Available online at www.sciencedirect.com
ScienceDirect
Developmental Biology 313 (2008) 398–407
DEVELOPMENTAL BIOLOGY
www.elsevier.com/developmentalbiology

Proliferation and patterning are mediated independently in the dorsal spinal cord downstream of canonical Wnt signaling

Jennifer Bonner [a,1], Suzanna L. Gribble [a,b], Eric S. Veien [a,b], O. Brant Nikolaus [a], Gilbert Weidinger [c], Richard I. Dorsky [a,*]

[a] Department of Neurobiology and Anatomy University of Utah School of Medicine Salt Lake City, UT 84132, USA
[b] Program in Neuroscience, University of Utah, Salt Lake City, UT, USA
[c] Biotechnological Center, Technical University of Dresden, Germany

Received for publication 6 September 2007; revised 23 October 2007; accepted 25 October 2007
Available online 11 December 2007

Abstract

Canonical Wnt signaling can regulate proliferation and patterning in the developing spinal cord, but the relationship between these functions has remained elusive. It has been difficult to separate the distinct activities of Wnts because localized changes in proliferation could conceivably alter patterning, and gain and loss of function experiments have resulted in both types of defects. To resolve this issue we have investigated canonical Wnt signaling in the zebrafish spinal cord using multiple approaches. We demonstrate that Wnt signaling is required initially for proliferation throughout the entire spinal cord, and later for patterning dorsal progenitor domains. Furthermore, we find that spinal cord patterning is normal in embryos after cell division has been pharmacologically blocked. Finally, we determine the transcriptional mediators of Wnt signaling that are responsible for patterning and proliferation. We show that *tcf7* gene knockdown results in dorsal patterning defects without decreasing the mitotic index in dorsal domains. In contrast, *tcf3* gene knockdown results in a reduced mitotic index without affecting dorsal patterning. Together, our work demonstrates that proliferation and patterning in the developing spinal cord are separable events that are regulated independently by Wnt signaling.

Keywords: Zebrafish; Wnt; *tcf7*; *tcf3*; Spinal cord; Patterning; Proliferation

Introduction

Proper organization and function of the spinal cord relies on a developmental program that coordinates proliferation and progenitor patterning. The regulation of progenitor number, coupled with the acquisition of positional identity within the spinal cord, leads to the production of specific neuronal populations of the correct size. Both the roof plate and floor plate are spinal cord organizing centers that secrete morphogens such as Wnt, bone morphogenetic protein (BMP), and Hedgehog family molecules. BMP signaling specifies dorsal positional identity, and Hedgehog signaling specifies ventral positional identity (Chizhikov and Millen, 2005). When either pathway is manipulated, the positional identity of progenitors is affected, as illustrated by an increase in size of particular gene expression domains at the expense of other neighboring domains. In contrast, while Wnts are known to be secreted from the roof plate, their roles in dorsal spinal cord development are less clear.

Our understanding of canonical Wnt signaling in the dorsal spinal cord has been complicated by seemingly contradictory results from multiple studies. A clear role for Wnt signaling in proliferation has been established (Chesnutt et al., 2004; Megason and McMahon, 2002; Zechner et al., 2003), as activation of the canonical pathway results in massive hypertrophy within the progenitor domain. However, disruption of the canonical pathway, using compound *Wnt1/Wnt3a* mutant mice, results in obvious patterning defects. In these

* Corresponding author. Fax: +1 801 581 4233.
E-mail address: richard.dorsky@neuro.utah.edu (R.I. Dorsky).
[1] Current address: Department of Biology, Skidmore College, Saratoga Springs, NY 12866, USA.

0012-1606/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2007.10.041

mice, both dorsal progenitors and dorsal neurons are lost as ventral cell types expand (Muroyama et al., 2002). It has been proposed that lack of localized proliferation is the root of observed patterning disruptions in *Wnt1/Wnt3a* knock out mice (Chesnutt et al., 2004). Recent studies have attempted to distinguish between proliferative and patterning defects following activation of the canonical Wnt signaling pathway. For example, dorsal spinal cord expression of activated β-catenin results in patterning defects without increases in proliferation (Ille et al., 2006). However, the patterning defects observed were only partly consistent with results from *Wnt1/Wnt3a* knockout phenotypes, and could be consistent with BMP pathway activation. Indeed, these authors observed an increase in BMP signaling, as evidenced by more widespread phospho-SMAD immunofluorescence, which could explain the observed defects. In contrast, another study used the same mutant mice and observed different effects on proliferation and phospho-SMAD activation within the spinal cord (Zechner et al., 2006). These differences could result from different stages of development examined, different rostro-caudal spinal cord levels examined, and the complex interplay between Wnt and BMP signaling. Furthermore, interpretation of these data are confounded by the potential activation of canonical Wnt signaling in domains that may not normally receive signaling, thus resulting in activities that do not reflect the endogenous roles of the canonical Wnt pathway.

To determine whether regulation of dorsal progenitor proliferation and patterning are separable outputs of canonical Wnt signaling, we examined the role of this pathway in both processes using zebrafish embryos. We employed loss of function approaches and temporal modulation of canonical Wnt signaling to examine multiple steps in the pathway. Interference with the canonical Wnt signaling pathway revealed disruptions in proliferation throughout the spinal cord and clear dorsal progenitor patterning defects. While proliferation and patterning defects were observed together in the dorsal domain, the ventral domain had reduced proliferation and intact patterning, indicating that reduced proliferation is not sufficient to induce patterning defects. Furthermore we found that these phenotypes were temporally separable, as proliferation required Wnt activity at an earlier developmental stage than dorsal patterning. In addition, when we specifically blocked proliferation throughout the spinal cord by application of cell cycle inhibitors, we did not observe any defects in dorsal progenitor patterning. To determine the mechanisms that underlie proliferative and patterning roles of Wnts, we examined the function of downstream Lef/Tcf transcription factors. Gene knockdown of *tcf7* resulted in dorsal patterning defects similar to loss of Wnt signaling without proliferative defects in dorsal domains. In contrast, knockdown of *tcf3* genes resulted in proliferative defects similar to loss of Wnt signaling without affecting dorsal patterning. We conclude that canonical Wnt signaling is responsible for dorsal patterning and proliferation, but that these activities are separable, and are differentially dependent upon Tcf7 and Tcf3, respectively.

Materials and methods

Fish strains

Wild-type embryos were collected from natural matings of (AB*) crosses. Transgenic fish that express Dkk1 under control of the heat shock promoter: *Tg(hsp70:dkk1-GFP)*$^{w32/+}$ (Stoick-Cooper et al., 2007) were intercrossed to generate Dkk1-expressing embryos.

ΔTcf-GFP embryos were obtained by outcrossing *Tg(hsΔTcf-GFP)*^{w26} (Lewis et al., 2004) heterozygote males to wild-type females.

Morpholino injections

The *tcf7* translation blocking morpholino (AGCTGCGGCATGATC-CAAACTTTCT), *tcf3a* splice blocking morpholino (TTTTTGCTTACTTCG-GAGTCTGATG), and *tcf3b* splice blocking morpholino (CATCCCT-GATTGGCTTACGTGTTAA) were obtained from Gene Tools, LLC. 2 ng of *tcf7* MO, and 5 ng each of *tcf3a* and *tcf3b* MO (diluted in 1× Danieau solution: 58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6 mM $Ca(NCO_3)_2$, 5.0 mM HEPES pH 7.6) were injected at the one-cell stage.

Western blots

For each sample, 5 embryos at 24 hours post fertilization (hpf) were homogenized in loading buffer (125 mM Tris pH 7.6; 4% SDS; 40% glycerol; 0.2 M DTT; 0.01% bromophenol blue), heated to 95 °C for 10 min and run on a 10% polyacrylamide gel. The gel was transferred to PDVF membrane and probed with anti-Tcf7 polyclonal antibody (Open Biosystems) at a dilution of 1:2000. Anti-rabbit IgG-HRP (Molecular Probes) was applied at 1:10,000, and the secondary antibody was visualized with an ECL reaction, using standard protocols.

In situ hybridization

Probe synthesis and in situ hybridization was performed as described by (Jowett and Lettice, 1994). Digoxigenin antisense RNA probes were visualized with BM Purple (Roche). The following probes were generated by our laboratory: *dbx1a*, *dbx1b*, *dbx2*, *nkx6.2*, *msxc*. The probes for *tcf3a*, *tcf3b*, *sox3* and *gfp* were used as previously described (Dorsky et al., 2003, 2002; Lee et al., 2006). The following probes were gifts: *pax3* (Seo et al., 1998), *iro3* (Lewis et al., 2005) and *wnt1* (Dorsky et al., 1998). The cDNA template for *radar* was obtained from Open Biosystems.

Immunohistochemistry

Anti-active β-catenin (Upstate) was used at 1:500 on 12 μm cryosections of embryos that had been fixed in 4% paraformaldehyde for 3 h. Sections were boiled in 10 mM citrate pH 6.0 for 20 min for antigen retrieval. Anti-HuC/D (Molecular Probes was used at 1:1000 on cryosections. Anti-phospho-histone H3 (Upstate) was used at a 1:500 dilution on whole-mount embryos. Cy3-conjugated secondary antibody (Jackson) was added at a concentration of 1:200 overnight at 4 °C.

Determination of mitotic indices

Using Nomarski optics on laterally mounted embryos, the mitotic index was determined for the dorsal, intermediate and ventral domains of the spinal cord at the level of the floor plate. The ventral extent of the dorsal domain was determined by counting three cells ventral to the roof plate and drawing a horizontal line. The ventral extent of the intermediate domain was determined by drawing a horizontal line three cells ventral to the dorsal extent of the intermediate domain. Mitotic index was generated by dividing the number of phospho-histone 3-positive cells by the total number of cells in the field.

Hydroxyurea–Aphidicholin (HUA) application

HUA in 4% DMSO (20 mM hydroxyurea and 150 μM aphidicholin) was added to 12 hpf embryos whose chorions had been manually torn to allow

maximal penetration. Embryos were cultured at 28.5 °C for 12–18 h, depending on the assay used.

Heat shock activation of transgenes

Tg(hsΔTcf-GFP)^{w26} embryos were heat-shocked at 18 hpf for 1 h at 37 °C. Embryos were sorted based upon GFP expression, and fixed at 24 hpf. *Tg(hsp70:dkk1-GFP)$^{w32/+}$* embryos were heat-shocked at 12 or 18 hpf for 1 h at 37 °C. Embryos were sorted based upon GFP expression and fixed at 24 hpf. For analysis of *dbx2* gene expression, ΔTcf embryos were heat-shocked at 22 hpf and fixed at 28 hpf, and Dkk1 embryos were heat-shocked at 12 hpf and fixed at 28 hpf.

Results

Canonical Wnt signaling is active in the zebrafish dorsal spinal cord

To determine whether Wnt activity is present during zebrafish spinal cord development, we first analyzed several markers of the canonical pathway. At 15 hours post-fertilization (hpf), when the neural keel is being formed, we examined the expression of *top:dgfp*, a transgenic reporter for β-catenin activity (Dorsky et al., 2002). We observed *gfp* expression throughout the neural keel, though with a clear dorsal bias, suggesting that at early stages of spinal cord development Wnt signaling is widespread (Fig. 1A). Later, at 24 hpf, dorso-ventral patterning markers are expressed in proliferating progenitors, and neuronal differentiation is ongoing. At this stage we found that canonical Wnt signaling is active in the dorsal spinal cord, as determined by expression of *top:dgfp* (Fig. 1B), and immunostaining for dephosphorylated β-catenin (Fig. 1C) (Staal et al., 2002). These results are consistent with previous reports of high β-catenin reporter activation in the dorsal chick spinal cord (Megason and McMahon, 2002). We also found that *wnt1* mRNA was localized to the roof plate of the spinal cord (Fig. 1D) consistent with previous reports in other vertebrates (Parr et al., 1993). Additionally, no expression of *dkk1* has been observed in the spinal cord at these stages, while the Wnt inhibitor *sfrp1a* is transiently expressed in the ventral spinal cord (ZFIN: www.zfin.org). These data demonstrate that Wnt signaling in the zebrafish spinal cord is initially broad, then later refines to a dorsal domain as observed in other vertebrates.

Wnt signaling regulates proliferation and patterning in the zebrafish spinal cord

To determine if blocking the canonical Wnt signaling pathway produces defects in proliferation and/or dorsal patterning, we induced global expression of a secreted Wnt inhibitor, Dickkopf1 (Dkk1), by heat shocking *tg(hsp70:dkk1-GFP)$^{w32/+}$* embryos at 12 hpf (Stoick-Cooper et al., 2007). Induction of Dkk1 in zebrafish embryos has been previously shown to block expression of *top:dgfp*, suggesting that it efficiently inhibits canonical Wnt signaling (Stoick-Cooper et al., 2007). To avoid affecting an early developmental requirement of Wnt signaling, the transgene was induced at 12 hpf, when embryos have completed gastrulation and the anterior–posterior axis has already formed. Analysis of Dkk1-expressing embryos was performed at 24 hpf and compared to heat-shocked non-transgenic siblings.

Consistent with previous reports of interference with canonical Wnt signaling (Zechner et al., 2003) the spinal cords of Dkk1-expressing embryos were smaller than heat-shocked siblings, suggesting a defect in proliferation. We therefore asked whether proliferation was generally reduced, or instead affected in a particular domain. To explore these possibilities, the mitotic indices of Dkk1-expressing embryos were determined by anti-phospho-histone H3 immunofluorescence and compared to heat-shocked non-transgenic siblings (Figs. 1E, F). We found that Dkk1 expression reduced the mitotic index by 79%, 60%, and 80% in dorsal, intermediate, and ventral domains, respectively (Fig. 1G). These data suggest that Wnt signaling acts as a mitogen throughout the entire dorso-ventral extent of the spinal cord, and not solely in dorsal progenitors.

To ask whether dorsal spinal cord patterning was affected following Dkk1 expression, we first analyzed markers expressed in the roof plate. We assayed expression of *radar*, which encodes a member of the BMP signaling family (Rissi et al., 1995), and *msxc*, which also labels dorsal neural progenitors. Both *radar* and *msxc* were completely eliminated in 100% ($n=16$, $n=19$) of Dkk1-expressing embryos (Figs. 1H–K). We next analyzed *pax3*, which is also expressed in the roof plate and 3–4 rows of dorsal progenitor cells. In 94% ($n=16$) of Dkk1-expressing embryos, *pax3* expression was restricted to the roof plate and only 1–2 more ventral cells (Figs. 1L, M). If *pax3* was reduced as a result of dorsal patterning defects, we expected to see a concomitant shift of the adjacent progenitor domain, labeled by *dbx2* expression. We observed a decreased distance between the roof plate and the dorsal boundary of *dbx2* in 91% ($n=30$) of Dkk1-expressing embryos (Figs. 1N, O), although the absolute size of the *dbx2* domain was variable. Finally, we examined ventral progenitors, labeled by expression of *nkx6.2*, which is expressed in the floor plate and 6 cells dorsal to it (Guner and Karlstrom, 2007; Hutchinson et al., 2007). We found that the extent of *nkx6.2* expression was indistinguishable from WT in all embryos examined ($n=27$) (Figs. 1P, Q), demonstrating that canonical Wnt signaling is not required to pattern the ventral spinal cord. Together, the dorsal loss of *radar* and *msxc* and reduction in *pax3*, combined with the dorsal shift in *dbx2* and normal expression of *nkx6.2*, are consistent with defects in dorsal progenitor positional identity, or patterning.

Alterations in the expression of progenitor markers could be caused by cell death or premature differentiation. To address the first possibility we performed TUNEL assays on wild-type and Dkk1-expressing embryos and found no significant apoptosis in either condition (not shown). To examine premature differentiation we performed immunohistochemistry for HuC/D proteins, which mark all postmitotic neurons (Figs. 1R, S). We observed 6.1±0.4 (S.E.M., $n=12$) Hu-positive cells per 12 μm cryosection in wild-type embryos, compared with 6.3±0.5 (S.E.M.,

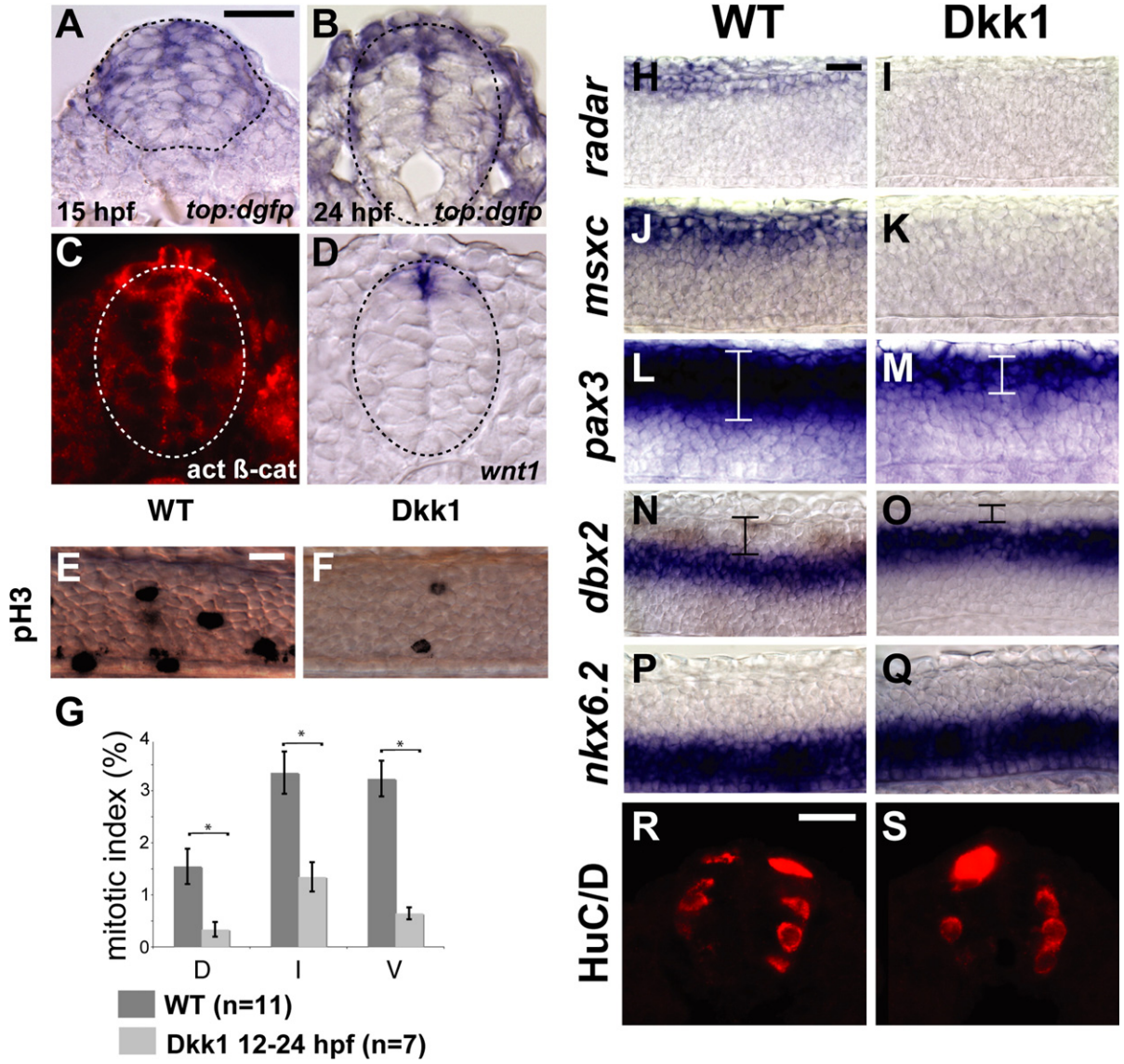

Fig. 1. Canonical Wnt signaling regulates proliferation and patterning in the developing spinal cord. (A) Expression of *top:dgfp*, a β-catenin-dependent reporter, is widespread in the neural keel (dotted line) at 15 hpf, with a dorsal bias. (B) Expression of *top:dgfp* is restricted to the dorsal spinal cord at 24 hpf. (C) Immunofluorescence of unphosphorylated β-catenin is localized to the dorsal spinal cord at 24 hpf. (D) Expression of *wnt1* is found in the roof plate of the spinal cord at 24 hpf. (E, F) Immunostaining for phospho-histone H3 is reduced at 24 hpf following Dkk1 overexpression at 12 hpf. (G) The mitotic index is reduced in dorsal, intermediate and ventral domains following Dkk1 overexpression. *$p<0.05$ by *T*-test; error bars indicate ±SEM. (H, I) Expression of *radar* and (J, K) *msxc* is eliminated following Dkk1 overexpression. (L, M) Expression of *pax3* is reduced in Dkk1-expressing embryos. (N, O) Expression of *dbx2* shifts into the dorsal domain in Dkk1-expressing embryos. (P, Q) Expression of *nkx6.2* is unaffected in Dkk1-expressing embryos. (R, S) HuC/D immunostaining is unaffected in Dkk1-expressing embryos. (A–D, R, S) show 12 μm cross-sections, and (E, F, H–Q) show lateral mounts. Scale bars = 10 μm.

$n=12$) cells in Dkk1-expressing embryos. When we specifically counted the dorsal third of the spinal cord, we observed 2.4±0.4 (S.E.M., $n=12$) cells per section in wild-types, compared with 2.75±0.4 (S.E.M., $n=12$) cells in Dkk1-expressing embryos. Because there was no significant difference in Hu labeling either throughout the spinal cord or specifically in dorsal regions, we conclude that progenitors do not undergo premature differentiation following Dkk1 expression. The lower mitotic index may instead represent arrest or slowing of the cell cycle, rather than cell cycle exit. Furthermore, these results suggest that changes in progenitor gene expression were most likely specifically due to defects in dorsal patterning.

Wnt signaling is required early for proliferation and later for patterning

To ask whether the effects of Dkk1 on proliferation and patterning could be separated temporally, we heat-shocked transgenic embryos at 18 hpf and analyzed them at 24 hpf. Under these conditions, we observed no significant change in mitotic index in any part of the spinal cord (Supplementary Fig. 1A). This result indicates that Wnt signaling is required for regulating proliferation between 12 and 18 hpf, at a time when pathway activity is present throughout the dorso-ventral extent of the spinal cord, and consistent with

the non-localized effects on mitotic index that we observed previously.

Heat shock at 18 hpf did not lead to loss of *radar* and *msxc* expression in the roof plate, however we did observe decreased expression of *msxc* and *pax3* in dorsal progenitors and a dorsal shift in the expression of *dbx2* (Supplementary Fig. 1B–I). While less severe than the phenotypes resulting from Dkk1 expression at 12 hpf, the reduction in dorsal markers and dorsal shift of the intermediate marker *dbx2* were consistent with defects in dorsal patterning. Together, these findings suggest that Wnt signaling is required after 18 hpf for dorsal spinal cord patterning, and this requirement may be independent from regulation of roof plate BMP signaling which occurs at an earlier step of development.

Blocking proliferation does not affect spinal cord patterning

To address whether decreases in cellular proliferation can secondarily cause patterning defects, cells were arrested in S-phase with hydroxyurea and aphidicholin (HUA), which have been used previously in zebrafish to reduce proliferation (Ikegami et al., 1999; Lyons et al., 2005). We applied HUA to embryos at 12 hpf, and analyzed spinal cord development at 24 hpf. Upon examination, the spinal cords of HUA embryos were considerably smaller than DMSO-treated embryos, while individual cells were larger, and far fewer anti-phospho-histone H3 positive cells were present (Figs. 2A, B). However, the domain of *pax3* expression was normal in HUA-treated embryos (Figs. 2C, D). In addition, we observed no shift of *dbx2* into the dorsal domain, although a decreased expression level of *dbx2* was found in 93% of embryos ($n=31$, Figs. 2E, F). Patterning of the ventral spinal cord was similarly unaffected, as determined by expression of *nkx6.2* (Figs. 2G, H). These results argue that the patterning defects that arise when canonical Wnt signaling is disrupted are not due to decreases in localized proliferation.

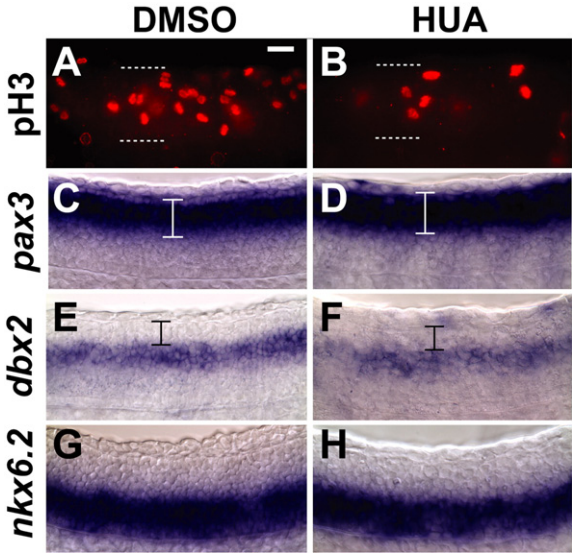

Fig. 2. Reduction of cell proliferation does not cause spinal cord patterning defects. (A, B) Anti-phospho histone H3 staining reveals a decrease in proliferative cells in the HUA-treated spinal cord. (C, D) Expression of *pax3* is unaffected in embryos treated with HUA for 12 h. (E, F) The *dbx2* expression domain is unchanged in HUA embryos although an overall decrease in signal was observed. Expression in the dorsal epidermis is non-specific. (G, H) Expression of *nxk6.2* is unaffected in HUA-treated embryos. Lateral mounts are shown. Scale bar=10 μm.

Tcf7 is required for dorsal progenitor patterning in the spinal cord

Since all studies to date have focused on either removal or overexpression of Wnt and β-catenin, we sought to identify which Lef/Tcf transcription factors mediate the distinct activities downstream of these molecules. At 15 hpf, prior to spinal cord formation, *tcf7* is expressed throughout the neural keel, though with a clear dorsal bias (Fig. 3A) similar to *top: dgfp* (Fig. 1A). At 24 hpf *tcf7* is expressed in the dorsal spinal cord in a domain similar to Wnt activity (Fig. 3B), consistent with observations in other vertebrates (Schmidt et al., 2004). To knock down *tcf7* function, a translation blocking antisense morpholino (*tcf7* MO) was injected at the one cell stage and embryos were analyzed at 24 hpf. Western blot analysis of *tcf7* morphants demonstrated knockdown of two Tcf7 protein isoforms at 44 and 46 kDa (Supplementary Fig. 2A). Morphant embryos had relatively normal overall morphology (Supplementary Fig. 2B), and neural plate specification occurred normally as assayed by *sox3* expression (Supplementary Figs. 2C, D).

To determine if Tcf7 can mediate the mitogenic role of canonical Wnts, the mitotic index of *tcf7* morphants was compared to uninjected controls (Fig. 3C). In dorsal and intermediate domains, no significant decrease in the mitotic index was observed in *tcf7* morphants. However, in the ventral domain, where no patterning defects were observed in Dkk1-expressing embryos, a significant decrease in the mitotic index was evident. This suggests that Tcf7 may play a separable role in regulating proliferation in the ventral spinal cord, but is not the primary mediator of Wnt signaling in regulating proliferation of dorsal and intermediate cells.

We observed normal expression of *wnt1* in 100% ($n=24$) of *tcf7* morphants (Figs. 3D, E), indicating that Wnt signals from the roof plate may still be present. In addition, *radar* expression was normal in 100% ($n=6$) of morphants (Figs. 3F, G), suggesting that BMP signaling is present. However, similar to Dkk1-expressing embryos, genes expressed in dorsal spinal progenitors exhibited reduced expression domains. We found that *msxc* expression was reduced to the roof plate and 1–2 cells ventral in 56% ($n=41$) of *tcf7* morphants (Figs. 3H, I). In *tcf7* morphants, *pax3* was similarly reduced in 67% ($n=27$) of embryos (Figs. 3J, K). Also consistent with Dkk1 overexpression, *dbx2* expression was shifted into the dorsal domain of the spinal cord in 65% ($n=60$) of morphants (Figs. 3L, M). In addition, the *dbx2* domain was expanded in size, suggesting a re-patterning of progenitors from dorsal to intermediate fates. We did not

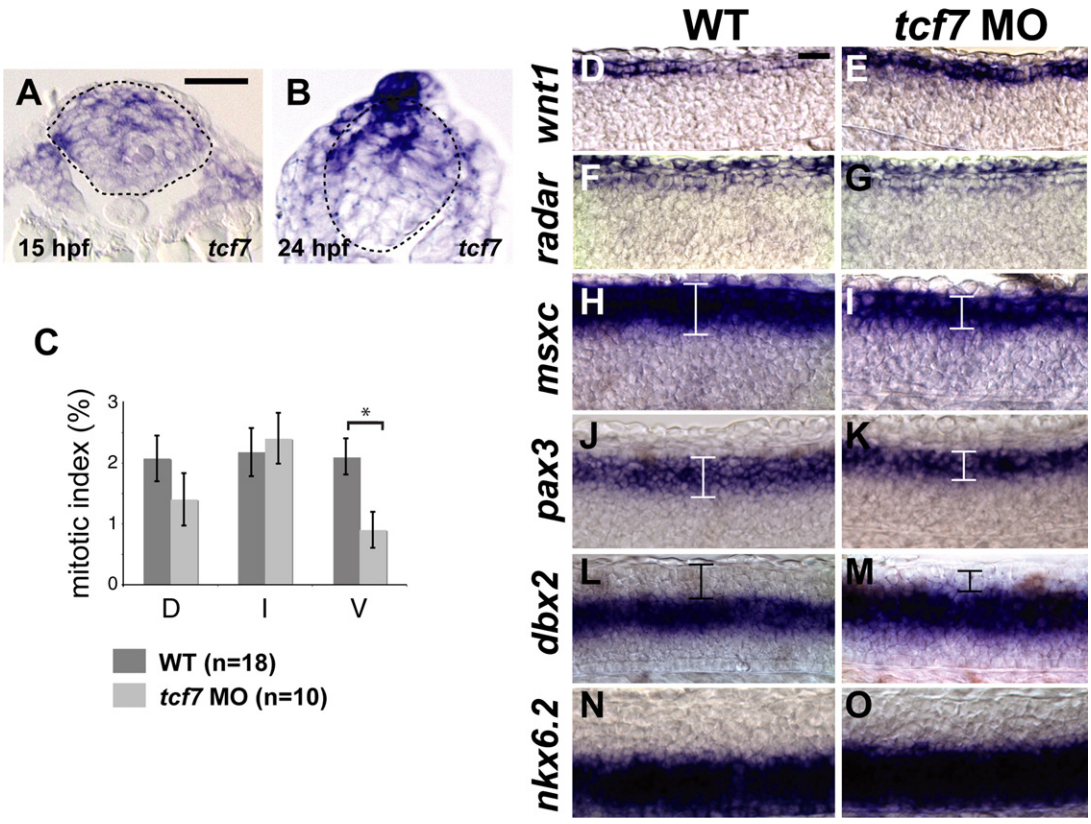

Fig. 3. Tcf7 is required for dorsal progenitor patterning. (A) At 15 hpf, *tcf7* is expressed widely throughout the neural keel (dotted line), with a dorsal bias. (B) At 24 hpf *tcf7* is expressed in the dorsal spinal cord as well as the median fin fold. (C) There is no significant decrease in the mitotic index of the dorsal and intermediate domains in *tcf7* morphants, but mitosis in the ventral domain is significantly reduced. *$p<0.05$ by *T*-test, error bars indicate ± SEM. (D, E) Expression of *wnt1* and (F, G) *radar* mRNA is unaffected in *tcf7* morphants. (H, I) Expression of *msxc* and (J, K) *pax3* is reduced in *tcf7* morphants. (L, M) Expression of *dbx2* shifts into the dorsal domain in *tcf7* morphants. (N, O) Ventral expression of *nkx6.2* is unaffected in *tcf7* morphants. (A, B) 12 μm cross-sections are shown. (D–O) Lateral mounts are shown. Scale bars = 10 μm.

analyze whether *dbx2* was co-expressed with dorsal markers such as *msxc* in *tcf7* morphants, and thus could not determine whether normal cross-repressive interactions between these factors were still functional. Similar to Dkk1-expressing embryos, *nkx6.2* was unaffected in all ($n=15$) *tcf7* morphants (Figs. 3N, O). Together, the defects observed in these morphants lead us to conclude that Tcf7 may mediate canonical Wnt signaling in dorsal progenitor patterning, and indicate distinct mechanisms for regulation of proliferation and patterning in these cells.

Tcf3 functions to regulate proliferation but not patterning

In the zebrafish spinal cord at 24 hpf, the only two Lef/Tcf factors expressed in addition to Tcf7 are Tcf3a and Tcf3b (unpublished observations). Since no deficit in *wnt1* expression was observed in *tcf7* morphants, we hypothesized that canonical signaling could still occur in the absence of *tcf7*. To determine if *tcf3a* and *tcf3b* could be compensating for the loss of *tcf7* in maintaining proliferation, we examined their expression in *tcf7* morphants. In WT embryos, *tcf3a* and *tcf3b* are co-expressed in part of the dorsal, and all of the intermediate and ventral spinal cord (Figs. 4A, C). In *tcf7* morphants, both *tcf3a* and *tcf3b* are not only expressed in their normal domains, but appear to be expressed at higher levels (Figs. 4B, D). To determine if *tcf3a* and *tcf3b* are required for proliferation, the mitotic indices of each domain were determined in *tcf3a*/*tcf3b* double morphant embryos. In dorsal, intermediate, and ventral domains, the mitotic index was reduced by 74%, 73% and 55%, respectively, compared to uninjected controls (Fig. 4E). These data indicate that canonical Wnt signaling may act through Tcf3a and Tcf3b to regulate proliferation.

In contrast, we observed no dorsal patterning defects consistent with a loss of Wnt activity in *tcf3a*/*tcf3b* double morphants. There was no change in the expression of the dorsal markers *msxc* and *pax3* (Figs. 4F–I). While we surprisingly did observe a specific loss of *dbx2* in these morphants (Figs. 4J, K), it appeared that the intermediate domain was patterned normally as assayed by another marker, *iro3* (Figs. 4L, M). While the loss of *dbx2* was intriguing and will be explored more fully in a future study, this phenotype was nevertheless inconsistent with the dorsal patterning defects observed following Dkk1 expression or in *tcf7* morphants. Therefore, we conclude that Tcf3a and Tcf3b do not act to mediate canonical Wnt signaling in dorsal spinal cord patterning, further supporting the idea that proliferation and patterning are separable events.

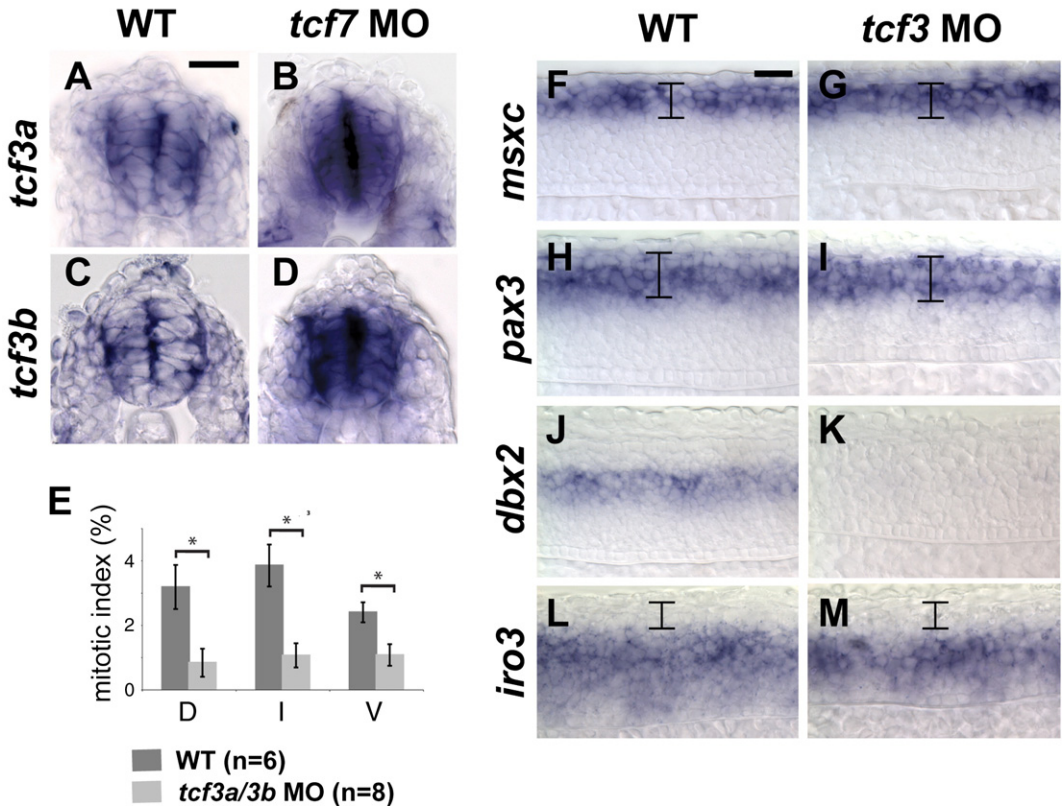

Fig. 4. Tcf3 is required for proliferation but not for dorsal progenitor patterning. (A–D) Both *tcf3a* (A, B) and *tcf3b* (C, D) are expressed at higher levels in *tcf7* morphants. (E) The mitotic index in dorsal, intermediate and ventral domains is significantly reduced in *tcf3a/tcf3b* double morphants. *$p<0.05$ by *T*-test, error bars indicate ±SEM. (F, G) Expression of *msxc* and (H, I) *pax3* is unaffected in *tcf3a/tcf3b* double morphants. (J, K) Expression of *dbx2* is eliminated in *tcf3a/tcf3b* double morphants, but the intermediate progenitor domain is not shifted dorsally as indicated by *iro3* expression (L, M). (A–D) 12 μm cross-sections are shown. (F–M) Lateral mounts are shown. Scale bars = 10 μm.

Transcriptional activation mediates the downstream effects of canonical Wnt signaling in dorsal spinal cord patterning

The fact that dorsal patterning defects in *tcf7* knockdown are similar to defects resulting from overexpression of Dkk1 suggest that Tcf7 acts downstream of Wnt signaling as a transcriptional activator of target genes. To directly test this, we expressed an inducible dominant repressor form of Tcf (ΔTcf) using transgenic embryos (Lewis et al., 2004). This transgene will act to repress targets of any Lef/Tcf molecule, and thus can mimic loss of Wnt or Tcf function. Embryos were heat-shocked at 18 hpf and assayed at 24 or 28 hpf, as earlier induction of the transgene was not possible due to deleterious effects of ΔTcf on development. In ΔTcf-expressing embryos, we found dorsal spinal cord patterning defects similar to those following Dkk1 overexpression and *tcf7* knockdown, as we observed reduction of *msxc* in 73% ($n=22$) of embryos (Figs. 5A, B). In addition, *pax3* was reduced in 41% ($n=32$) of ΔTcf-expressing embryos (Figs. 5C, D). Unlike in our other experiments, *dbx2* was ectopically expressed in the somites of transgenic embryos, precluding whole mount analysis. However, upon inspection of *dbx2* expression in cross section, expansion was observed into the dorsal spinal cord (Figs. 5E, F). In the ventral progenitor domain, *nkx6.2* expression was unaffected (Figs. 5G, H). These data suggest that Wnts signal through an activator, possibly Tcf7, which regulates downstream target genes to promote dorsal progenitor patterning.

Discussion

Canonical Wnt signaling is refined to the dorsal spinal cord from an initially broad domain

We determined that canonical Wnt signaling in the dorsal spinal cord is evolutionarily conserved between zebrafish and amniotes by demonstrating the expression of multiple components of the pathway. First, we show that *wnt1* is expressed in the roof plate in zebrafish, where it has been shown to regulate proliferation and patterning through the canonical pathway in amniotes (Megason and McMahon, 2002; Muroyama et al., 2002). Second, *tcf7*, which encodes a transcriptional mediator of Wnt signaling, is expressed in the dorsal spinal cord. Third, both activated β-catenin as well as *top:dgfp* are present in the dorsal spinal cord, in about 3 cell diameters ventral to the roof plate, indicative of the extent of detectable canonical signaling at 24 hpf. At 15 hpf, *top:dgfp* expression is distributed throughout the spinal cord indicating that Wnt signaling is more widespread at this time. Importantly, broad regulation of proliferation requires this early phase of Wnt signaling, while patterning phenotypes that result from Dkk1 overexpression

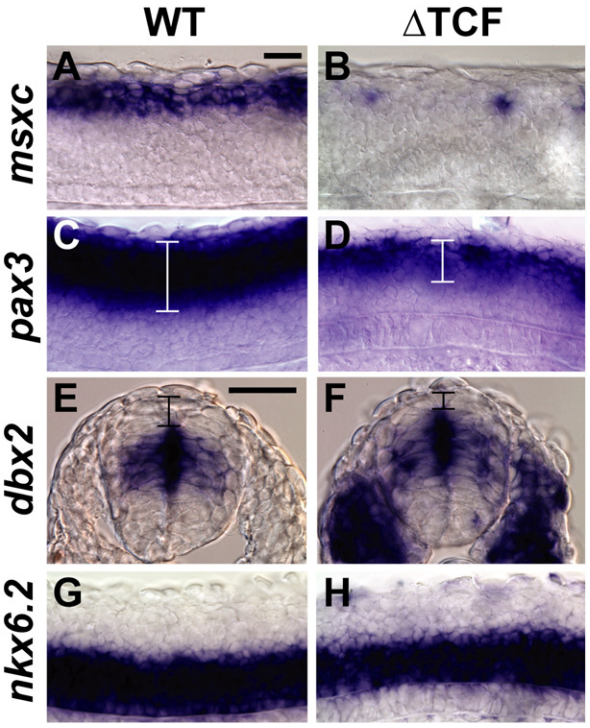

Fig. 5. Transcriptional activation by Lef/Tcf factors mediates progenitor patterning in the spinal cord. (A, B) Expression of *msxc* and (C, D) *pax3* is reduced in ΔTcf-expressing embryos. (E, F) Expression of *dbx2* shifts into the dorsal domain of the spinal cord in ΔTcf-expressing embryos, and is ectopically present in the somites. (G, H) Expression of *nkx6.2* is unaffected in ΔTcf-expressing embryos. (A–D, G, H) Lateral mounts are shown. (E, F) 12 μm cross-sections are shown. Scale bars = 10 μm.

and *tcf7* knockdown occur within the domain of observed Wnt activity at 24 hpf.

Canonical Wnt signaling controls dorsal progenitor patterning and proliferation

Previous studies have shown that in *Wnt1/Wnt3a* knockout mice, the *Mash1* domain is expanded at the expense of the more dorsal *Math1* domain (Muroyama et al., 2002), and in conditional β-catenin gain of function (GOF) mice, dorsal markers *Math1* and *Olig3* expand (Ille et al., 2006; Zechner et al., 2006). However, with few exceptions, when Wnt signaling is activated in the spinal cord, proliferation also increases significantly (Chesnutt et al., 2004; Ille et al., 2006; Megason and McMahon, 2002; Zechner et al., 2003, 2006). Two recent studies have indicated a separation of these two roles of Wnt signaling. First, when β-catenin is overexpressed in the *Olig3* mutant background, dorsalizing patterning defects are suppressed, while proliferation is still affected (Zechner et al., 2006). Patterning in these embryos is identical to *Olig3* mutant mice (Muller et al., 2005) suggesting that the patterning role of β-catenin is dependent upon *Olig3*, and that the proliferative role is dependent upon an unidentified factor. Second, a recent report of β-catenin overexpression in the dorsal spinal cord demonstrated that progenitor proliferation was not increased, but the dorsal *Math1* domain was expanded (Ille et al., 2006), consistent with Wnt activity patterning this domain. However, these authors found an increase in BMP signaling, and also demonstrated that BMP signaling inhibits the proliferative role of Wnts.

Our study confirms the dual role of canonical Wnts in regulating proliferation and patterning in the dorsal spinal cord. The rapid development of zebrafish spinal progenitors has allowed us to analyze these two processes separately, as the entire time period encompassed by our study likely only represents 1–2 cell cycles (Kimmel et al., 1994). In Dkk1-expressing embryos we found that the mitotic index was significantly reduced in all domains. In addition, dorsal *pax3* expression was reduced, and the intermediate marker *dbx2* shifted into the domain where *pax3* expression was absent. These phenotypes are consistent with previous examples of patterning defects, where individual progenitor domains are reduced concomitant with expansion of neighboring domains. Decreased proliferation is not sufficient to cause patterning defects in the spinal cord, as both *dbx2* and the ventral marker *nkx6.2* were not reduced in Dkk1-expressing embryos in spite of decreased proliferation in these domains. In addition, when we specifically reduced proliferation throughout the spinal cord by treating with HUA, no patterning defects were observed. These data strongly suggest that distinct molecular pathways mediate the dual function of Wnts. Interestingly, decreased expression of the BMP ligand *radar* was not correlated with all perturbations that affected patterning, suggesting that while the two pathways may be linked they are not necessarily interdependent.

Wnt signaling can regulate dorsal progenitor patterning independently from differentiation

Several of our observations indicated that Dkk1 expression affects dorsal progenitor gene expression directly, rather than through a general effect on progenitor cell number. First, we could decouple effects on proliferation from dorsal patterning defects. Second, neighboring progenitor markers were affected in opposite ways, suggesting a change in dorso-ventral positional identity. Third, there was no increase in cell death following Dkk1 overexpression. Finally, the number of postmitotic neurons was unaffected by Dkk1 under conditions where progenitor markers were changed. We propose that while Wnt signaling can regulate the rate of progenitor proliferation, it is not absolutely required for regulating cell cycle exit, and that dorsal Wnt activity acts directly to specify progenitor identity.

We were unable to monitor the ultimate neuronal fates of dorsal progenitors in this study, because the dorsal-most interneurons of known origin in zebrafish at 24 hpf probably arise from the intermediate *Dbx* expression domain (Gribble et al., 2007). It is not surprising that more dorsal interneurons are absent in zebrafish at this time, as they primarily serve as targets for dorsal root ganglia neurons which are not present until later in development (An et al., 2002; Williams et al., 2000). At 24 hpf, sensory information is conveyed by Rohon–Beard neurons, which arise from the neural plate/neural crest border (Cornell and Eisen, 2000). Intriguingly, we did observe an

increase in *chx10*$^{+}$ neurons in both Dkk1-expressing embryos and *tcf7* morphants, consistent with an expansion in more ventral (V2) intereuron domains (data not shown).

Different Lef/Tcf factors mediate separate aspects of spinal cord development

Our data provide evidence that the diverse biological consequences of canonical Wnt signaling may be mediated by specific Lef/Tcf molecules. When we knocked down *tcf7* function, we observed very similar dorsal patterning defects to those observed by Dkk1 overexpression, especially at 18 hpf. Interestingly, we never observed a complete loss of *pax3* or misexpression of *dbx2* in the most dorsal domain of the spinal cord, possibly due to residual BMP signaling in dorsal progenitors (Ille et al., 2006). Importantly, in *tcf7* morphants, proliferation in dorsal and intermediate domains was unaffected, demonstrating that changes in proliferation can occur without affecting dorsal patterning, and patterning defects can occur without changes in proliferation. To further support this idea, we also observed decreased proliferation in the ventral domain of *tcf7* morphants, while patterning in this domain was unaffected as determined by *nkx6.2* expression.

What is the mechanism through which Wnt signaling promotes progenitor proliferation? Our data suggest that Tcf3a/3b can serve this function. We found that *tcf3a/3b* morphants exhibit significant reductions in proliferation in the dorsal, intermediate and ventral domains without effects on dorsal patterning. Additionally, in *tcf7* morphants, *tcf3a* and *tcf3b* are strongly expressed in part of the dorsal spinal cord and may account for the maintained mitotic index in this domain. These data do not rule out the possibility that Tcf7 may play a partially redundant role in regulating proliferation, and it clearly has a separate required function in ventral progenitors. Our results indicate that the mitogenic effects of Wnts take place earlier in development when signaling and Tcf7 expression are more widespread, and may in fact involve Wnts expressed outside of the roof plate.

Evolutionary conservation and divergence of Wnt function in spinal cord development

Our data suggest that the functions of canonical Wnt signaling in spinal cord proliferation and dorsal patterning are conserved throughout vertebrates. While the degree of conservation is significant, it is also informative to consider the differences between Wnt targets in different species. For example, our data show that the dorsal limit of *Dbx* expression is regulated by Wnt activity, while in amniotes this domain appears to be beyond the reach of roof plate-derived Wnt signals (Ille et al., 2006; Zechner et al., 2006). In addition, at 24 hpf zebrafish spinal progenitors do not express bHLH family genes in specific dorsal domains (S.L.G., unpublished observations), suggesting that Wnt targets may vary between organisms and developmental stages. We also found that *msxc* expression responded differently to early loss of Wnt signaling or ΔTcf expression than to loss of *tcf7*, while *pax3* behaved similarly in all assays. These data suggest that individual dorsal patterning markers may have different requirements for BMP signaling or may be regulated by different mechanisms downstream of Wnt activity. It is intriguing to speculate that the interchangeability in enhancer-mediated Wnt target genes may underlie evolutionary differences in spinal cord anatomy and function across species.

Acknowledgments

We thank Randall Moon and Hee Chan Seo for providing plasmids and the University of Utah zebrafish facility for fish husbandry. R.I.D. is supported by NIH RO1NS053897. J.B. and E.S.V. were supported by National Institutes of Health Developmental Biology Training Grant T32HD007491, and S.L.G. is supported by National Institutes of Health Genetics Training Grant T32 GM0764. G.W. was funded by the Howard Hughes Medical Institute and by the Deutsche Forschungsgemeinschaft grant "Collaborative Research Center 655: Cells into tissues: stem cell and progenitor commitment and interactions during tissue formation" (SFB 655, Dresden).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2007.10.041.

References

An, M., Luo, R., Henion, P.D., 2002. Differentiation and maturation of zebrafish dorsal root and sympathetic ganglion neurons. J. Comp. Neurol. 446, 267–275.

Chesnutt, C., Burrus, L.W., Brown, A.M., Niswander, L., 2004. Coordinate regulation of neural tube patterning and proliferation by TGFbeta and WNT activity. Dev. Biol. 274, 334–347.

Chizhikov, V.V., Millen, K.J., 2005. Roof plate-dependent patterning of the vertebrate dorsal central nervous system. Dev. Biol. 277, 287–295.

Cornell, R.A., Eisen, J.S., 2000. Delta signaling mediates segregation of neural crest and spinal sensory neurons from zebrafish lateral neural plate. Development 127, 2873–2882.

Dorsky, R.I., Moon, R.T., Raible, D.W., 1998. Control of neural crest cell fate by the Wnt signalling pathway. Nature 396, 370–373.

Dorsky, R.I., Sheldahl, L.C., Moon, R.T., 2002. A transgenic lef1/beta-catenin-dependent reporter is expressed in spatially restricted domains throughout zebrafish development. Dev. Biol. 241, 229–237.

Dorsky, R.I., Itoh, M., Moon, R.T., Chitnis, A., 2003. Two *tcf3* genes cooperate to pattern the zebrafish brain. Development 130, 1937–1947.

Gribble, S.L., Nikolaus, O.B., Dorsky, R.I., 2007. Regulation and function of *Dbx* genes in the zebrafish spinal cord. Dev. Dyn. 236, 3472–3483.

Guner, B., Karlstrom, R.O., 2007. Cloning of zebrafish *nkx6.2* and a comprehensive analysis of the conserved transcriptional response to Hedgehog/Gli signaling in the zebrafish neural tube. Gene Expr. Patterns 7, 596–605.

Hutchinson, S.A., Cheesman, S.E., Hale, L.A., Boone, J.Q., Eisen, J.S., 2007. Nkx6 proteins specify one zebrafish primary motoneuron subtype by regulating late islet1 expression. Development 134, 1671–1677.

Ikegami, R., Hunter, P., Yager, T.D., 1999. Developmental activation of the capability to undergo checkpoint-induced apoptosis in the early zebrafish embryo. Dev. Biol. 209, 409–433.

Ille, F., Atanasoski, S., Falk, S., Ittner, L.M., Marki, D., Buchmann-Moller, S., Wurdak, H., Suter, U., Taketo, M.M., Sommer, L., 2006. Wnt/BMP signal

integration regulates the balance between proliferation and differentiation of neuroepithelial cells in the dorsal spinal cord. Dev. Biol. 304, 394–408.

Jowett, T., Lettice, L., 1994. Whole-mount in situ hybridizations on zebrafish embryos using a mixture of digoxigenin- and fluorescein-labelled probes. Trends Genet. 10, 73–74.

Kimmel, C.B., Warga, R.M., Kane, D.A., 1994. Cell cycles and clonal strings during formation of the zebrafish central nervous system. Development 120, 265–276.

Lee, J.E., Wu, S.F., Goering, L.M., Dorsky, R.I., 2006. Canonical Wnt signaling through Lef1 is required for hypothalamic neurogenesis. Development 133, 4451–4461.

Lewis, J.L., Bonner, J., Modrell, M., Ragland, J.W., Moon, R.T., Dorsky, R.I., Raible, D.W., 2004. Reiterated Wnt signaling during zebrafish neural crest development. Development 131, 1299–1308.

Lewis, K.E., Bates, J., Eisen, J.S., 2005. Regulation of *iro3* expression in the zebrafish spinal cord. Dev. Dyn. 232, 140–148.

Lyons, D.A., Pogoda, H.M., Voas, M.G., Woods, I.G., Diamond, B., Nix, R., Arana, N., Jacobs, J., Talbot, W.S., 2005. *erbb3* and *erbb2* are essential for Schwann cell migration and myelination in zebrafish. Curr. Biol. 15, 513–524.

Megason, S.G., McMahon, A.P., 2002. A mitogen gradient of dorsal midline Wnts organizes growth in the CNS. Development 129, 2087–2098.

Muller, T., Anlag, K., Wildner, H., Britsch, S., Treier, M., Birchmeier, C., 2005. The bHLH factor Olig3 coordinates the specification of dorsal neurons in the spinal cord. Genes Dev. 19, 733–743.

Muroyama, Y., Fujihara, M., Ikeya, M., Kondoh, H., Takada, S., 2002. Wnt signaling plays an essential role in neuronal specification of the dorsal spinal cord. Genes Dev. 16, 548–553.

Parr, B.A., Shea, M.J., Vassileva, G., McMahon, A.P., 1993. Mouse Wnt genes exhibit discrete domains of expression in the early embryonic CNS and limb buds. Development 119, 247–261.

Rissi, M., Wittbrodt, J., D'Elot, E., Naegeli, M., Rosa, F.M., 1995. Zebrafish Radar: a new member of the TGF-beta superfamily defines dorsal regions of the neural plate and the embryonic retina. Mech. Dev. 49, 223–234.

Schmidt, M., Patterson, M., Farrell, E., Munsterberg, A., 2004. Dynamic expression of Lef/Tcf family members and beta-catenin during chick gastrulation, neurulation, and early limb development. Dev. Dyn. 229, 703–707.

Seo, H.C., Saetre, B.O., Havik, B., Ellingsen, S., Fjose, A., 1998. The zebrafish Pax3 and Pax7 homologues are highly conserved, encode multiple isoforms and show dynamic segment-like expression in the developing brain. Mech. Dev. 70, 49–63.

Staal, F.J., Noort Mv, M., Strous, G.J., Clevers, H.C., 2002. Wnt signals are transmitted through N-terminally dephosphorylated beta-catenin. EMBO Rep. 3, 63–68.

Stoick-Cooper, C.L., Weidinger, G., Riehle, K.J., Hubbert, C., Major, M.B., Fausto, N., Moon, R.T., 2007. Distinct Wnt signaling pathways have opposing roles in appendage regeneration. Development 134, 479–489.

Williams, J.A., Barrios, A., Gatchalian, C., Rubin, L., Wilson, S.W., Holder, N., 2000. Programmed cell death in zebrafish Rohon Beard neurons is influenced by TrkC1/NT-3 signaling. Dev. Biol. 226, 220–230.

Zechner, D., Fujita, Y., Hulsken, J., Muller, T., Walther, I., Taketo, M.M., Crenshaw III, E.B., Birchmeier, W., Birchmeier, C., 2003. beta-Catenin signals regulate cell growth and the balance between progenitor cell expansion and differentiation in the nervous system. Dev. Biol. 258, 406–418.

Zechner, D., Muller, T., Wende, H., Walther, I., Taketo, M.M., Crenshaw III, E. B., Treier, M., Birchmeier, W., Birchmeier, C., 2006. Bmp and Wnt/beta-catenin signals control expression of the transcription factor Olig3 and the specification of spinal cord neurons. Dev. Biol. 303, 181–190.