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Tcf3 inhibits spinal cord neurogenesis by regulating *sox4a* expression

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The Lef/Tcf factor Tcf3 is expressed throughout the developing vertebrate central nervous system (CNS), but its function and transcriptional targets are uncharacterized. Tcf3 is thought to mediate canonical Wnt signaling, which functions in CNS patterning, proliferation and neurogenesis. In this study, we examine Tcf3 function in the zebrafish spinal cord, and find that this factor does not play a general role in patterning, but is required for the proper expression of Dbx genes in intermediate progenitors. In addition, we show that Tcf3 is required to inhibit premature neurogenesis in spinal progenitors by repressing *sox4a*, a known mediator of spinal neurogenesis. Both of these functions are mediated by Tcf3 independently of canonical Wnt signaling. Together, our data indicate a novel mechanism for the regulation of neurogenesis by Tcf3-mediated repression.

KEY WORDS: Zebrafish, Tcf3, Spinal progenitors, Wnt

INTRODUCTION

The central nervous system (CNS) begins as a population of neural progenitor cells that will give rise to postmitotic neurons and glia during development. This process has been extremely well characterized in the vertebrate spinal cord, where important regulatory genes have been identified for progenitor cell specification and neuronal differentiation (Lee and Pfaff, 2001). However, several important unanswered questions remain, including the mechanisms underlying progenitor cell maintenance and neurogenesis.

Previous work has shown that secreted signaling molecules such as sonic hedgehog (Shh) and bone morphogenetic protein (BMP) act to pattern the spinal cord, instructing progenitors of their position in the dorsoventral (DV) axis (Briscoe and Ericson, 2001; Lee and Jessell, 1999). More recently, it has been shown that Wnt signaling from the dorsal spinal cord also specifies dorsal progenitor fates (Alvarez-Medina et al., 2008; Bonner et al., 2008; Zechner et al., 2007). Interestingly, the mechanisms underlying specification of intermediate spinal cord progenitors are less clear. Neither Shh nor BMP signaling are required for the expression of Dbx genes in intermediate progenitors (Gribble et al., 2007), and we and others have shown that Wnt signaling acts to prevent these genes from being expressed in more dorsal regions (Alvarez-Medina et al., 2008; Bonner et al., 2008). Our laboratory has also shown that another candidate inductive signal, retinoic acid, is not absolutely required for Dbx expression in the spinal cord (Gribble et al., 2007). This leaves an open question regarding the mechanism responsible for inducing Dbx expression, a topic of considerable interest due to the speculation that Dbx-positive progenitors may represent a stem-cell population (Fogarty et al., 2005).

Canonical Wnt signaling can also promote proliferation in spinal progenitors (Bonner et al., 2008; Ille et al., 2007; Megason and McMahon, 2002; Zechner et al., 2003), but the source of this signal is less clear. By contrast, relatively little is known about the role of Wnt signaling in the process of spinal cord neurogenesis. Manipulation of the Wnt pathway can affect the ultimate number of spinal neurons produced (Ille et al., 2007; Megason and McMahon, 2002; Zechner et al., 2003), but it is unclear whether this is primarily a downstream result of altered progenitor proliferation, or instead represents a direct response of progenitors to initiate a program of neurogenesis. In other regions of the CNS, Wnt signaling has been shown to directly regulate the expression of genes that control neurogenesis (Lee et al., 2006; Lie et al., 2005; Machon et al., 2007; Van Raay et al., 2005), suggesting that this process is conserved in the spinal cord.

Lef/Tcf proteins are responsible for mediating the transcriptional output of canonical Wnt signals. These proteins can act as transcriptional activators or repressors (Bienz, 1998; Brantjes et al., 2002), and have been shown to regulate specific target genes during CNS development downstream of Wnt signaling (Lee et al., 2006; Takemoto et al., 2006). All vertebrates examined express *Tcf7* and *Tcf3* in the spinal cord (Alvarez-Medina et al., 2008; Merrill et al., 2004; Schmidt et al., 2004; Veien et al., 2005), while *Tcf4* is expressed in chick and mouse (Alvarez-Medina et al., 2008; Lei et al., 2006) but not in zebrafish spinal cord (Young et al., 2002). We and others have described a role for Tcf7 in mediating the dorsal patterning activity of Wnts from the roof plate (Alvarez-Medina et al., 2008; Bonner et al., 2008). By contrast, Tcf3 and Tcf4 are expressed in a complementary pattern to canonical Wnt signals (Alvarez-Medina et al., 2008; Bonner et al., 2008), suggesting that they may act to antagonize Wnt function. Consistent with this model, Tcf4 appears to act primarily as a repressor in the absence of Wnt signaling, helping to refine the dorsal boundary of the ventral progenitor gene *nkx2.2* (Lei et al., 2006). To date, no study has examined the function of Tcf3 in the spinal cord.

All in vivo evidence thus far describes Tcf3 as a transcriptional repressor. The *Tcf3* knockout mouse exhibits defects in rostrocaudal neural patterning and a duplicated node and notochord (Merrill et al., 2004), which are consistent with increased Wnt function. Mouse embryonic skin progenitors require Tcf3 to repress terminal differentiation programs (Nguyen et al., 2006), and in *Xenopus*, depletion of Xtcf3 upregulates known Wnt target genes in the

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organizer such as *siamois*, *goosecoid*, *Xnr3* and *chordin* (Houston et al., 2002). Importantly, forebrain phenotypes in zebrafish *tcf3a* (*headless*) mutants can be rescued by injection of truncated Tcf3a fused to the Engrailed repressor domain (Kim et al., 2000). It is therefore likely that Tcf3 primarily functions to repress target genes in the absence of Wnt activity.

Zebrafish have two *tcf3* genes that are redundantly expressed, required cooperatively in early zebrafish development, and encode proteins with 82% identity (Dorsky et al., 2003). Here we demonstrate that both *tcf3* genes are expressed in overlapping domains in the spinal cord, but are not generally required for DV patterning. We do find, however, that Tcf3 function is required specifically for the expression of Dbx genes in intermediate spinal progenitors. In addition, we find that Tcf3 function prevents spinal progenitors from prematurely undergoing neurogenesis. In the absence of Tcf3 function, proliferating progenitors ectopically express neuronal markers, but do not produce specified neuronal subtypes. We show that Tcf3 functions as a repressor of *sox4a*, a gene known to regulate neurogenesis, and that *sox4a* acts downstream of Tcf3 in the spinal cord. Collectively, these results establish a novel biological role for Tcf3 in maintaining a progenitor state in the spinal cord.

MATERIALS AND METHODS

Fish strains and staging

Embryos were obtained from natural spawning of wild-type (AB*) or *Tg(hsp70l:Δtcf-GFP)*^{w26} (Lewis et al., 2004) zebrafish lines. *tcf3a*$^{-/-}$;*tcf3b*$^{-/-}$ double mutants were produced from a heterozygous incross carrying two mutations: *tcf7l1a*^{m881} and a retroviral insertion in the first exon of *tcf3b* (Znomics). Double mutants were confirmed by PCR genotyping. All developmental stages in this study are reported in hours post-fertilization (hpf) at 28.5°C, according to Kimmel et al. (Kimmel et al., 1995).

Morpholino injections

The sequences of *tcf3a* and *tcf3b* translation and splice-blocking morpholinos have been published previously (Bonner et al., 2008; Dorsky et al., 2003). The sequence of the *sox4a* translation-blocking morpholino is: 5′-CATGCACTACAACAGTCTCAACTTT-3′. Translation-blocking morpholinos at 1 ng/nl (*tcf3a/b*) or 2 ng/nl (*sox4a*) and splice-blocking morpholinos at 5 ng/nl were injected into one-cell stage embryos. The *p53* morpholino (Robu et al., 2007) was co-injected at 5 ng/nl.

RT-PCR

Total RNA from 75 embryos at 18 hpf was isolated using Trizol reagent and was reverse transcribed by random hexamers using the SuperScript First-Strand Synthesis System (Invitrogen). PCR was performed for 30 cycles using an annealing temperature of 57°C (*tcf3a*) and 60°C (*tcf3b*), and reactions were visualized on 1% agarose gels in Tris-acetate-EDTA. The spliced product for *tcf3a* is 257 bp and the unspliced product is 354 bp. The spliced product in *tcf3b* is 205 bp and the unspliced product is approximately 3 kb.

In situ hybridization

Probe synthesis and in situ hybridization were performed as described previously (Oxtoby and Jowett, 1993), using digoxigenin-labeled antisense RNA probes and BM Purple (Roche). Double in situ hybridizations were carried out using digoxigenin- and fluorescein-labeled antisense RNA probes (Jowett, 2001) and visualized using BM Purple (Roche) and Fast Red (Roche). The following probes were made by our lab: *tcf3a* (*tcf7l1a* – Zebrafish Information Network), *tcf3b* (*tcf7l1b*), *tubb5*, *lhx1a*, *dbx1a*, *dbx2*, *sox4a*, *sox11a* and *zic2b*. We were given the following probes: *iro3* (*irx3a*) (Lewis et al., 2005), *pax2a* (Krauss et al., 1991), *pax3* (Seo et al., 1998), *nkx6.1* (Cheesman et al., 2004), *olig2* (Park et al., 2002), *nkx2.2* (Barth and Wilson, 1995), *pcna* (Lee and Gye, 1999), *cdkn1c* (Park et al., 2005), *isl1* (Okamoto et al., 2000), *evx1* (Thaeron et al., 2000), *sox3* (Kudoh et al., 2004) and *en1b* (*eng1b* – Zebrafish Information Network) (Higashijima et al., 2004). For whole-mount photography after all staining methods, embryos were de-yolked and mounted laterally or cryosectioned.

Immunohistochemistry

Cryosections (12 μm) were incubated in Alexa Fluor 594-conjugated (1:1000, Molecular Probes) or unconjugated BrdU (1:1000, DSHB), phospho-histone H3 (1:1000, Upstate), and HuC/D (1:1000, Molecular Probes) antibodies for 2 hours at room temperature. Sections were washed and incubated in anti-mouse Alexa Fluor 488 or Cy3 secondary antibodies (1:200, Molecular Probes) for 1 hour at room temperature. Slides were washed and mounted in Vectashield (Vector Laboratories). Whole-mount embryos were incubated in phospho-histone H3 antibody at 1:500 overnight at room temperature, then incubated in anti-mouse HRP-conjugated secondary antibody (1:200, Molecular Probes) overnight, followed by DAB staining.

BrdU labeling

Embryos at 18 or 24 hpf were incubated in 10 mM BrdU solution for 20 minutes, then fixed immediately (for determination of labeling index), or incubated for 6 hours (for HuC/D double-labeling) then fixed. Following cryosectioning, embryos were incubated for 1 hour in 2M HCl followed by immunohistochemistry. For determination of labeling index, sections were counterstained with propidium iodide.

ΔTcf3 heat-shock experiments

Tg(hsp70l:Δtcf-GFP)^{w26} heterozygous males were mated with wild-type females and embryos were injected with the *tcf3* morpholinos. All embryos were heat-shocked at 18 hpf for 1 hour at 37-39°C and fixed at 24 hpf for analysis.

mRNA injections

sox4a mRNA was synthesized from a *sox4a*-pCS2+ plasmid, using the SP6 mMESSAGE mMACHINE Transcription Kit (Ambion). Approximately 1 ng *sox4a* mRNA was injected into one-cell-stage wild-type embryos.

Western blotting

Dechorionated wild-type, morphant and heat-shocked *Tg(hsp70l:Δtcf-GFP)*^{w26} embryos were homogenized in 4× sample buffer, subjected to 8% SDS-PAGE, and blotted onto polyvinylidene fluoride membrane. Affinity-purified rabbit anti-Tcf3a serum (Open Biosystems) was applied at 1:150 dilution, and anti-rabbit IgG-HRP (Molecular Probes) was applied at 1:10,000. The secondary antibody was visualized with an electrogenerated chemiluminescence reaction, using standard protocols.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) analysis was performed as described previously (Lee et al., 2006), using a polyclonal Tcf3a antibody (Open Biosystems). We used chromatin lysates from whole 24 hpf embryos, and following cross-linking the DNA was sonicated to <500 bp. Following immunoprecipitation, PCR was performed on eluted DNA, total input chromatin, and no antibody controls, for 38-40 cycles using the following primers: –4.8 kb fragment-L, 5′-TCCAAGAATCTAT-CACTTTTCTTGTTT-3′; –4.8 kb fragment-R, 5′-TCAATCCAAGGT-GATGTAGCC-3′; +1.6 kb fragment-L, 5′-TGGTTGTTTTGCTTC-GAGTG-3′; +1.6 kb fragment-R, 5′-AAAGCCAGCCAATTGTGTC-3′; +7.4 kb fragment-L, 5′-GCAGGCGCACTAAAACTACC-3′; and +7.4 kb fragment-R, 5′-AGTGCATGATATCGGACAAGG-3′. Each assay was performed in triplicate to confirm positive and negative signals.

RESULTS

Expression of *tcf3a* and *tcf3b* suggests redundant function in spinal cord development

The expression and function of zebrafish *tcf3* in early development has been reported previously (Dorsky et al., 2003; Kim et al., 2000). To determine the expression of *tcf3* in the developing spinal cord we performed in situ hybridization for *tcf3a* and *tcf3b* during the period when spinal cord progenitors are proliferating, patterned and producing postmitotic neurons. We found that at similar axial levels *tcf3a* and *tcf3b* expression remained consistent from 15-24 hpf (Fig. 1A-F). Both genes were expressed in the intermediate and ventral spinal cord but were restricted from the most dorsal region until at

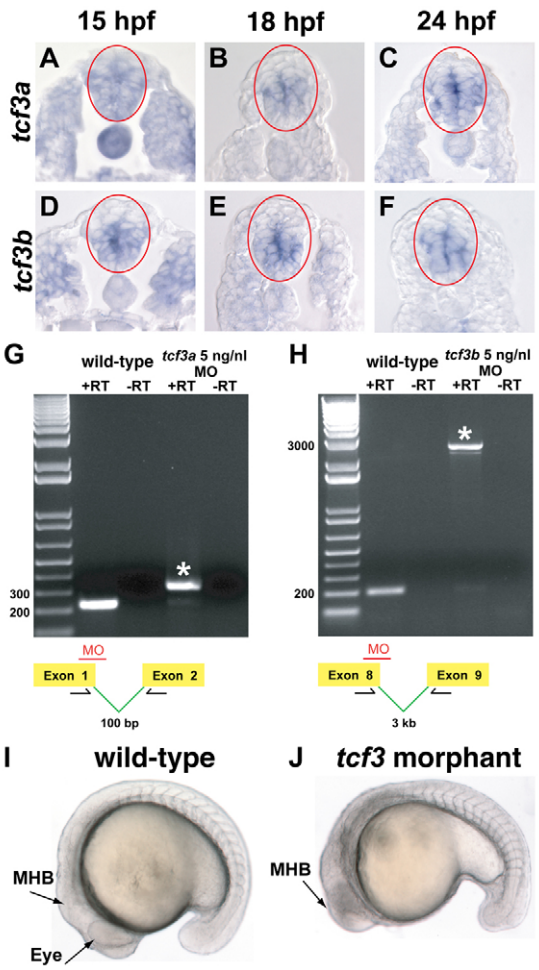

Fig. 1. Analysis of *tcf3a* and *tcf3b* spinal cord expression and effectiveness of morpholinos. (**A-F**) Cross-sections with the spinal cord outlined in red. *tcf3a* and *tcf3b* are co-expressed in the intermediate and ventral spinal cord at 15 hpf (A,D), 18 hpf (B,E) and 24 hpf (C,F). (**G**,**H**) RT-PCRs for both *tcf3a* and *tcf3b* splice morphants show unspliced products (the asterisk in each gel) at 5 ng/nl for each morpholino. (**I**,**J**) *tcf3* splice morphant embryos lack eyes, produce minimal brain tissue rostral to the midbrain-hindbrain boundary (MHB) and have a shortened rostral-caudal axis.

least 30 hpf, the latest timepoint analyzed (not shown). This pattern is complementary to expression of dorsal Wnt genes, activated β-catenin and a Wnt-responsive reporter transgene (Bonner et al., 2008) and suggests that Tcf3 may function as a repressor in the absence of canonical Wnt signaling.

Both *tcf3* genes are required zygotically for embryonic patterning

In order to examine Tcf3 loss-of-function phenotypes we knocked down zygotic transcription with splice-blocking morpholino oligonucleotides for *tcf3a* and *tcf3b*. RT-PCR showed complete block of splicing at a concentration of 5 ng/nl for each morpholino (Fig. 1G,H). Injection of the *tcf3a* morpholino alone resulted in a mild truncation of forebrain structures (not shown), a phenotype significantly more severe than seen in *hdl* zygotic mutants (Kim et al., 2000), suggesting that the *m881* allele may not represent a null mutation. Injection of the *tcf3b* morpholino alone produced no significant morphological phenotypes (not shown). Injection of both *tcf3a* and *tcf3b* morpholinos resulted in embryos with minimal brain tissue rostral to the midbrain-hindbrain boundary and a shortened rostrocaudal axis (Fig. 1I,J). The morphological phenotypes generated with the splice-blocking morpholinos were similar to those generated with translation-blocking morpholinos (Dorsky et al., 2003), suggesting that zygotic expression of both genes is required cooperatively for normal patterning.

Because of the overlapping spinal cord expression patterns and the compound effect of injecting both morpholinos simultaneously, all subsequent phenotypes were analyzed following co-injection of *tcf3a* and *tcf3b* morpholinos, and we refer to these embryos as '*tcf3* morphants'. We also observed similar spinal cord phenotypes after co-injecting translation-blocking morpholinos for *tcf3a* and *tcf3b* (Dorsky et al., 2003), despite more severe morphological defects (not shown). Furthermore, embryos doubly homozgyous for mutations in *tcf3a* and *tcf3b* (see Materials and methods for further details) showed similar morphological defects to *tcf3* morphants, as well as similar defects in spinal cord development (not shown). Finally, neither *tcf3a* nor *tcf3b* splice morpholinos produced any phenotypes in the spinal cord when injected alone (not shown). Together, these results suggest that the spinal cord phenotypes produced by the splice-blocking morpholinos are specific, and thus all assays carried out in this study were performed using these reagents. We observed that the *tcf3b* splice-blocking morpholino produced one non-specific effect: widespread cell death throughout the embryos at 18 hpf and beyond (not shown). This apoptosis was due to p53-dependent off-target effects (Robu et al., 2007), and was completely abolished by co-injection with a *p53* morpholino. We therefore injected both *tcf3* morpholinos with a *p53* morpholino for all subsequent analysis.

Tcf3 is not generally required for dorsoventral spinal cord patterning

Because previous work has shown that canonical Wnt signaling is essential for dorsal progenitor patterning and interneuron specification (Alvarez-Medina et al., 2008; Bonner et al., 2008; Ille et al., 2007; Zechner et al., 2007), we asked whether *tcf3* morphant spinal cords were mis-patterned. In a previous study, we concluded that Tcf3 function was not required for DV patterning of several markers at 24 hpf (Bonner et al., 2008). To confirm and extend these findings, we assayed for expression of multiple genes at 18 hpf, a time at which there are some differentiated neurons but the majority of cells are still progenitors. By 18 hpf many well-characterized transcription factors, which define regions of the spinal cord, are expressed, including *zic2b*, *pax3*, *iro3*, *nkx6.1*, *olig2* and *nkx2.2*. We did not observe a dorsoventral shift in the expression of any of these markers, indicating that patterning in this axis was unaffected (Fig. 2). As each of these markers demarcates a specific region of the spinal cord and collectively they encompass the dorsoventral axis, we can conclude from these data that Tcf3 is not generally required for DV patterning of spinal progenitors.

Tcf3 is required for *dbx1a* expression in spinal progenitors

In our previous work, we noted that although Tcf3 did not generally affect DV patterning, it was specifically required for *dbx2* expression at 24 hpf (Bonner et al., 2008). To extend these observations, we examined the expression of *dbx1a*, which normally arises earlier than *dbx2* (Gribble et al., 2007). In addition to marking a specific domain in the spinal cord, in mouse embryos *Dbx1* is expressed in a unique class of progenitors that gives rise to neurons, astrocytes and oligodendrocytes (Fogarty et al., 2005; Pierani et al.,

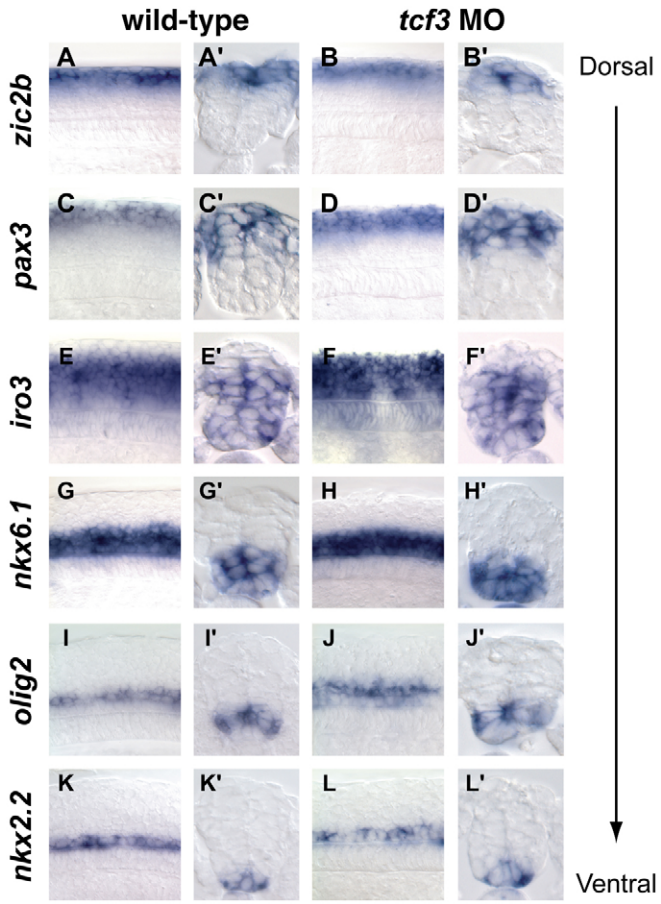

Fig. 2. Dorsoventral patterning is unaffected in *tcf3* morphants. Whole-mount lateral and cross-section views of 18 hpf wild-type and *tcf3* morphant embryos hybridized for *zic2b* (**A-B′**), *pax3* (**C-D′**), *iro3* (**E-F′**), *nkx6.1* (**G-H′**), *olig2* (**I-J′**) and *nkx2.2* (**K-L′**). None of these expression domains was unaffected in *tcf3* morphant embryos.

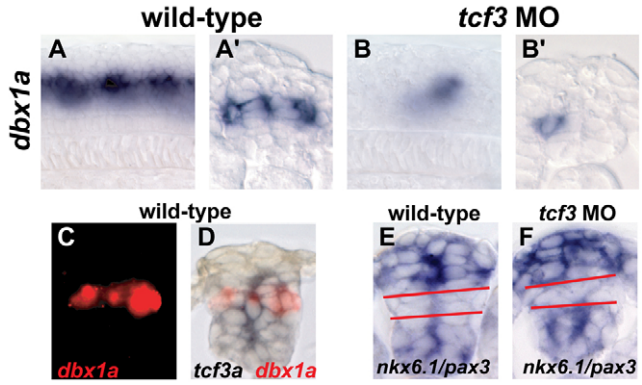

Fig. 3. *dbx1a* expression is absent in *tcf3* morphants. (**A**,**A′**) Whole-mount lateral and cross-section views of 18 hpf wild-type embryos hybridized for *dbx1a* mRNA. (**B**,**B′**) *tcf3* morphant embryos hybridized for *dbx1a*, showing loss of expression throughout intermediate spinal progenitors. Residual expression is likely to be in postmitotic neurons. (**C**,**D**) Double in situ hybridization for *dbx1a* (red) and *tcf3* (blue), showing overlap between the two genes. (**E**,**F**) Both wild-type (E) and *tcf3* morphants (F) have a similar gap (red lines) between the *pax3* (dorsal) and *nkx6.1* (ventral) expression domains, suggesting that a general dorsoventral shift in patterning has not occurred.

2001). We found that *tcf3* morphants have discontinuous expression of *dbx1a* in spinal cord progenitors at 18 hpf throughout the rostrocaudal axis (Fig. 3A,B′). Double in situ hybridization in wild-type embryos shows that the dorsal limit of *tcf3* expression overlaps with *dbx1a* expression (Fig. 3C,D). Because *dbx1a* marks a specific domain of spinal progenitors, we wanted to confirm that the cells had not adopted an alternative positional identity in *tcf3* morphants. We determined whether the affected cells were still present by examining expression of two adjacent genes unaffected in *tcf3* morphants: *pax3* and *nkx6.1* (Fig. 2). In wild-type embryos a clear two-cell gap in expression of *pax3* and *nkx6.1* was observed (Fig. 3E). In *tcf3* morphants, we still observed a gap between *pax3* and *nkx6.1* (Fig. 3F), suggesting that these cells are physically present and occupy the proper DV position. In addition, we previously showed that another dorsal marker adjacent to the *Dbx* domain, *msxc*, was unaffected in *tcf3* morphants (Bonner et al., 2008). We conclude that intermediate progenitors in *tcf3* morphants have the appropriate positional identity and specifically fail to express multiple *Dbx* genes.

Tcf3 controls the rate of progenitor proliferation

Previous work from our laboratory and others has demonstrated a role for Wnt signaling in regulating the proliferative state of spinal progenitors (Bonner et al., 2008; Ille et al., 2007; Megason and McMahon, 2002; Zechner et al., 2003). In addition, we previously showed that loss of Tcf3 function results in a significant reduction in mitotic index at 24 hpf (Bonner et al., 2008). To determine whether progenitors lacking Tcf3 are forced to exit the cell cycle, we performed several assays. First, we analyzed the mitotic index at 18 hpf, when we observed a loss of *dbx1a* expression. Staining with the M-phase marker phospho-histone H3 (pH3) showed a small but significant decrease in mitotic index in *tcf3* morphants (Fig. 4A-C), indicating that the phenotype at 18 hpf is less severe than the one we observed at 24 hpf. In addition we determined the BrdU labeling index at 18 hpf for wild-type and *tcf3* morphants using a short (20 minute) pulse of BrdU labeling. We found that the BrdU labeling index was also slightly but significantly decreased in *tcf3* morphants compared with controls (Fig. 4D-F). We next performed in situ hybridization for the proliferative marker *pcna* (Lee and Gye, 1999) and *cdkn1c* (*p57*), which is upregulated upon cell cycle exit (Park et al., 2005). These two assays allowed us to detect gross changes in cell cycle state. Importantly, we did not observe loss of *pcna* or ectopic *cdkn1c* in medial progenitor cells (Fig. 4G-J), demonstrating that the majority of progenitors remain proliferative in *tcf3* morphants. Together, these results suggest that loss of Tcf3 results in a slower overall cell cycle time and/or arrest before the G2/M transition.

Spinal progenitors precociously express neuronal markers in *tcf3* morphants

We next asked whether Tcf3 function was required for the regulation of neurogenesis in spinal progenitors, using both general and subtype-specific postmitotic neuronal markers. We first examined *tubulin beta 5* (*tubb5*), which is expressed in cells as they undergo neurogenesis, and is the closest homolog to mammalian *Tubb3* with respect to expression pattern (Oehlmann et al., 2004). At 18 hpf, *tcf3* morphants displayed ectopic *tubb5* expression in the medial spinal cord, which normally exclusively contains progenitor cells (Fig. 5A,B). Overall, 12/30 sections from morphants showed ectopic *tubb5* expression compared with 1/30 sections from wild-type embryos.

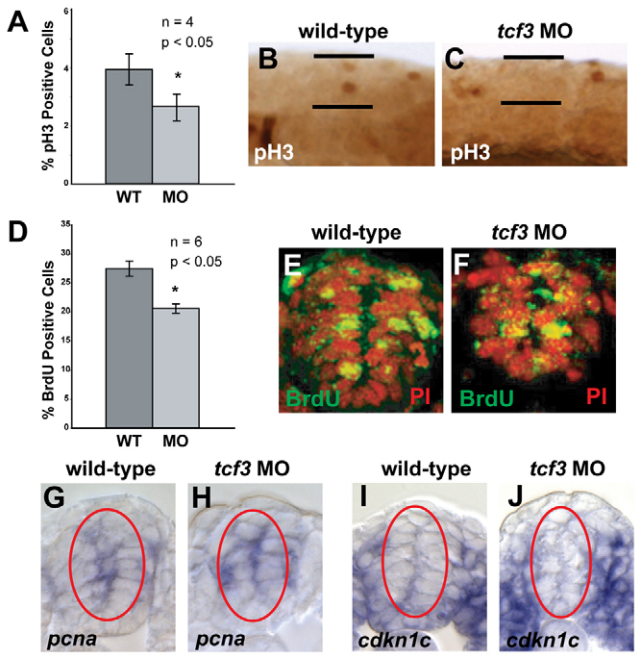

Fig. 4. Tcf3 controls the rate of spinal progenitor proliferation. (**A-F**) The mitotic index (A-C) and the BrdU-labeling index (D-F) of *tcf3* morphant (MO) embryos are slightly decreased, yet significantly different (*), as compared with wild-type (WT) embryos. Error bars show s.e.m. and *P*-values were calculated using unpaired *t*-tests. (B,C) Whole-mount views of anti-pH3 staining, with the spinal cord demarcated by the black lines. (E,F) Cross-sections, with BrdU staining in green and propidium iodide (PI) counterstaining in red. (**G-J**) In situ hybridization on cross-sections shows that proliferative cell nuclear antigen (*pcna*) and *cdkn1c* (*p57*) are expressed normally in *tcf3* morphants at 18 hpf.

Additionally, at 24 hpf we examined HuC/D protein expression, a pan-neuronal marker (Park et al., 2000), in embryos treated with BrdU at 18 hpf. As with *tubb5*, we observed ectopic HuC/D expression in the medial spinal cord (Fig. 5C,D). We found that 18/30 morphant sections showed medial Hu-positive cells, compared with 2/30 wild-type sections. In addition, 15.6±2.6% (s.e.m., *n*=30 sections) of Hu-positive cells per section were also BrdU-positive. Significantly, we never observed cells to be double-positive for HuC/D and BrdU in sections from wild-type embryos (*n*=30 sections). To confirm that this phenotype was specific to loss of *tcf3a* and *tcf3b* function, we analyzed embryos homozygous for mutations in both *tcf3* genes. After the genotype of individual embryos was confirmed by PCR, they were sectioned and stained for HuC/D expression. We found that 16/40 sections from mutant embryos showed ectopic HuC/D-positive cells in the medial spinal cord (not shown). Together, these results suggest that progenitor cells lacking Tcf3 precociously adopt neuronal characteristics.

To determine whether progenitors lacking Tcf3 were being specified as particular classes of neurons, we assayed for multiple subtype-specific markers previously characterized in zebrafish and other vertebrates. At 18 hpf we first examined expression of *lhx1a* and *pax2a*, which mark broad populations of interneurons (Mikkola et al., 1992; Park et al., 2002). We found that expression of both markers was substantially reduced in *tcf3* morphants (Fig. 5E-H), suggesting that fewer specified interneurons were present. We next examined *evx1*, *en1b* and *isl1*, which are expressed earlier than *lhx1a* and *pax2a*, and mark specific classes of zebrafish spinal neurons (Higashijima et al., 2004; Okamoto et al., 2000; Thaeron et al., 2000). We found that *tcf3* morphants possessed fewer *evx1*$^+$, *en1b*$^+$ and *isl1*$^+$ neurons than controls (Fig. 5I-N and Table 1). Together, these data suggest that although loss of Tcf3 results in precocious neurogenesis, it does not drive progenitors into specific postmitotic fates. We therefore conclude that Tcf3 normally acts to coordinate neurogenesis with cell cycle exit and fate specification, and maintains cells in a progenitor state until they receive the necessary signals for differentiation.

Tcf3 negatively regulates *sox4a* expression in spinal progenitors

The phenotype of *tcf3* morphants was strikingly similar to chick embryos in which the group-C *Sox* genes *Sox4* and *Sox11* were overexpressed (Bergsland et al., 2006). In zebrafish, *sox4a* is normally expressed very weakly in the spinal cord at 24 hpf (Fig. 6A). By contrast, we found that in *tcf3* morphants *sox4a* is ectopically expressed throughout the intermediate and ventral spinal cord progenitor domain at 24 hpf (Fig. 6B), consistent with precocious expression of neuronal markers. To test the function of *sox4a* in zebrafish neurogenesis, we injected *sox4a* mRNA into embryos at the one-cell stage and analyzed HuC/D expression at 24 hpf. We observed ectopic HuC/D-positive cells in the medial spinal cord (Fig. 6C), similar to the phenotype in *tcf3* morphants (Fig. 5D). We found that 11/30 sections from *sox4a*-injected embryos showed medial Hu-positive cells, compared with 2/30 sections from controls. In addition, following BrdU labeling at 18 hpf, we found that 8.2±1.5% (s.e.m., *n*=30 sections) of Hu-positive cells per section were also BrdU-positive at 24 hpf. These results confirm that the function of *sox4a* is conserved between zebrafish and other vertebrates, and suggest that Tcf3 acts upstream of *sox4a* to prevent precocious expression of neuronal markers.

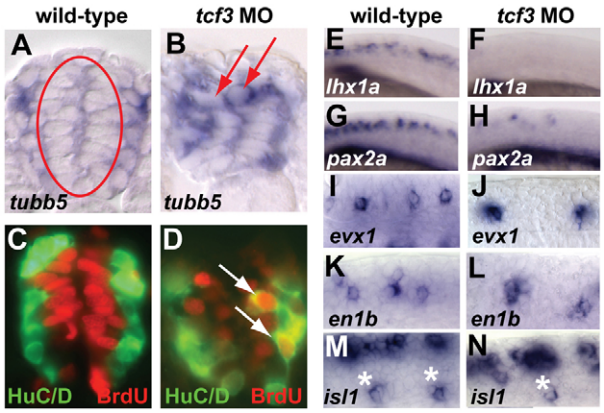

Fig. 5. Spinal progenitors lacking Tcf3 precociously express neuronal markers. (**A**,**B**) Cross-sections of *tubb5* in situ hybridization at 18 hpf show that *tcf3* morphants have ectopic *tubb5*-positive cells in the medial progenitor domain (arrows). (**C**,**D**) Double labeling for HuC/D (green) and BrdU (red) at 24 hpf shows BrdU-positive Hu-positive cells (arrows) in *tcf3* morphants, some of which are in the medial domain. (**E-N**) Lateral whole-mount views of postmitotic neuronal markers at 18 hpf. (E-H) *lhx1a* and *pax2a*, which mark multiple classes of spinal interneurons, are drastically reduced in *tcf3* morphants. (I-N) *evx1*$^+$ interneurons, *en1b*$^+$ interneurons and *isl1*$^+$ primary motoneurons (asterisks) are also reduced in *tcf3* morphants.

In addition, we examined the expression of the group-B *Sox* gene *sox3*, which marks mitotic neural progenitors in the spinal cord and is expressed reciprocally with *Sox4* and *Sox11* in other vertebrates (Bergsland et al., 2006). Consistent with the ectopic expression of *sox4a*, we observed a dramatic reduction in *sox3* expression in *tcf3* morphants (Fig. 6D,E). We also examined the expression of other group-C *Sox* genes to determine which factors other than Sox4a might normally regulate neurogenesis in the zebrafish spinal cord. We found that *sox11a* was normally expressed in both the progenitor zone and postmitotic neurons (Fig. 6F), but was unaffected in *tcf3* morphants (not shown). Together, these results suggest that Tcf3 regulates the normal balance between group-B and group-C *Sox* gene expression, thus preventing precocious neurogenesis.

To determine whether all of the phenotypes in *tcf3* morphants could be reproduced by *sox4a* overexpression, we analyzed the expression of *dbx1a* in injected embryos. In contrast to our observations of precocious neurogenesis, we found no changes in *dbx1a* expression at 18 hpf (Fig. 6G,H). In addition, we found that *dbx2* expression was unaffected following *sox4a* overexpression (not shown). Therefore, we conclude that Tcf3 does not act through *sox4a* in regulating *Dbx* genes, and must function through an independent pathway.

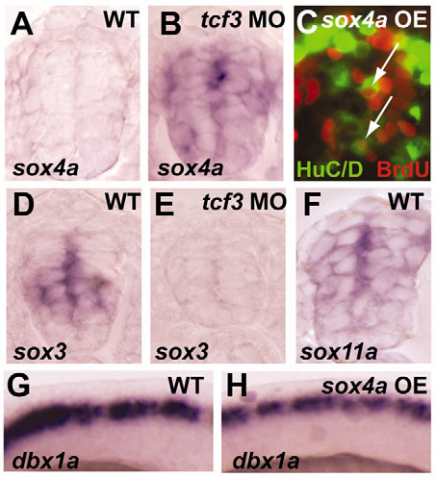

Fig. 6. *sox4a* is repressed by Tcf3 in spinal progenitors. (**A**,**B**) *sox4a* is weakly expressed in the spinal cord of wild-type embryos at 24 hpf and is ectopically expressed throughout the intermediate and ventral spinal cord of *tcf3* morphants. (**C**) Overexpression of *sox4a* mRNA leads to ectopic HuC/D BrdU double-positive cells in medial progenitors (arrows). (**D**,**E**) *sox3* is expressed in intermediate and ventral spinal progenitors of wild-type embryos at 24 hpf, and is drastically reduced in *tcf3* morphants. (**F**) *sox11a* is expressed in both spinal progenitors and postmitotic neurons at 24 hpf. (**G**,**H**) *sox4a* overexpression does not affect the expression of *dbx1a* in spinal progenitors.

Tcf3 functions as a repressor upstream of *sox4a* and neurogenesis

To test whether Tcf3 acts as a transcriptional repressor in spinal progenitors, we used a transgenic line, *Tg(hsp70l:tcf3-GFP)*^{w26}, which inducibly expresses a dominant-repressor form of Tcf3 (Lewis et al., 2004). We hypothesized that if Tcf3 functions as a repressor, then expression of ΔTcf should epistatically antagonize the morphant phenotypes. We found that induction of ΔTcf was able to repress the expression of *sox4a*, both alone and in the presence of *tcf3* morpholinos (Fig. 7A,B). Analysis of proliferation in embryos expressing ΔTcf showed that this transgene completely inhibits BrdU uptake (Fig. 7C,D). No BrdU-positive cells were observed in ΔTcf-expressing embryos at 24 hpf, following a 20 minute labeling period.

In addition, expression of ΔTcf inhibited the normal expression of HuC/D throughout the spinal cord, alone and in *tcf3* morphants (Fig. 7E,F). We found that uninjected ΔTcf-expressing embryos had 2.3±0.5 (s.e.m., n=15 sections) Hu-positive cells per section, while ΔTcf-expressing embryos injected with *tcf3* MO had 1.9±0.8 (s.e.m., n=15 sections) Hu-positive cells per section. By contrast, wild-type controls had 6.2±0.9 (s.e.m., n=15 sections) Hu-positive cells per section. These results indicate that Tcf3-mediated repression can block cell proliferation, but does not result in premature neurogenesis. Furthermore, they suggest that Tcf3 functions as a repressor of *sox4a*, and that this repressor activity is required to prevent progenitor cells from precociously expressing neuronal markers.

To determine whether *sox4a* expression is sufficient to cause ectopic neurogenesis in the presence of Tcf3-mediated repression, we overexpressed ΔTcf in transgenic embryos injected with *sox4a* mRNA. Because ΔTcf overexpression blocks proliferation, we were not able to assess whether the absolute number of Hu-positive cells was restored following *sox4a* injection. However, we did observe ectopic medial Hu-positive cells in embryos expressing both ΔTcf and *sox4a* (Fig. 7G). We found that 5/30 sections from *sox4a*-injected embryos showed medial Hu-positive cells, compared with 0/30 sections from uninjected ΔTcf-expressing controls. To test whether *sox4a* is required for ectopic neurogenesis in the absence of Tcf3, we simultaneously injected *tcf3* morpholinos with a translation-blocking morpholino for *sox4a*. Whereas the *sox4a* morpholino did not have any effect on spinal cord neurogenesis when injected alone (not shown), we found that 0/30 sections from *tcf3/sox4a* morphants contained ectopic Hu-positive cells (Fig. 7H). Together, these data indicate that ectopic expression of *sox4a* is both necessary and sufficient to promote precocious neurogenesis in progenitors downstream of Tcf3.

Table 1. Subtype-specific neuronal markers in *tcf3* morphants

	evx1$^+$	*en1b*$^+$	*isl1*$^+$
Wild type	24.78±0.78	14.60±0.70	15.33±1.00
tcf3 morphant	11.00±0.82	9.80±0.29	10.10±0.48
P	<0.0001	<0.0001	<0.05

sox4a is a target of Tcf3 in vivo

Using ChIP analysis we asked whether enhancer regions of *sox4a* interact with Tcf3 protein in vivo. For immunoprecipitation, we used a polyclonal antibody against zebrafish Tcf3a that recognizes a single band of 53 kDa in lysates from wild-type 24 hpf embryos, which is absent in *tcf3* morphant embryo lysates (Fig. 8A). The Tcf3 antibody is also able to detect the ΔTcf3-GFP protein in lysates from heat-shocked transgenic embryos (Fig. 8A). We first identified two regions of *sox4a* genomic sequence containing Lef/Tcf consensus sites within evolutionarily conserved elements (Fig. 8B), using the UCSC Genome Browser (Kent et al., 2002). One site was 4.8 kb upstream of the *sox4a* start codon, and the other was 1.6 kb downstream of the start codon, near the 3′ end of the cDNA. After sonication and immunoprecipitation of 24 hpf chromatin extracts with the Tcf3a antibody, we performed PCR to amplify DNA fragments near these putative binding sites, along with a negative control fragment that did not contain any consensus sites. We were able to immunoprecipitate fragments near both conserved sites, but

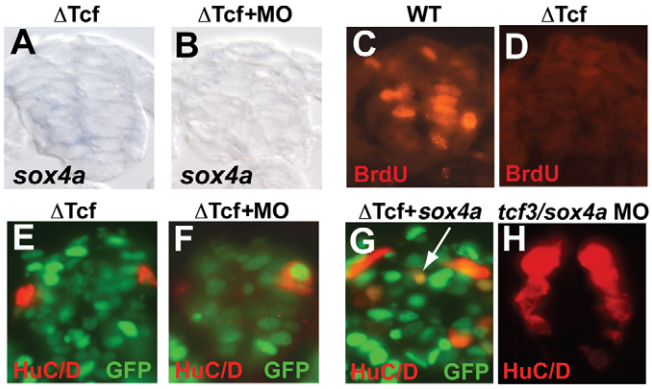

Fig. 7. A repressor form of Tcf3 can block neurogenesis upstream of *sox4a*. (**A**,**B**) Heat shock-induced expression of ΔTcf at 18 hpf eliminates expression of *sox4a*, both alone and in the presence of *tcf3* morpholinos. (**C**,**D**) ΔTcf expression at 18 hpf completely blocks BrdU uptake at 24 hpf. (**E**,**F**) Expression of ΔTcf (green, visualized by GFP fusion) blocks expression of HuC/D (red), both alone and in the presence of *tcf3* morpholinos. (**G**) Simultaneous overexpression of *sox4a* mRNA and the ΔTcf transgene leads to ectopic Hu-positive cells in the medial spinal cord (arrow). (**H**) Co-injection of *tcf3* and *sox4a* morpholinos does not lead to ectopic Hu-positive cells in the medial spinal cord.

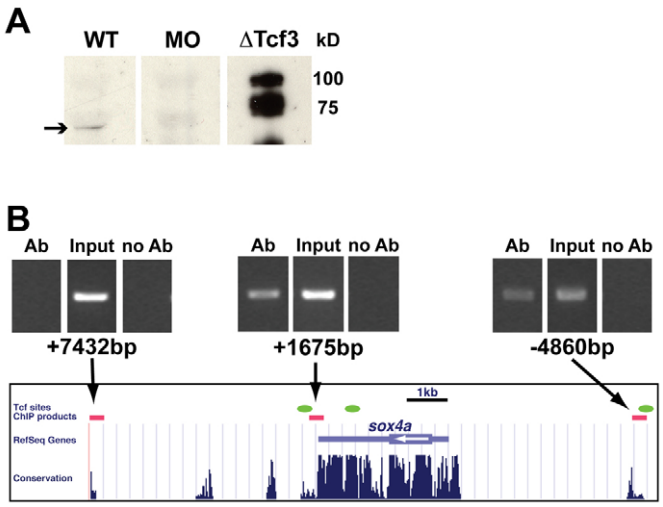

Fig. 8. Tcf3 binds to regulatory regions of *sox4a* in vivo. (**A**) A Tcf3a polyclonal antibody detects a specific band that is absent from *tcf3* morphants, and detects larger bands in ΔTcf3-expressing embryo lysates. Multiple bands are most likely to be due to rearranged copies of the transgene. (**B**) ChIP analysis of whole-embryo lysates shows specific immunoprecipitation of two phylogenetically conserved DNA fragments in regulatory regions of *sox4a*. A third fragment, lacking nearby Tcf sites, does not precipitate. The conservation plot is adapted from the UCSC Genome Browser. ChIP fragments are indicated by red bars and potential Tcf-binding sites in conserved regions are indicated by green ovals

not our negative control (Fig. 8B). These experiments indicate that Tcf3 interacts with putative regulatory regions of *sox4a* in 24 hpf zebrafish embryos. Therefore, Tcf3 may be a direct transcriptional repressor of *sox4a* in the spinal cord.

DISCUSSION

The canonical Wnt pathway is clearly important for spinal cord development, but the role of Tcf3, which has previously been shown to antagonize Wnt function during development, has not been studied. We show that from the earliest stages of spinal cord development, *tcf3* expression is restricted to intermediate and ventral regions. In addition, we find that expression is maintained through the time when cells are proliferating and expressing progenitor-specific genes. Whereas we have previously shown that Tcf7 mediates dorsal spinal patterning (Bonner et al., 2008), and other studies have suggested that Tcf4 regulates the dorsal limit of the ventral progenitor gene *nkx2.2* (Lei et al., 2006), our data indicate that Tcf3 is not required for these functions. We show instead that Tcf3 is required to inhibit progenitor differentiation in the spinal cord. Our data indicate that in the absence of Tcf3, a gene implicated in neuronal differentiation (*sox4a*) is ectopically expressed, leading to premature expression of *tubb5* and HuC/D in progenitor cells. In addition, we find that Tcf3 is required for expression of Dbx genes in intermediate spinal progenitors. Work by ourselves and others has shown that Dbx expression is required for the proper specification of spinal interneurons (Gribble et al., 2007; Pierani et al., 2001), and several of the cell-type-specific markers analyzed here are known to depend on Dbx function. Thus the simultaneous loss of Dbx expression and premature neurogenesis could lead to the production of 'generic' or unspecified neurons. Other factors may be downstream of Tcf3 in the ventral spinal cord, possibly accounting for the decrease in primary motoneurons we observed in morphants. Together, these findings suggest that Tcf3 normally acts to coordinate multiple processes during neurogenesis, and maintains cells in a progenitor state until they receive all the necessary signals for differentiation.

We found that although the majority of progenitor cells lacking Tcf3 function appeared to remain in the cell cycle, there was a small but significant decrease in both mitotic index and BrdU labeling index. This result could reflect either a longer overall cell cycle time, or cell cycle arrest. Because we did not observe ectopic expression of *cdkn1c*, it is unlikely that cells were exiting the cell cycle prematurely, although we cannot conclusively rule out this possibility. Although we observed cells double-labeled with Hu and BrdU in both *tcf3* morphants and following *sox4a* overexpression, these cells were labeled 6 hours before analysis, and thus it is not clear whether they were still cycling, were arrested, or had recently exited the cell cycle. By contrast, inhibition of Wnt signaling by Dkk1 expression (Bonner et al., 2008), or ΔTcf expression (Fig. 7C,D), drastically reduces cell proliferation in the spinal cord, but neither manipulation results in increased neurogenesis. Therefore our data best fit a model in which Tcf3 is not directly required for cell proliferation, but may affect cell cycle time and/or progression. In this model, Tcf3-mediated repression of *sox4a* coordinates neurogenesis with other signals regulating cell cycle exit. In the absence of Tcf3 or the presence of Sox4a, neuronal markers are expressed prematurely in progenitors that are either still in, or have recently exited, the cell cycle.

A recent study (Bergsland et al., 2006) determined that electroporation of *Sox4* and *Sox11* into chick spinal cord induces expression of neuronal markers. Because our experiments showed ectopic *tubb5* and HuC/D expression in *tcf3* morphants, we hypothesized that group-C Sox genes may function downstream of Tcf3 in zebrafish spinal progenitors. Our data show that Tcf3 acts as a transcriptional repressor of *sox4a* in the intermediate and ventral spinal cord. Group-B *Sox* genes are known targets of canonical Wnt signaling (Lee et al., 2006; Takemoto et al., 2006; Van Raay et al., 2005), but we have now identified a member of the group-C Sox

family that is normally repressed by Tcf3. Through epistasis experiments we show that ectopic *sox4a* expression is both necessary and sufficient for ectopic neurogenesis in the absence of Tcf3. However, as *sox4a* is normally expressed very weakly in the spinal cord at 24 hpf, and knockdown of *sox4a* did not affect spinal neurogenesis (not shown), other group-C Sox factors may play this role during normal development. Interestingly, we found that *sox11a* was expressed in both progenitors and postmitotic neurons (Fig. 6F) but was unaffected in *tcf3* morphants (not shown), suggesting that not all group-C Sox genes are targets of Tcf3. It is possible that the normal balance between group-B and group-C Sox factors is sufficient to keep progenitor cells from differentiating, and the excess group-C Sox activity present in *tcf3* morphants upsets this balance, leading to precocious differentiation.

We have also shown that Tcf3 functions as a regulator of Dbx genes in spinal progenitors. Previous work from our laboratory and others has shown that canonical Wnt signaling is not required for the expression of Dbx genes, but instead acts to restrict the dorsal boundary of Dbx expression (Alvarez-Medina et al., 2008; Bonner et al., 2008). Furthermore, in these studies expression of the dominant-repressor ΔTcf molecule led to expansion, rather than loss, of Dbx genes. We therefore conclude that Tcf3 acts as a repressor to allow normal *Dbx* expression, presumably through an unknown intermediate transcriptional repressor. Further examination of this regulatory mechanism is warranted, due to the speculation that Dbx-expressing progenitors may represent a putative stem-cell population (Fogarty et al., 2005).

Although Lef/Tcf proteins can function in the presence and absence of Wnts (Dorsky et al., 2002; Labbe et al., 2000; Travis et al., 1991; van de Wetering et al., 1991), the lack of Wnt activity in the intermediate and ventral spinal cord (Bonner et al., 2008) suggests that Tcf3 primarily acts in a Wnt-independent manner to permit Dbx expression, as well as to repress *sox4a* and inhibit neurogenesis. Our data further indicate that Tcf3 does not normally function to antagonize canonical Wnt signals from the roof plate, but instead acts on a separate set of targets in neural progenitors. We have no evidence that other Lef/Tcf proteins (for example, Tcf7) are capable of activating Wnt targets in regions where Tcf3 is present, and the phenotypes we observed do not suggest that canonical Wnt signaling is activated in the absence of Tcf3. At this point it is unclear whether a Wnt signal or a parallel pathway acts to antagonize Tcf3 and initiate neurogenesis in spinal progenitors. The restriction of Tcf3 function to specific regions of the spinal cord may reflect the fact that in zebrafish at 24 hpf all spinal interneurons are derived from regions at or ventral to the Dbx expression domain (Gribble et al., 2007). It is therefore possible that neurogenesis in the dorsal spinal cord is controlled by a separate mechanism.

It is known that Tcf3 is similarly expressed in the spinal cord of other vertebrates (Alvarez-Medina et al., 2008; Schmidt et al., 2004); however, due to early lethality of Tcf3 knockout mice its function in the spinal cord has not been studied. Importantly, because Tcf3 normally functions as a repressor, only loss-of-function experiments can be used to determine its required role, whereas overexpression of 'dominant-negative' forms will mimic its normal function. Although dominant-activator forms of Tcf could theoretically be used to approximate loss of Tcf3 function, these reagents suffer from a lack of specificity, as they may activate non-physiological targets. In chick and mouse, it is possible that significant functional redundancy exists between Tcf3 and Tcf4, suggesting that loss-of-function of both genes must be examined to reveal a severe phenotype. In zebrafish, Tcf3 is the only Lef/Tcf factor expressed in the intermediate and ventral spinal cord; therefore, our studies have potentially revealed an evolutionarily conserved function of these proteins in spinal cord development. Here we have provided evidence that Tcf3 is required for preventing precocious neurogenesis in spinal progenitors, and have identified a new transcriptional target of Tcf proteins in this process.

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