *tcf7l1* morphants (Fig. 5G,H). The reduction in OPC marker expression did not correlate with a general developmental delay, as dorsal root ganglion formation was normal in 72 hpf *tcf7l1* morphant embryos (Fig. 5I,J). Together, these results suggest that Tcf7l1 is required for the continued production of multiple late derivatives of ventral spinal progenitors.

Loss of ventral cell types is not due to patterning defects

While our previous study showed that Tcf7l1 is not required for dorsal/ventral patterning in the spinal cord before 24 hpf, our current results indicate that at 36 hpf *tcf7l1a* deficient embryos have reduced numbers of two ventral spinal cord cell types: sMNs and OPCs. To test whether Tcf7l1 knockdown affects dorsal/ventral patterning of the spinal cord at this embryonic stage, we performed in situ hybridization with several region-specific patterning markers, *msxC*, *pax3*, *dbx2* and *nkx6.1* (listed dorsal to ventral). We found that the expression of all of these markers was unaffected in *tcf7l1* morphants (Supplementary fig. 2A–H), suggesting that the reductions in sMNs and OPCs were not a result of patterning defects.

Discussion

Tcf7l1 acts uniquely in CNS development, independent from other characterized functions of Wnt/β-catenin signaling and Tcf/Lef-mediated activation in regulating neural progenitor proliferation and dorsal/ventral neural tube patterning. Our previous work identified a role for Tcf7l1 (formerly named Tcf3) in blocking premature differentiation of spinal progenitors (Gribble et al., 2009). We found that the two zebrafish orthologs *tcf7l1a/b* are expressed in cells with low levels of Wnt activity, where their gene products function primarily as transcriptional repressors. In the absence of Tcf7l1 function, a direct target gene, *sox4a*, is ectopically expressed and promotes neuronal differentiation. While these data provided a partial mechanism for the *tcf7l11* morphant phenotype, they left some key questions unaddressed. First, it was not clear whether Tcf7l1 plays an additional role in the maintenance of progenitor markers. This possibility was raised by our observations that morphant cells sometimes remained proliferative even while they expressed differentiated neuronal markers, suggesting that they might have a dual progenitor/neuron identity. Second, the effects on successive phases of spinal progenitor derivatives were unexplored. While we had observed a general decrease in spinal interneuron differentiation, it was not clear whether this was due to a failure in fate specification or rather a loss of progenitor maintenance. Answering these questions would lead to a more complete understanding of how spinal progenitors are maintained throughout development and even into adult stages.

Tcf7l1 maintains the spinal progenitor state

In this study we used several approaches to determine the role of Tcf7l1 in spinal progenitor maintenance. We first demonstrated that *tcf7l1a/b* gene expression in the spinal cord persists beyond primary neurogenesis, as progenitors continue to proliferate and generate differentiated neurons (Fig. 1). While the expression patterns of the two genes are not completely identical, all our phenotypic analyses suggest that their zygotic function is redundant. Single homozygous mutants for each gene are viable and fertile, indicating that any non-redundant functions throughout life of the animal are not essential. Next, we used transgenic and immunohistochemical markers to show that Tcf7l1 is required for maintenance of multiple spinal progenitor phenotypes (Figs. 2–3). Our data also indicate that the proliferative state can be uncoupled from progenitor marker expression, as cells lacking *gfap:gfp* and Sox3 are still able to incorporate BrdU. Together, these results suggest

that Tcf7l1 is required to simultaneously maintain the progenitor state and inhibit differentiation.

Failure of progenitor maintenance results in loss of multiple secondary pMN derivatives

Because the spinal progenitor pool must be maintained in order to produce later-born fates, loss of Tcf7l1 function would be predicted to influence the number of these progeny. Most primary neurons in the zebrafish spinal cord have differentiated by 18 hpf (Bernhardt et al., 1990), and neurons born after this point are secondary derivatives. Because few specific markers exist for secondary interneurons, we focused our analysis on motoneuron progenitors. We found that *tcf7l1* mutants and morphants produced significantly fewer secondary motoneurons and OPC's (Figs 4–5). Both of these cell types arise from an *olig2*-expressing pMN progenitor population (Park et al., 2007), and we observed a decreased number of proliferating *olig2:gfp*+ cells in *tcf7l1a* morphants (Fig. 4). These data suggest that fewer pMN progenitors are present when Tcf7l1 function is lost, resulting in a general decrease in all progeny. Our findings reveal a different role for Tcf7l1 in progenitor maintenance than has been previously found for Notch signaling. Careful analysis of pMN fates following manipulation of the Notch pathway has shown that alternative fates are increased when Notch signaling is lost (Kim et al., 2008). For example, early inhibition of Notch leads to increased GABAergic interneurons at the expense of motoneurons (Shin et al., 2007), while later inhibition results in more secondary motoneurons at the expense of radial glial progenitors and OPC's. In contrast, we do not observe increases in alternative fates when Tcf7l1 is lost. Rather, progenitors prematurely differentiate as HuC/D+ neurons and presumably die due to lack of proper fate specification. Together, our work suggests a general role for Tcf7l1 in progenitor maintenance, rather than in mediating binary cell fate decisions. We predict that our findings should extend to other secondary neuron populations throughout the expression domain of *tcf7l1* genes, but confirmation will require the use of specific markers for these cell types.

A conserved function for Tcf7l1 in epithelial progenitor maintenance

In some epithelial tissues, continued progenitor self-renewal is a necessary part of homeostasis. In epithelial progenitors of the skin and hair follicles, Tcf7l1 plays a critical role in maintaining the progenitor state, as a transcriptional regulator of progenitor-specific genes (Nguyen et al., 2006; Cole et al., 2008; Yi et al., 2008). While progenitor self-renewal is much more limited in tissues of the central nervous system, there are specific instances in which analogous processes may function. During embryogenesis, radial glial neural progenitors proliferate and generate progeny throughout the brain and spinal cord, and these populations must be maintained long enough to generate the full complement of neurons and glia necessary for function. Following embryogenesis, discrete populations of neural stem cells are maintained in the brain, but the mechanisms responsible for promoting self-renewal and multipotency of these progenitors are largely uncharacterized. Our work here suggests that the maintenance of epithelial progenitors in different tissues may require a common gene program, which includes transcriptional repression by Tcf7l1.

Potential implications for maintenance of spinal cord progenitors beyond embryogenesis

Radial glial-like ependymal cells in adult fish and amphibians express GFAP and maintain processes that span the spinal cord to the pial surface (Tomizawa et al., 2000). Because regenerating motoneurons in zebrafish are thought to arise from these persistent radial glial progenitors (Reimer et al., 2009), these cells may represent an endogenous stem cell population capable of post-injury repair in the zebrafish spinal cord. Our data suggest that Tcf7l1 may be a key component of the mechanism underlying the persistence of radial glial progenitors beyond embryogenesis. To test this hypothesis, it will be necessary to determine the expression and functional requirement for Tcf7l1 at larval and adult stages.

Experimental Procedures

Zebrafish maintenance

Embryos were obtained from natural spawning of wild-type (AB*) or transgenic and mutant zebrafish lines listed below, and were staged according to Kimmel et al., (Kimmel et al., 1995). *tcf7l1* mutants were generated using *tcf7l1a*^{m881} and *tcf7l1b*^{zd10} alleles and mutant embryos were identified as described previously (Gribble et al., 2009). The *Tg(olig2:EGFP)*^{vu12} transgenic line was obtained from Dr. Bruce Appel (Park et al., 2007; Takada et al., 2010). *Tg(gfap:GFP)*^{mi2001} transgenic zebrafish were obtained from Dr. Pamela Raymond (Bernardos and Raymond, 2006). *Tg(top:dgfp)*^{w25} fish have been described and characterized previously (Dorsky et al., 2002).

Morpholino injections and in situ hybridization

tcf7l1a/b (splice-blocking) + *p53* (translation-blocking) morpholino injections were performed as described previously (Gribble et al., 2009). Antisense digoxigenin-labeled RNA probes for *tcf7l1a*, *tcf7l1b*, *msxC*, *pax3*, *dbx2* and *nkx6.1* were produced using a DIG-RNA labeling kit (Roche) according to the manufacturer's instructions. All probes were described previously in (Gribble et al., 2009), except *msxC* which was described in (Bonner et al., 2008). Whole mount in situ hybridization was performed with digoxigenin labeled probes as described previously (Thisse et al., 1993).

BrdU labeling and immunostaining

26, 36, and 48 hpf zebrafish embryos were incubated in a 10 mM BrdU solution containing 15% DMSO in Ringer's solution. At 26 and 36 hpf, embryos were fixed after a 20 minute incubation and embryos at 48 hpf were fixed after a 1 hour incubation. All embryos were fixed in 4% paraformaldehyde for 2 hrs in room temperature. For cryosectioning, embryos were cryoprotected in a 30% sucrose/PBS solution, then mounted in OCT compound (Tissue-Tek). Blocks were sectioned at 10μm thickness for immunohistochemistry.

Immunostaining on whole mount embryos and cryosections was performed as described previously (Gribble et al., 2009). For BrdU detection, slides were incubated for 1 hour in 2N HCl. The following primary antibodies were used: rat polyclonal anti-BrdU (AbD Serotec), mouse monoclonal anti-Isl1/2 (DSHB), rabbit polyclonal Sox3 (Zhang et al., 2003), mouse monoclonal anti-Neurolin (zn8, ZIRC), mouse monoclonal anti-HuC/D (Invitrogen). We used secondary antibodies conjugated to Alexa Fluor 568 or Alexa Fluor 647 (Invitrogen). For whole mount DAB immunohistochemistry, we used the Vectastain ABC kit (Vector Laboratories).

Imaging

Fixed whole-mount embryos and sections were mounted in 80% glycerol and Fluoromount-G (Southern Biotech), respectively, and imaged using conventional (bright-field) or confocal (fluorescent) microscopy. Confocal images were acquired using an Olympus FV1000 microscope. For live imaging, embryos were manually dechorionated at appropriate embryonic stages and transferred into embryo media containing tricaine for anesthesia. Embryos were then embedded in 0.8 % low-melting temperature agarose and mounted in 35-mm Petri dishes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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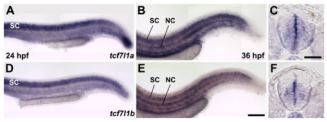

Figure 1. Expression of *tcf7l1a* and *tcf7l1b* is maintained in the zebrafish trunk following primary neurogenesis

(A–C) Expression of *tcf7l1a* mRNA at 24 hpf **(A)**, and 36 hpf **(B,C). (D–F)** Expression of *tcf7l1b* mRNA at 24 hpf **(D)**, and 36 hpf **(E,F)**. Lateral whole-mount views are shown in **(A,B,D,E)** and cross-sections through the spinal cord are shown in **(C,F)** SC: spinal cord; NC: notochord. Scale bars = 20μM in C, 80μM in E.

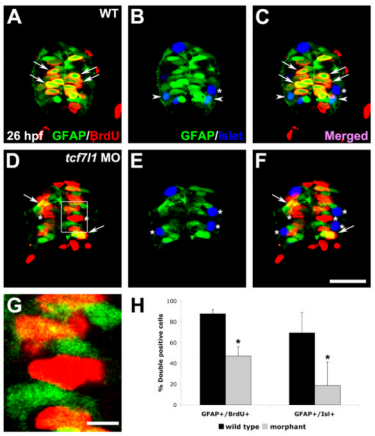

Figure 2. *gfap:gfp* expression is lost in *tcf7l1* morphants

(A–F) Cross-sections through the spinal cord at 26 hpf. **(A–C)** *Tg(gfap:GFP)*^{mi2001} embryo co-labeled for BrdU and Isl1/2, following 20 minutes of BrdU incubation. **(D–F)** *tcf7l1a/b* morpholino-injected *Tg(gfap:GFP)*^{mi2001} embryo labeled as described above. Arrows indicate GFP+/BrdU+ cells, arrowheads indicate GFP+/Isl+ cells, and asterisks indicate GFP −/BrdU+ or GFP−/Isl+ cells. **(G)** Higher magnification view of box in **(D). (H)** Percent of double-positive cells/section in wild-type and *tcf7l1* morphant spinal cords. Error bars indicate SD and asterisks indicate statistical significance. $p < 0.001$ by Student's unpaired t-test. n=9 sections from 3 individual embryos for each bar. Scale bars = 20μM in F, 5μM in G.

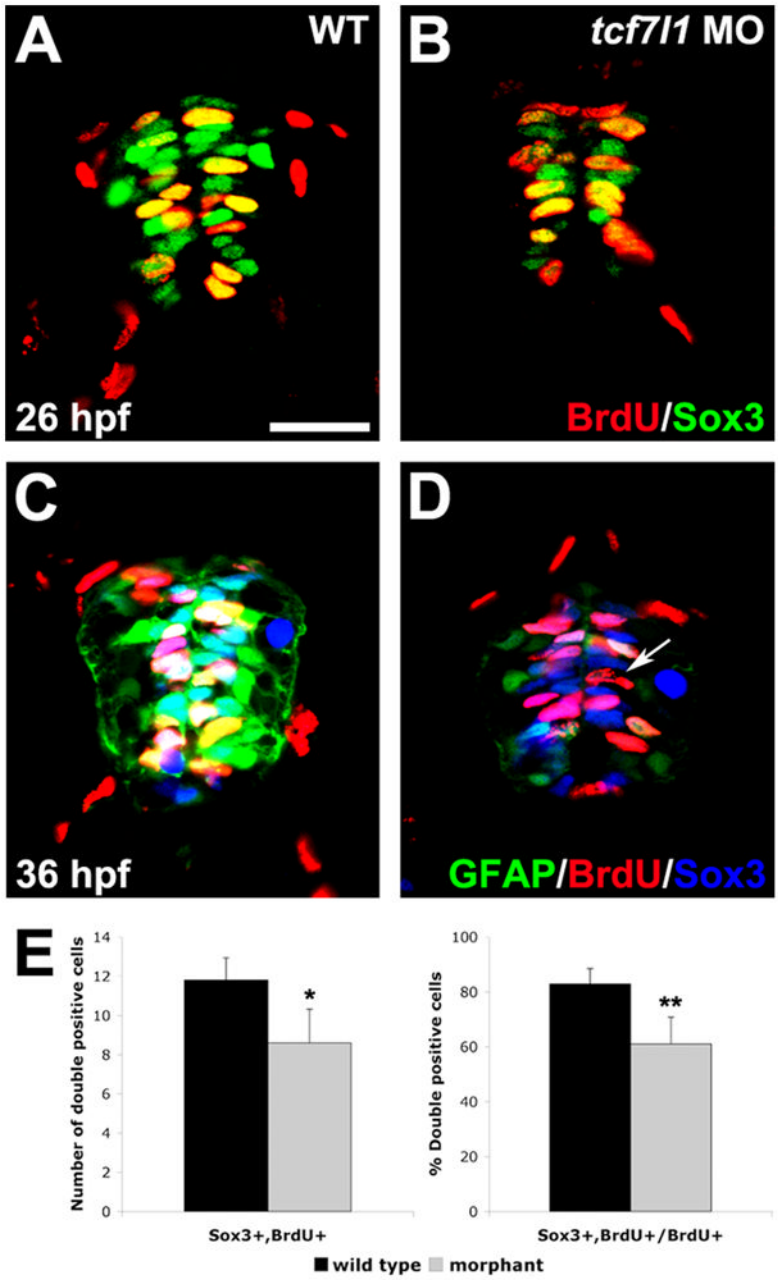

Figure 3. Sox3 expression is lost in *tcf7l1* morphants

(A,B) BrdU and Sox3 co-immunohistochemistry in spinal cross-sections of wild-type and *tcf7l1* morphant embryos. Sox3 expression is reduced in *tcf7l1* morphants at 26 hpf. **(C,D)** BrdU, Sox3 and *gfap:GFP* triple-labeling at 36 hpf. Expression of Sox3 and GFAP are greatly reduced in *tcf7l1* morphants **(D)** compared to wild-type embryos **(C)**. Arrow indicates a BrdU+ cell without expression of GFAP or Sox3. **(E)** Number and percent of BrdU/Sox3 double-positive cells/section in wild-type and *tcf7l1* morphant spinal cords. Error bars indicate SD and asterisks indicate statistical significance. *p < 0.05, **p<.0.001 by Student's unpaired t-test. n=12 sections from 5 individual embryos for each bar. Scale bar = 20μM.

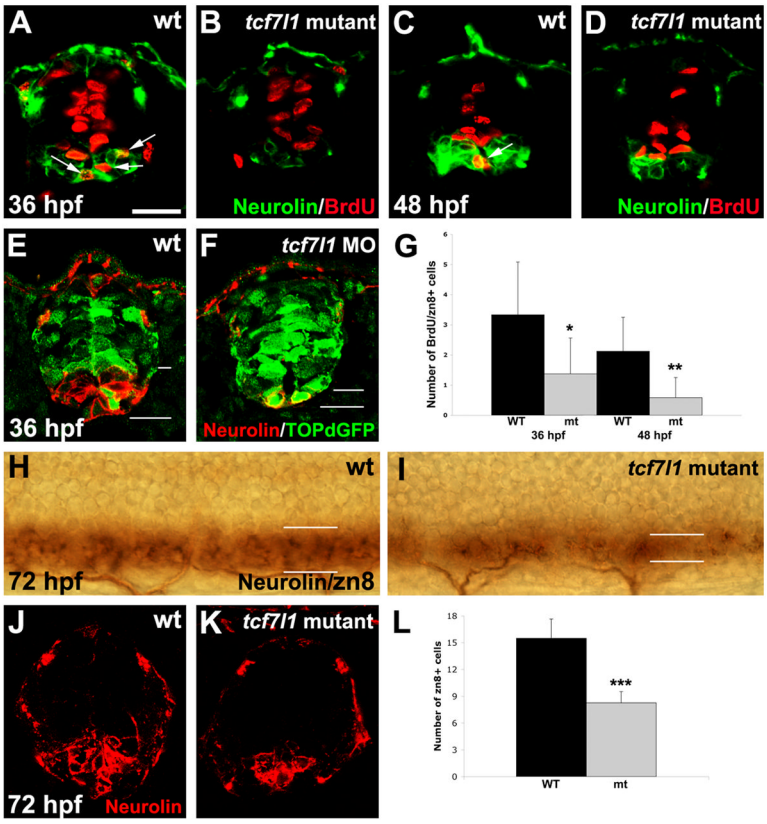

Figure 4. Production of secondary motoneurons is decreased in *tcf7l1* morphants and mutants

(A–D) BrdU and Neurolin double immunohistochemistry on cryosections of wild-type and *tcf7l1* mutant embryos. Arrows indicate double-labeled cells. BrdU+/Neurolin+ secondary motoneurons are reduced in *tcf7l1* mutants compared to wild-type embryos at 36 and 48 hpf. **(E,F)** GFP and Neurolin double immunohistochemistry on cryosections of wild-type and *tcf7l1* morphants expressing the *top:dgfp* Wnt reporter. In morphants, GFP expression is extended ventrally into the zone of decreased Neurolin expression. **(G)** Number of BrdU/Neurolin double-positive cells per cryosection in wild-type and *tcf7l1* mutant embryos. Error bars indicate SD and asterisks indicate statistical significance. *$p < 0.05$, **$p < 0.005$ by Student's unpaired t-test. n=12 sections from 5 individual embryos for each bar. **(H–K)** Whole-mount **(H,I)** and cryosection (**J,K)** immunostaining for Neurolin, showing reduction of secondary motoneurons in *tcf7l1* mutants compared to wild-type controls at 72 hpf. **(L)** Number of Neurolin double-positive cells per cryosection in wild-type and *tcf7l1* mutant embryos. Error bars indicate SD and asterisks indicate statistical significance. ***$p < 0.001$ by Student's unpaired t-test. n=12 sections from 5 individual embryos for each bar. Scale bar = 20μM.

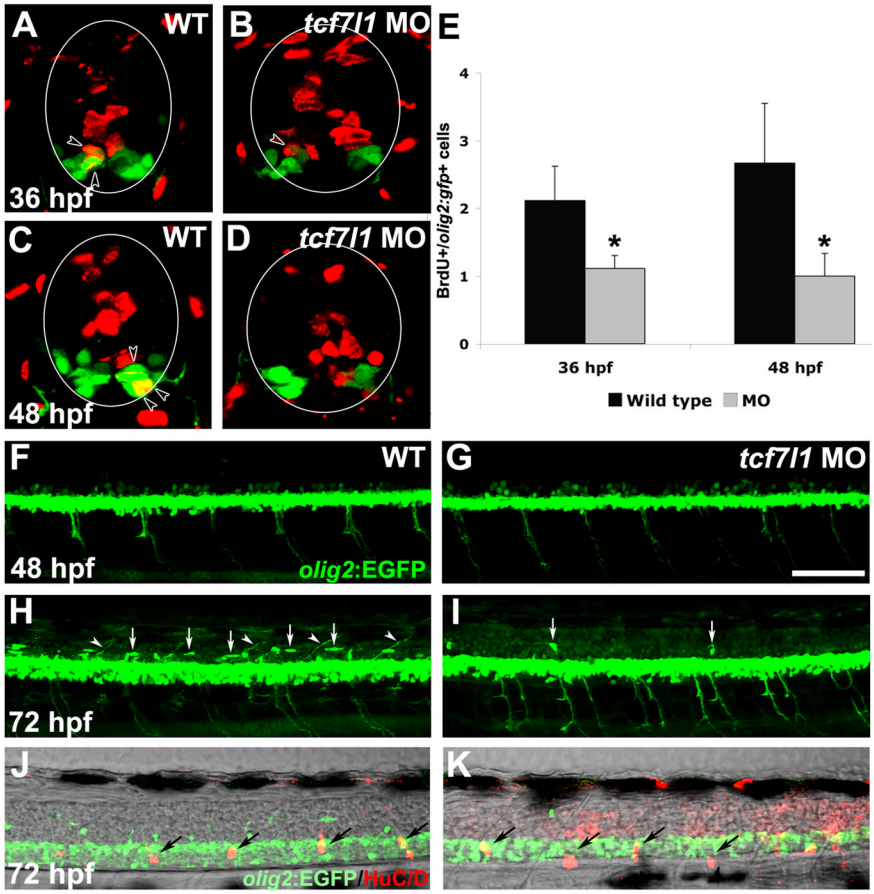

Figure 5. OPC development is reduced in *tcf7l1* morphants

(A–D) BrdU and GFP double immunohistochemistry on cryosections of wild-type and *tcf7l1* morphant *Tg(olig2:EGFP)*^{vu12} embryos. Spinal cord is outlined in circles and arrowheads indicate double-labeled cells. In *tcf7l1* morphants, fewer BrdU+ cells are observed within the GFP+ population. **(G)** Number of BrdU+/*olig2:gfp*+ cells per cryosection in wild-type embryos and *tcf7l1* morphants at 36 and 48 hpf. Error bars indicate SD and asterisks indicate statistical significance. *$p < 0.05$ by Student's unpaired t-test. n=9 sections from 3 different embryos for each bar. **(F–K)** Confocal projection images showing lateral views of the spinal cord in *Tg(olig2:EGFP)*^{vu12} embryos. **(F,G)** GFP-positive cells are reduced in *tcf7l1* morphants at 48 hpf. **(H,I)** At 72 hpf, dorsally migrating OPCs (arrows) and dorsally projecting motoneurons (arrowheads) are absent in *tcf7l1* morphants. **(J,K)** The decrease in OPCs is not due to a general developmental delay, as normal dorsal root ganglion development (arrows) is observed in *tcf7l1* morphants. Scale bars = 20μM in D, 40μM in F.