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Two *tcf3* genes cooperate to pattern the zebrafish brain

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SUMMARY

Caudalizing factors operate in the context of Wnt/β-catenin signaling to induce gene expression in discrete compartments along the rostral-caudal axis of the developing vertebrate nervous system. In zebrafish, basal repression of caudal genes is achieved through the function of Headless (Hdl), a Tcf3 homolog. In this study, we show that a second Tcf3 homolog, Tcf3b, limits caudalization caused by loss of Hdl function and although this Lef/Tcf family member can rescue *hdl* mutants, Lef1 cannot. Wnts can antagonize repression mediated by Tcf3 and this de-repression is dependent on a Tcf3 β-catenin binding domain. Systematic changes in gene expression caused by reduced Tcf3 function help predict the shape of a caudalizing activity gradient that defines compartments along the rostral-caudal axis. In addition, Tcf3b has a second and unique role in the morphogenesis of rhombomere boundaries, indicating that it controls multiple aspects of brain development.

Key words: Tcf3b, Headless, Zebrafish, Wnt, Neural patterning, Morphogen gradient

INTRODUCTION

The blastoderm margin is the source of secreted molecules that regulate gene expression along the rostral-caudal axis of zebrafish (Woo and Fraser, 1997) and *Xenopus* embryos. Multiple signals, including Wnts, FGFs and Activin/Nodal-related factors, cooperate to establish a gradient of caudalizing activity in the gastrula with its high end around the blastoderm margin and its low end near the animal pole (McGrew et al., 1997; Thisse et al., 2000). By the end of gastrulation the isthmic organizer at the midbrain-hindbrain boundary (MHB) becomes a source of additional Wnts and FGFs and the prechordal plate and anterior neural ridge become sources of Wnt antagonists (Eroshkin et al., 2002; Hashimoto et al., 2000; Houart et al., 2002; Kim et al., 2002; Shinya et al., 2000). These additional sources of caudalizing factors and their antagonists are thought to further modulate the shape of the caudalizing activity gradient in the anterior neural plate.

Analysis of zebrafish maternal-zygotic *headless* (hereafter simply called *hdl*) mutants has suggested that caudalizing factors, in particular Wnts, operate in the context of basal repression provided by this Tcf3 homolog (Kim et al., 2000). Canonical Wnt signaling facilitates the expression of downstream target genes through β-catenin, which associates with Lef/Tcf proteins that bind to DNA regulatory elements (Barker et al., 2000). When β-catenin levels are low, Lef/Tcf proteins maintain target genes in a repressed state (Brannon et al., 1997). Although Lef/Tcf transcription factors can have dual roles in activation or repression of target genes, it appears that in vivo Lef1 has a primary role in activation, whereas Tcf3 has a primary role as a repressor (Kengaku et al., 1998; Houston et al., 2002).

Several studies have converged to provide evidence for the role of Wnt/β-catenin activity in defining discrete domains of gene expression along the rostral-caudal axis of the neural plate and in the subsequent establishment of rostral-caudal compartments of the vertebrate neural tube (Domingos et al., 2001; Erter et al., 2001; Hashimoto et al., 2000; Lekven et al., 2001; McGrew et al., 1995; Nordstrom et al., 2002; Shinya et al., 2000; van de Water et al., 2001; Houart et al., 2002; Kiecker and Niehrs, 2001; Kim et al., 2002). These studies have shown that exaggerated Wnt signaling leads to loss of rostral neural domains and expansion of more caudal neural domains, whereas reduced Wnt signaling leads to expansion of rostral neural domains and loss of more caudal domains. However, in some contexts β-catenin is not primarily required for activation of target genes but rather for antagonizing repression mediated by Tcf homologs (Chan and Struhl, 2002; Houston et al., 2002). The primary role for Wnt/β-catenin signaling in rostral-caudal patterning thus remains unclear.

Consistent with the role of *hdl* in repressing genes that define relatively caudal domains, *hdl* mutants are characterized by expanded expression of genes that define the MHB domain. At the same time, the forebrain, whose specification is most

dependent on the repression of caudal genes, is lost in *hdl* mutants. Interestingly, patterning defects are restricted to the rostral neurectoderm, leaving the hindbrain and spinal cord relatively unaffected. Many zygotic *hdl* mutants survive to adulthood, suggesting that other *lef/tcf* genes may limit the severity of phenotypes observed in these fish.

Previously, we identified a partial cDNA clone of a second zebrafish *tcf3* gene, which we named *tcf3b* (Dorsky et al., 1999). We report here the full-length sequence of *tcf3b*, and show that although both *hdl* and *tcf3b* are expressed maternally and throughout development, there are important differences in their expression patterns, most notably during early gastrulation. By examining loss-of-function phenotypes and performing mRNA rescue experiments, we determine that both genes have unique and cooperative roles in early zebrafish development. By comparing the abilities of *tcf3b* and *lef1* to suppress the caudalization in *hdl* mutants, we reveal functional differences between these *lef/tcf* family members in repressing caudal target genes. In addition, we show that Wnt8 function is primarily required in the neurectoderm for de-repression of caudal genes rather than for their activation. Finally, by analyzing changes in the shape of gene expression domains caused by reduction of *tcf3b* function in *hdl* mutants, we make specific predictions about the shape of the caudalizing activity gradient in the neurectoderm.

MATERIALS AND METHODS

Cloning of *tcf3b*

A partial cDNA clone of *tcf3b* from a phage library was used to design primers for 5′ RACE, which was performed using the SMART PCR Kit (Clontech). To obtain a full-length cDNA, we amplified the SMART cDNA library with primers corresponding to the 5′ and 3′ ends of the coding sequence using Expand polymerase (Roche) and inserted the PCR product into the vector pCS2p+ (Turner and Weintraub, 1994). This clone was sequenced and submitted to GenBank (#AY221031).

RT-PCR

PCR was performed on cDNA from various developmental stages, using the following primers and an annealing temperature of 50°C for 30 cycles. Products were run on a 2% agarose gel and stained with ethidium bromide.

tcf3b-F 5′ AGGTGGCATTCGCTATCACG 3′
tcf3b-R 5′ TTTGGTGGTCAGGGACAACG 3′
max-L 5′ GCCGAAGAATGAGCGACAAC 3′
max-R 5′ CTGCTGTGTGTGTGGTTTTTC 3′

In situ hybridization

In situ hybridization with digoxigenin-labeled mRNA probes was performed as described previously (Oxtoby and Jowett, 1993). Probes for *hdl*, *tcf3b*, *lef1* and *wnt1* were made from full-length cDNAs. Digital images were processed with Adobe Photoshop software.

Other plasmids used to make in situ probes have been published previously: *opl* (Grinblat et al., 1998), *pax2.1* (Krauss et al., 1991a), *pax6* (Krauss et al., 1991b), *gbx1* (Itoh et al., 2002), *gsc* (Stachel et al., 1993), *krox20* (Oxtoby and Jowett, 1993), *en2* (Ekker et al., 1992), *isl1* (Inoue et al., 1994), and *mar* (Popperl et al., 2000). Double in situs using digoxigenin- and fluorescein-labeled RNA probes were performed as described (Jowett, 2001).

Phalloidin staining

Fixed embryos were soaked in 0.1 mg/ml AlexaFluor 594 phalloidin (Molecular Probes) for one hour at room temperature and rinsed in PBS. Embryos were mounted in glass coverslips and imaged on a Nikon PCM2000 confocal microscope.

TNT reactions

We added 200 ng of *hdl* and *tcf3b* cDNAs in pCS2+ (Turner and Weintraub, 1994) to TNT Quick Coupled reactions (Promega, Madison, WI). Morpholinos (MOs) were added to the reactions as indicated, and reactions were labeled with ^{35}S Methionine. Following incubation, reactions were run on 10% acrylamide gels, dried and exposed overnight for autoradiography.

Zebrafish maintenance and hdl mutant embryos

Zebrafish were raised and maintained under standard conditions. To collect maternal zygotic *headless*^{m881} mutant embryos, heterozygous males and homozygous females were crossed (Kim et al., 2000).

MO and mRNA injections

MO antisense oligonucleotides were designed by and purchased from Gene Tools (Philomath, OR). The MO sequences are as follows:

hdl: 5′ CTCCGTTTAACTGAGGCATGTTGGC 3′
tcf3b: 5′ CGCCTCCGTTAAGCTGCGGCATGTT 3′

For both MOs, doses ranging from 500 pg-5 ng were injected. After examining phenotypes and embryo survival, 1 ng was chosen as the optimal dose for producing specific phenotypes. *wnt8* MOs were kindly provided by Arne Lekven (Lekven et al., 2001).

For mRNA injections, transcripts were synthesized using the mMessage mMachine kit (Ambion). Expression constructs were made by inserting full-length cDNAs into pCS2+ (Turner and Weintraub, 1994). For rescue experiments, we injected approximately 1 ng MO with 10 pg *hdl*, *tcf3b*, Δ*tcf3b*, *lef1* or Δ*lef1* mRNA and 100 pg *wnt1* mRNA.

RESULTS

Cloning of full-length *tcf3b*

We used 5′ RACE to screen a cDNA library from somitogenesis-stage zebrafish embryos with primers derived from a partial clone of *tcf3b*. By performing RT-PCR on the same cDNA library, we obtained a full-length open reading frame for *tcf3b* (Fig. 1A). The predicted Tcf3b protein is 82% identical to Hdl, its closest homolog. The overall homology between the two proteins suggests that Hdl and Tcf3b are both orthologs of Tcf3 in other vertebrates. By RT-PCR analysis, we showed that *tcf3b* is expressed maternally and throughout gastrulation and somitogenesis, similarly to *hdl* (Fig. 1B). Based on their substantial homology and similar temporal expression patterns, we hypothesized that the two genes could play cooperative roles during zebrafish development.

Embryonic expression of *hdl* and *tcf3b*

To examine possible sites of cooperation between the two genes, we performed in situ hybridizations for *hdl* and *tcf3b* at multiple developmental stages (Fig. 2). Although both genes are widely expressed maternally (not shown), we observed a sharp difference in *hdl* and *tcf3b* expression soon after zygotic transcription begins. At shield stage, *hdl* is expressed broadly throughout the epiblast, whereas *tcf3b* shows very low expression in this tissue (Fig. 2A,B). Following gastrulation, both *hdl* and *tcf3b* show specific expression in the rostral neurectoderm (Fig. 2C,D). Although both genes are expressed at a low level in the notochord, only *hdl* expression was seen in the tailbud (Fig. 2E,F). During somitogenesis, we observed

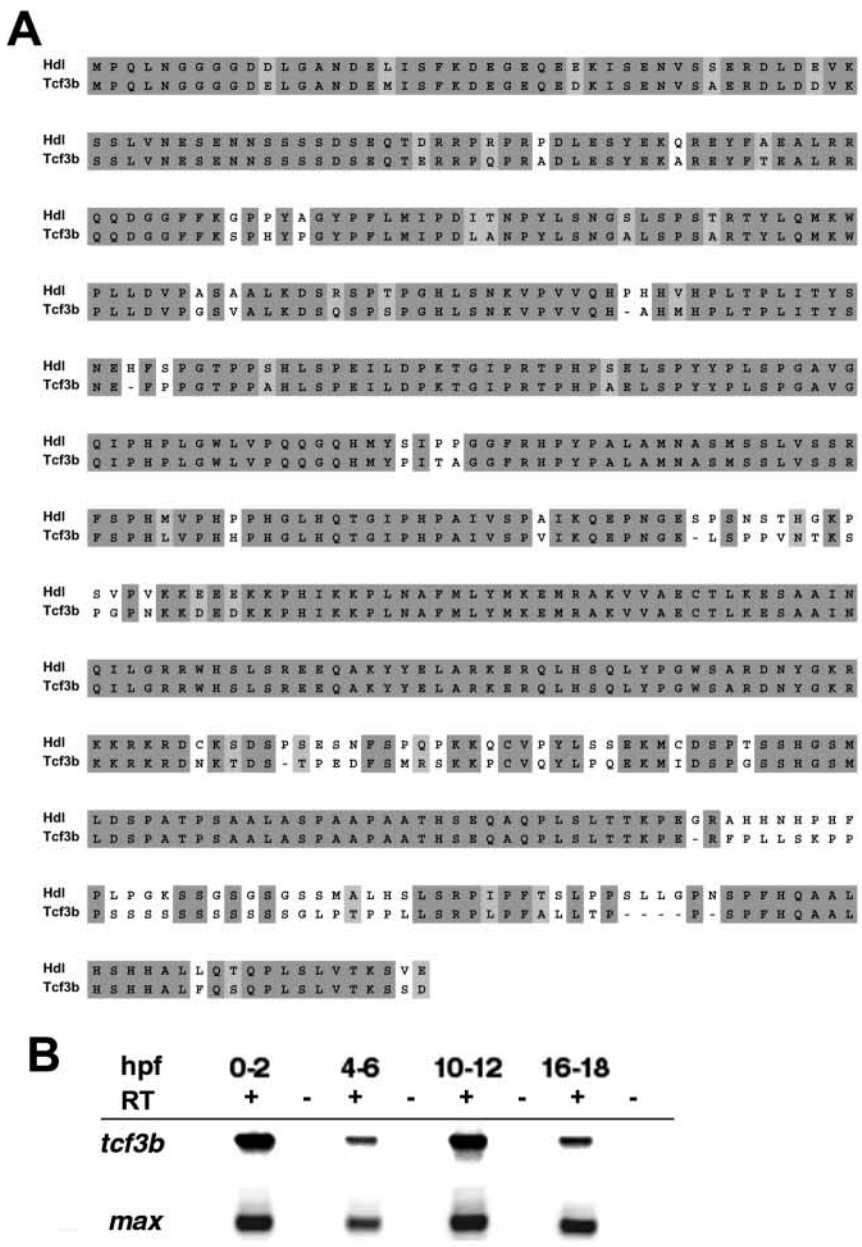

Fig. 1. *tcf3b* encodes a protein highly homologous to Hdl and is expressed throughout zebrafish embryogenesis. (A) Clustal alignment of Hdl and Tcf3b amino acid sequences. Dark-gray shading indicates identity and light gray indicates conservative substitutions. Homology is distributed throughout the proteins, but they are most divergent near the C terminus. (B) RT-PCR analysis shows that *tcf3b* is expressed maternally [0-2 hours post-fertilization (hpf)] and zygotically. *max*, which is expressed constantly throughout development (Schreiber-Agus et al., 1993), was used as a loading control.

expression of both genes throughout the developing brain (Fig. 2G,H). Caudally, we once again detected only *hdl* in the tailbud and presomitic mesoderm (Fig. 2I,J). At late somitogenesis stages, both genes are still expressed throughout the brain (Fig. 2K,L).

Morpholino antisense inhibition of *hdl* and *tcf3b*

To investigate the function of *tcf3b* we used MO antisense oligonucleotides to inhibit mRNA translation (Heasman et al., 2000; Nasevicius and Ekker, 2000). By knocking down the function of both *hdl* and *tcf3b*, we hoped to uncover functional overlap between the two genes. Using expression plasmids that contained sequences complementary to the MOs, we found that each MO could specifically block translation of the targeted gene in vitro (Fig. 3A). Importantly, the sequences of the two genes differ by only four bases in the targeted region.

We first examined the phenotype produced by a MO targeted against *hdl*, and found that injection of 1 ng of *hdl* MO at the one-cell stage can completely reproduce the *hdl* mutant phenotype (Fig. 3C). No obvious phenotypes were observed in trunk or tail regions, again consistent with genetic loss-of-function data. We conclude that our *hdl* MO can specifically block *hdl* gene function.

We next examined the phenotype following injection of one-cell embryos with 1 ng of a MO targeted against *tcf3b*. In contrast to *hdl*, we observed no gross morphological abnormalities through 72 hours post-fertilization (h.p.f.), except that the brain appeared slightly smaller and there was minor cardiac edema (Fig. 3D).

We then co-injected embryos with 1 ng each of *hdl* and *tcf3b* MOs. The size of the brain in many of these embryos was substantially smaller than with either MO alone, especially when examined from the ear to the rostral limit of the brain (Fig. 3E). These results suggest that the two genes may have cooperative roles in these tissues.

The *hdl* phenotype can be rescued by *tcf3b* overexpression in a *wnt1*-reversible manner

To determine whether *hdl* and *tcf3b* encode proteins with similar functions, we attempted to rescue the *hdl* phenotype by overexpressing *tcf3b*. We first titrated the dose of *tcf3b* mRNA to find a concentration that was insufficient to produce a phenotype when overexpressed. When 100 pg of *hdl* or *tcf3b* mRNAs were injected at the one-cell stage, we observed identical phenotypes that included cyclopia, short tails and somite defects (not shown). Because Wnt signaling can regulate the activity of Tcf3, it is difficult to interpret such overexpression phenotypes. Nevertheless, we hypothesized that at a gross level, the two genes may encode proteins with equivalent functions. At a dose of 10 pg, we observed no obvious defects in injected embryos, so we chose this amount for our rescue experiments.

Injecting 10 pg of *tcf3b* mRNA with 1 ng of *hdl* MO resulted

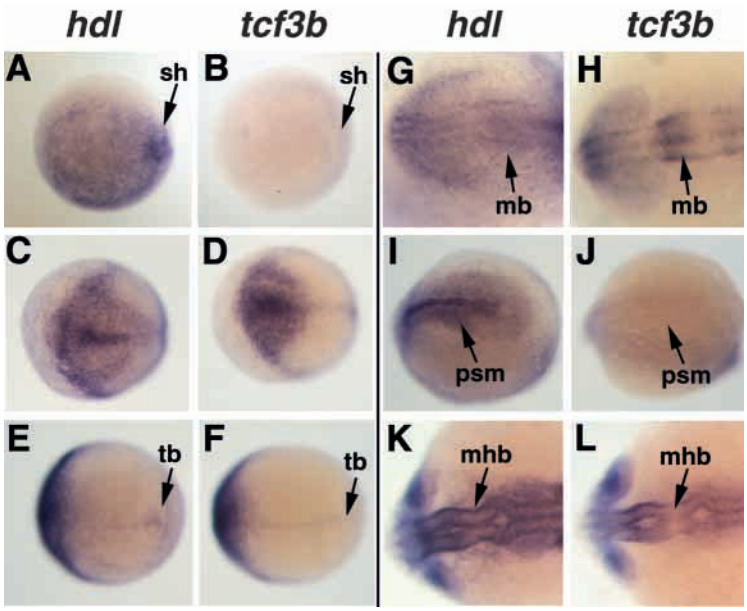

Fig. 2. *hdl* and *tcf3b* show redundant and unique domains of expression during development. (A,B) Shield stage, animal pole view, dorsal towards the right. Expression of *hdl* is much higher than *tcf3b* throughout the epiblast at this stage. (C,D) Bud stage, rostral view, dorsal is towards the right. Both genes are expressed in the presumptive forebrain and midbrain and at the ventral midline. (E,F) Bud stage, caudal view, dorsal towards the left. Both genes are expressed at a low level in the notochord, but only *hdl* is expressed in the tailbud (tb). (G,H) Closer view of head region at six somites. Both genes are expressed throughout the CNS, but *tcf3b* levels are higher in stripes corresponding to telencephalon and midbrain (mb). (I,J) Six somites, caudal view, dorsal towards the left. Only *hdl* is expressed in the tailbud and presomitic mesoderm (psm). (K,L) Eighteen somites. Both genes continue to be expressed throughout the brain, with *tcf3b* showing more specific domains of localization. There is a noticeable gap in *tcf3b* expression at the midbrain-hindbrain boundary (mhb). sh, shield.

in rescue of the *hdl* phenotype. Expression of *pax2.1*, a marker for the MHB, was expanded rostrally in 89% (25/28) of embryos injected with the *hdl* MO alone (Fig. 3F,G). Co-injection of *tcf3b* mRNA eliminated the expansion of *pax2.1* in 78% (25/32) of embryos examined (Fig. 3H). Injection of *tcf3b* was also able to rescue *hdl* morphology at 24 h.p.f. (Fig. 3I). Although only 8% of embryos injected with the *hdl* MO had eyes, this fraction increased to 91% when *tcf3b* mRNA was co-injected (Table 1). To further demonstrate that *tcf3b* mRNA suppressed the defects caused by loss of *hdl* function, we injected *tcf3b* mRNA into *hdl* mutant embryos. Again, injection of *tcf3b* mRNA increased the number of embryos with eyes from 0% to 91% (57/64). We therefore conclude that ectopic expression of *tcf3b* can functionally replace *hdl* in patterning the embryonic brain.

Low-level overexpression of *wnt1* causes caudalization of the neurectoderm resulting in a phenotype similar to *hdl* mutants (Dorsky et al., 1998). To test whether *tcf3b* function is sensitive to Wnt signaling, we examined how co-injection of *wnt1* mRNA affected the ability of *tcf3b* to suppress the eyeless phenotype induced by the *hdl* MO. Co-injection of *wnt1* and *tcf3b* mRNAs and *hdl* MO resulted in only 4% of embryos having eyes (Table 1), indicating a lack of rescue. A truncated form of *tcf3b* that lacks the β-catenin binding domain (Δ*tcf3b*) decreases expression of a β-catenin-dependent reporter transgene (Dorsky et al., 2002) (data not shown) and rescues the *hdl* MO phenotype in 78% of injected embryos (Table 1). However, rescue by Δ*tcf3b* mRNA was not reversible by *wnt1*, as 79% of embryos injected with both mRNAs had eyes (Table 1). These data suggest that Hdl and Tcf3b both act as repressors

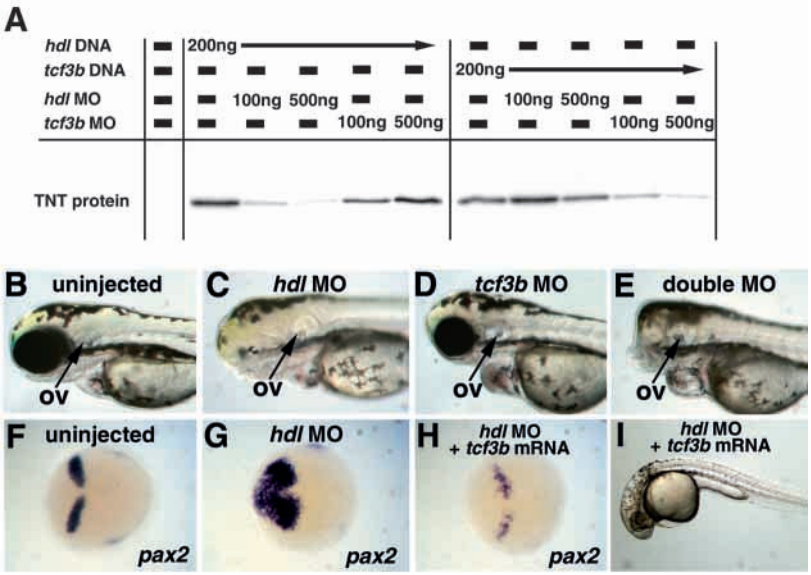

Fig. 3. *hdl* and *tcf3b* morpholinos (MOs) produce different phenotypes and ectopic expression of *tcf3b* can rescue the *hdl* MO phenotype. (A) The *hdl* and *tcf3b* MOs can specifically block translation of expression constructs in vitro, in a dose-dependent manner. The *hdl* MO has no effect on a *tcf3b* expression construct, and the *tcf3b* MO has no effect on a *hdl* construct. (B) Uninjected embryo at 72 hours post-fertilization (hpf). (C) Embryos injected with 1 ng of *hdl* MO have no eyes or telencephalon, similar to *hdl* mutant embryos. (D) Embryos injected with 1 ng of *tcf3b* MO have normal early brain patterning, but overall head size appears smaller (compare with B). (E) When 1 ng of both MOs are injected simultaneously, embryos develop with a much smaller head than with either MO alone (compare with C,D). (F) Rostral view of *pax2.1* expression at 90% epiboly in an uninjected embryo, dorsal towards the right. (G) In *hdl* MO-injected embryos, *pax2.1* expression is expanded rostrally. (H) When *tcf3b* mRNA is co-injected with the *hdl* MO, *pax2.1* expression is restored to the normal domain of two stripes. (I) Normal morphology is observed at 24 hpf after co-injection of *tcf3b* mRNA and *hdl* MO. ov, otic vesicle.

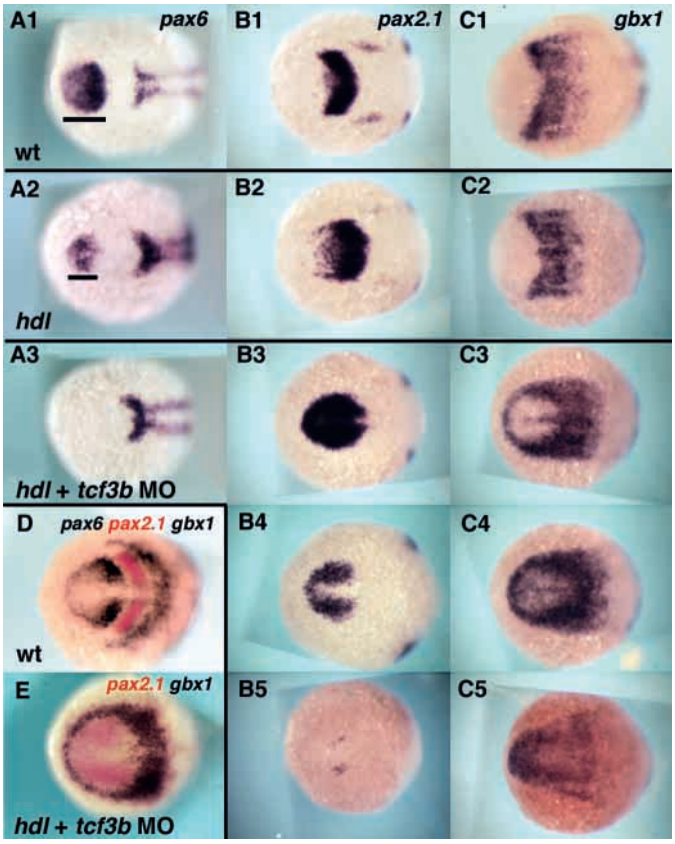

Fig. 4. Loss of *hdl* and *tcf3b* function leads to progressive loss of rostral gene expression domains and concomitant expansion of more caudal domains. All embryos are shown in rostral view with dorsal towards the right. (A1-A3) *pax6* expression at 1-3 somites. (B1-B5) *pax2.1* and (C1-C5) *gbx1* expression at tailbud stage. In *hdl* mutants, rostral *pax6* expression occupies a smaller domain (A2, bar), *pax2.1* shows moderate expansion (B2), whereas *gbx1* is unchanged (C2). *hdl* embryos injected with *tcf3b* MO show a range of phenotypes, suggesting further caudalization of the embryos. Moderately caudalized embryos show complete loss of rostral *pax6* (A3), further expansion of *pax2.1* (B3) and expansion of *gbx1* (C3) expression. In more severely caudalized embryos, *pax2.1* expression begins to be lost (B4) as *gbx1* expands further (C4). In the most severely caudalized embryos, *pax2.1* expression is completely lost (B5) and *gbx1* expression shifts to a more rostral domain (C5). (D) Simultaneous examination of *pax6* (rostral purple), *pax2.1* (red) and *gbx1* (caudal purple) expression at the tailbud stage. (E) *gbx1* (purple) expression expands rostrally around the *pax2.1* (red) expression domain in moderately caudalized embryos.

in vivo, and that target genes can be de-repressed by Wnt signaling via the Tcf3 β-catenin binding domain.

Different Lef/Tcf factors are known to play distinct roles in development (Roel et al., 2002). To further characterize the functions of these proteins in activating and repressing target genes, we attempted to suppress the *hdl* MO phenotype with a third family member, *lef1*. In our experiments, neither *lef1* (3% with eyes) nor Δ*lef1*, a form lacking the β-catenin binding domain, (5% with eyes) could compensate for the loss of *hdl* function (Table 1). The failure of *lef1* to suppress the *hdl* MO phenotype is consistent with its suggested primary role in activating rather than repressing target genes (Kengaku et al., 1998; Merrill et al., 2001).

Cooperative functions of *hdl* and *tcf3b* in early brain patterning

Analysis of *hdl* mutant embryos showed that this gene plays an essential role in forebrain specification. However, *hdl* and *tcf3b* double MO injections resulted in a more severe phenotype than that produced by the *hdl* MO alone (Fig. 3E), suggesting a cooperative role for *hdl* and *tcf3b* in early development. Furthermore, *tcf3b* is expressed both maternally and zygotically, indicating that it may function in the early embryo. We therefore explored the possibility that the two *tcf3* genes contribute to a common function during embryogenesis.

To examine rostral-caudal neural patterning, we compared the expression of *pax6*, *pax2.1* and *gbx1*, which respectively mark the future eye field and dorsal diencephalon, the MHB and the rostral hindbrain (Fig. 4). In *hdl* mutants, the size of the rostral *pax6* expression domain was reduced in 83% (20/24) of the embryos (Fig. 4A2). Our observations of *pax6* expression were restricted to the rostral neurectoderm, because mechanisms that determine its expression in the caudal neurectoderm remain poorly defined at this stage. There was also, as described earlier, a rostral expansion of the MHB domain marked by *pax2.1* expression in 26% (7/27) of the embryos (Fig. 4B2). There was no obvious change, however, in the size of the *gbx1* expression domain in *hdl* mutants (Fig. 4C2).

When 1 ng *tcf3b* MO was injected into wild-type embryos there was no significant change in the expression of *pax6*, *pax2.1* and *gbx1* (data not shown). However, when the same amount was injected into *hdl* mutants a range of phenotypes was seen that reflected further caudalization of the brain. In all *hdl* embryos injected with *tcf3b* MO, the rostral *pax6* expression domain was completely lost (100%, 34/34) (Fig. 4A3). We observed a variable change in *pax2.1*, first extending rostrally to define a broad oval expression domain in the rostral neurectoderm, then becoming restricted to the rostral edge of this domain (84%, 21/25) (Fig. 4B3-4) or in more severely caudalized embryos lost completely (8%, 2/25) (Fig. 4B5). Initially, *gbx1* expression expanded rostrally in an arc-like manner to enclose an oval domain (Fig. 4C3). In more severely caudalized embryos, *gbx1* expression spread into the oval domain (43%, 9/21) (Fig. 4C4-5) and was eventually restricted to an arc-like domain near the rostral edge of the neural plate (Fig. 4C5).

Table 1. Rescue of *hdl* phenotype by *lef/tcf* gene expression

Constructs injected	*hdl* MO	*hdl* MO+ *tcf3b* mRNA	*hdl* MO+ *tcf3b,wnt1* mRNA	*hdl* MO+ Δ*tcf3b* mRNA	*hdl* MO+ Δ*tcf3b,wnt1* mRNA	*hdl* MO+ *lef1* mRNA	*hdl* MO+ Δ*lef1* mRNA
Embryos with eyes	11/134 (8%)	139/153 (91%)	4/90 (4%)	70/90 (78%)	84/106 (79%)	2/78 (3%)	5/93 (5%)

Embryos were scored at 24 hpf for presence of eye pigment, lens and retina.
With all injections, only embryos with normal mesoderm development and dorsal/ventral patterning were scored.

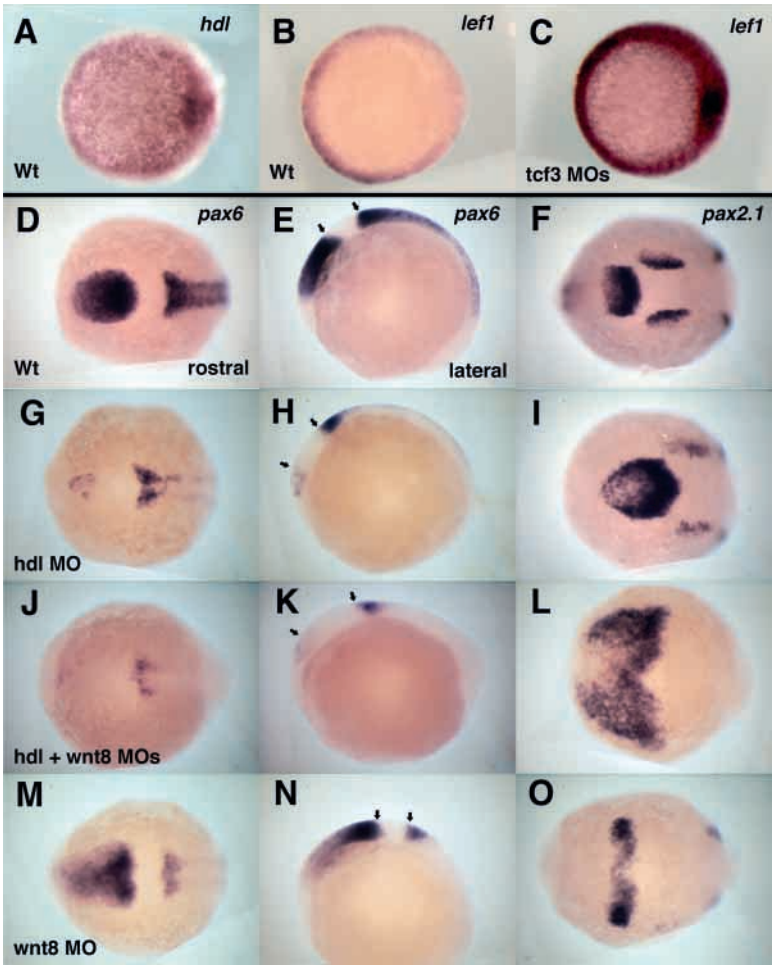

Fig. 5. Wnt8 is required to antagonize Tcf3-mediated repression but not to activate caudal genes. (A-C) Animal pole views, dorsal towards the right. (A) At shield stage, *hdl* is expressed in the neurectoderm where Wnt signaling is low. (B) By contrast, *lef1* is expressed in the ventrolateral mesoderm where Wnt signaling is high. (C) Reduction of Tcf3 function results in expanded *lef1* expression. (D,E) Wild-type *pax6* expression at one to three somites. Arrowheads in E define the caudal limit of *pax6* in the forebrain and rostral limit of *pax6* in the hindbrain. (F) Wild-type *pax2.1* expression at one to three somites. (G-I) In the absence of *hdl* function, *pax6* is shifted rostrally (arrowheads) and rostral expression is reduced, whereas *pax2.1* is expanded rostrally. Changes in the intensity of caudal *pax6* expression did not correlate with changes in other caudally expressed genes (see Fig. 4). (J-L) In the absence of both *hdl* and *wnt8* function, *pax6* is still reduced and shifted rostrally (arrowheads) and *pax2.1* is still expanded. The neural plate is wider in these embryos because of the role of *wnt8* in dorsoventral patterning. (M-O) Loss of *wnt8* function results in caudal expansion of *pax6* (arrowheads) and a caudal shift in *pax2.1*.

A striking feature of caudalized embryos is the systematic manner in which expression of caudal genes expands rostrally to resemble the wild-type gene expression in these compartments. For example, *pax2.1* expression expands in *hdl* mutants from its normal domain at the MHB to a rostral domain that resembles wild-type *pax6* expression (compare Fig. 4A1 and Fig. 4B2). At tailbud stage, the diencephalic marker *pax6* is expressed in a compartment that extends rostrally to enclose an unlabelled area (Fig. 4D). At the same time, *pax2.1* and *gbx1* are expressed in more caudal compartments where they define the MHB domain and rostral hindbrain, respectively (Fig. 4D). In caudalized embryos, *pax6* expression is lost and *pax2.1* expands rostrally within an oval domain that is surrounded by *gbx1* expression (Fig. 4E, also compare Fig. 4B3 and Fig. 4C3). As described earlier, in many caudalized embryos *pax2.1* is most prominently expressed in a rostral crescent within this oval domain (Fig. 4B4, Fig. 4E), resembling the wild-type expression of genes such as *emx1* that define the prospective telencephalon (Houart et al., 2002).

Loss of Hdl and Tcf3 function leads to changes in patterning that are evident by the shield stage

We showed that although *tcf3b* can functionally replace *hdl*, a third family member, *lef1*, which is more likely to have a role in gene activation, cannot. The respective roles of *hdl* and *lef1* in repressing and activating target genes correlates with their complementary expression in the blastoderm at the shield stage (Fig. 5A,B). *lef1* is expressed in a domain that overlaps with *wnt8* at the ventrolateral blastoderm margin and where Wnt/β-catenin signaling is expected to be high. In contrast, *lef1* expression is excluded from the prospective rostral neurectoderm, where *hdl* is expressed and where Wnt/β-catenin signaling is expected to be low. In embryos injected with *hdl* and *tcf3b* MOs, *lef1* expression expands to cover most of the blastoderm at the shield stage (Fig. 5C). This indicates that caudalization of neurectoderm following loss of Tcf3 function is preceded by expanded *lef1* expression at early gastrulation. Furthermore, it indicates that one role of Tcf3 is to restrict *lef1* expression to the blastoderm margin during normal development. It is important to note that *hdl* and *tcf3b* MOs do not cause increased activation of a β-catenin-dependent reporter transgene (Dorsky et al., 2002) (data not shown). This suggests that Wnt/β-catenin signaling may be able to antagonize repression by Tcf3, but it may not play a role in the direct activation of caudal genes.

Wnts may have a primary role in de-repression of caudal neurectoderm genes

To determine whether Wnts are essential for de-repression of caudal genes but not for their activation, we examined whether *wnt8* is required for caudal gene expression in the absence of *hdl* function. Embryos injected with *hdl* MO showed a

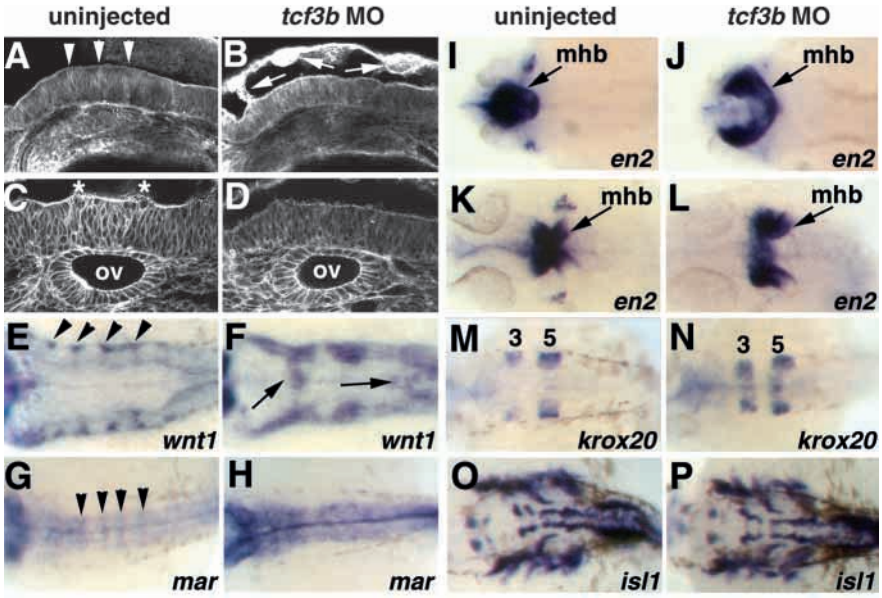

Fig. 6. Loss of *tcf3b* function affects brain morphogenesis. All embryos are shown at 24 hours post-fertilization (hpf), rostral towards the left. (A) Alexa Fluor 594 phalloidin staining reveals forming rhombomere boundaries (arrowheads). (B) In a *tcf3b* MO-injected embryo, no boundaries are visible and ectopic tissue is present dorsally (arrows). (C) At higher magnification, cell shape changes are apparent at rhombomere boundaries (asterisks) in an uninjected embryo. (D) These features are absent in an injected embryo. (E) *wnt1* is expressed at rhombomere boundaries (arrowheads). (F) This expression is disorganized following *tcf3b* MO injection, and ectopic expression is present medially (arrows). (G) *mariposa* is normally expressed at ventral rhombomere boundaries (arrowheads). (H) In injected embryos, *mariposa* expression is uniform and no boundary expression is visible. (I) *en2* expression is present throughout the entire midbrain-hindbrain boundary (mhb) when viewed as an optical cross-section. Lateral expression is in mesoderm. (J) After *tcf3b* MO injection, the mhb fails to close at the dorsal (left) side. (K,L) When viewed dorsally, the rostrocaudal size and position of *en2* expression is normal in injected embryos. (M,N) The positions of rhombomeres 3 and 5 are also normal, as marked by *krox20* expression. (O,P) *isl1* expression indicates that neurogenesis is grossly normal in *tcf3b* MO-injected embryos. ov, otic vesicle.

significant reduction in rostral *pax6* expression, an expansion of the *pax2.1* expression domain and a rostral shift in the position of these expression domains (Fig. 5D-I). In contrast, injection of *wnt8* MO expanded rostral *pax6* expression and shifted both *pax6* and *pax2.1* expression domains caudally (Fig. 5M-O), confirming previous studies that have shown loss of this Wnt homolog leads to expansion of rostral domains and reduction of caudal domains (Erter et al., 2001; Lekven et al., 2001). Reduction of Wnt8 function also caused dorsalization and subsequent broadening of the neurectoderm. When embryos were co-injected with *hdl* and *wnt8* MOs they were still mildly dorsalized, however, *pax6* expression was reduced and *pax2.1* was expanded, showing that *wnt8* is not required for caudalization in the absence of *hdl* function (Fig. 5J-L). These observations support the conclusion that Wnts primarily contribute to de-repression of caudal genes in the neurectoderm.

Analysis of the late *tcf3b* MO phenotype

The analysis of embryos lacking both *hdl* and *tcf3b* function revealed the cooperative roles of these genes in rostral-caudal neural patterning. To determine the unique function of *tcf3b* we examined molecular markers for brain development following *tcf3b* MO injection. At the earliest stages of brain development, the basic patterning of injected embryos appeared normal. We examined the expression of *pax2.1* at bud stage and found no changes compared to uninjected embryos (not shown), indicating that rostral-caudal patterning is unaffected by the *tcf3b* MO. In addition, other MHB markers (*wnt1* and *en2*) and dorsal/ventral patterning markers (*pax2.1* and *axial*) appear normal at 18 somites (not shown). In fact, we were unable to detect any effects on brain development until 24 h.p.f., when we examined hindbrain and MHB morphology.

We observed a severe loss of hindbrain rhombomere segmentation in *tcf3b* MO-injected embryos at 24 h.p.f. This phenotype was never observed in either *hdl* mutant or MO-injected embryos, suggesting a unique function for *tcf3b* in hindbrain development. Analysis of hindbrain morphology by phalloidin staining revealed a lack of physical boundaries (Fig. 6A-D). At the molecular level, *wnt1* is normally expressed at inter-rhombomere boundaries along the dorsolateral edge of the hindbrain (Fig. 6E). However, in injected embryos, *wnt1* expression is noticeably absent from boundary areas and appears uniform or patchy along the hindbrain margin (Fig. 6F). The *mariposa* gene is expressed ventrally in the hindbrain, again localized to rhombomere boundaries (Fig. 6G). Following *tcf3b* MO injection, there is a uniform level of expression throughout the hindbrain (Fig. 6H). We also observed a defect in the closure of the dorsal MHB (Fig. 6I,J), although the position and identity of the MHB appear normal as marked by the expression of *en2* (Fig. 6K,L). Rhombomere identity also does not appear to be affected in these embryos, because patterning markers such as *krox20* (rhombomeres 3 and 5) are expressed normally (Fig. 6M,N). Neurogenesis is also normal, as indicated by *isl1*, which marks ventral neurons of the hindbrain (Fig. 6O,P). These phenotypes indicate defects in brain morphogenesis, rather than in patterning or differentiation. We attempted to rescue these hindbrain defects by co-injection of both *hdl* and *tcf3b* mRNA with the MO, but were unable to restore normal morphology because of the fact that both mRNAs produced similar defects when overexpressed (not shown).

DISCUSSION

A second zebrafish *tcf3* gene, *tcf3b*, has a role in zebrafish embryogenesis

We have cloned a second zebrafish *tcf3* gene and have shown that it functions with *hdl* in early brain patterning and later on

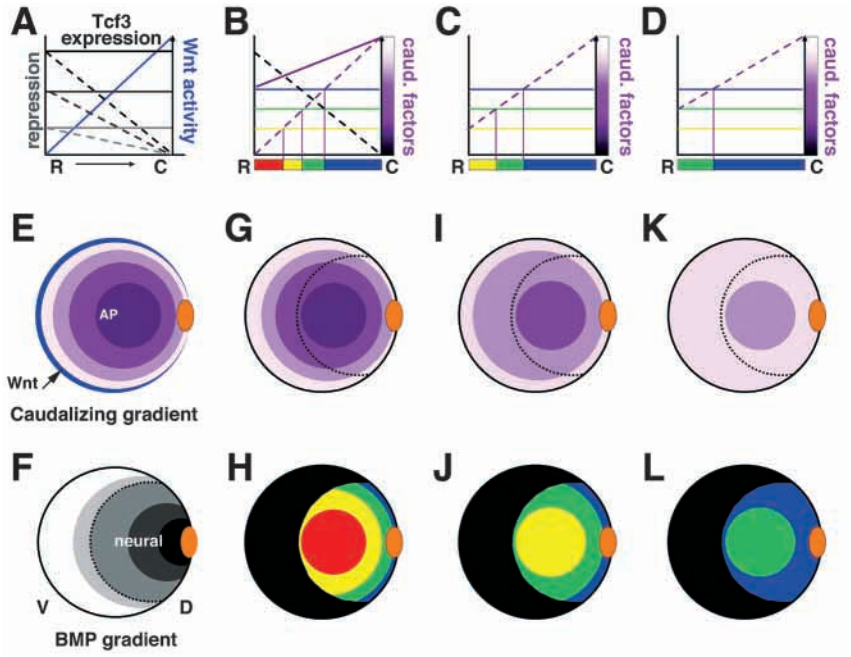

Fig. 7. Models illustrate how Tcf3 function shapes gene expression in the neurectoderm. (A) By antagonizing Tcf3-mediated repression, a gradient of Wnt/β-catenin signaling (blue line) transforms early broad expression of Hdl/Tcf3b (unbroken black line) to a rostrocaudal gradient of effective repression (broken black line). Progressive loss of Hdl/Tcf3b (unbroken gray lines) results in changes in the effective repression gradient (broken gray lines). (B) The gradient of Tcf3-mediated repression (broken black line) alters the efficacy of caudalizing factors that are distributed in a gradient (unbroken purple line) to define a gradient of effective caudalizing activity (broken purple line). Discrete windows of effective caudalizing activity regulate the expression of genes that define blue, green, yellow and red compartments. Moderate (C) to severe (D) reduction in Tcf3 function alters the gradient of effective caudalizing activity and expands caudal domains at the cost of rostral domains. (E) A ventrolateral source of Wnts and other caudalizing factors establishes a caudalizing activity gradient with its low end just dorsal to the animal pole. Darker shades of purple represents lower levels of caudalizing activity. (F) A gradient of BMP activity [black (highest) to white (lowest)] is established by the shield (orange) and determines the neurectoderm (broken line). (G,H) In the neurectoderm the gradient of caudalizing activity is interpreted to define discrete neural compartments (blue, green, yellow and red). (I-L) Illustrations of how gene expression domains would be altered with progressively higher effective caudalizing activity. AP, animal pole; V, ventral.

its own in establishment of morphological boundaries in the neural tube. Although both *hdl* and *tcf3b* are expressed maternally and in the rostral neurectoderm by late gastrulation, only *hdl* is widely expressed in the epiblast at the shield stage. This finding may explain why rostral-caudal neural patterning that takes place during early gastrulation is specifically affected when *hdl* function is lost.

Both *tcf3* genes are required for rostral-caudal brain patterning

Reduction of *tcf3b* function does not significantly affect early patterning in wild-type embryos, suggesting that *hdl* plays a more prominent role in this process. However, *tcf3b* can rescue the *hdl* mutant phenotype and reduction of *tcf3b* function leads to further caudalization of *hdl* mutant embryos. This suggests that low levels of Tcf3b present in the embryo during early gastrulation help limit the degree of caudalization caused by loss of Hdl function. Indeed, in zygotic *hdl* mutants, the loss of rostral neural structures is minimal and persistence of maternal *hdl* and *tcf3b* transcripts permits some mutants to be grown up to adulthood.

Hdl and Tcf3b function in the context of Wnt signaling

The essential function of Hdl protein revealed in zebrafish (Kim et al., 2000) and *Xenopus* (Brannon et al., 1997; Houston et al., 2002) is as a repressor. In contrast, Lef1 appears to be a more effective activator of target genes in vivo (Kengaku et al., 1998; DasGupta and Fuchs, 1999). Complementary roles of Hdl/Tcf3b and Lef1 in mediating repression and activation of target genes, respectively, are consistent with their complementary expression during early zebrafish development (Fig. 5A,B) (Dorsky et al., 2002), as well as their complementary roles in mouse and *Xenopus* development (Merrill et al., 2001; Roel et al., 2002).

Our experiments support a mechanism in which genes that are Hdl and Tcf3b targets in the neurectoderm do not require Wnt/β-catenin signaling for either their repression or their endogenous activation. Furthermore, in the absence of Tcf3 function we observe an increase in *lef1* expression without a corresponding increase in expression of a β-catenin-responsive reporter (Dorsky et al., 2002). Other Lef/Tcf factors have been shown to activate targets in lymphocytes (Travis et al., 1991; van de Wetering et al., 1991), and *Xenopus* embryos (Labbe et al., 2000) in a Wnt/β-catenin-independent manner. Through this evidence, it is possible to conclude that Hdl and Tcf3b function in a Wnt-independent manner as well. However, data from our studies indicates that the developmental roles of these factors are closely linked to Wnt signaling in the embryo. First, we show that rescue of *hdl* mutants is reversible by Wnt signaling in a manner that requires β-catenin binding. Second, loss of Wnt8 function results in expansion of the same rostral genes that require Tcf3 function for their expression. Although Wnt8 may act through Lef1 to activate target genes in the ventrolateral mesoderm, we conclude that it primarily antagonizes Tcf3 function in the neurectoderm.

Wnt signals are only a part of the system that determines gene expression along the rostral-caudal axis. Multiple caudalizing factors, including Wnts, FGFs and Activin/Nodal-related factors, contribute to rostral-caudal patterning (McGrew et al., 1997; Altmann and Brivanlou, 2001; Thisse et al., 2000). Wnt and TGFβ signals can operate synergistically through Lef1 to activate target genes (Nishita et al., 2000; Riese et al., 1997). In addition, the Wnt and MAPK pathways work synergistically to reduce repression of target genes mediated by Lef/Tcf homologs (Behrens, 2000; Meneghini et al., 1999;

Rocheleau et al., 1999). Together these studies suggest a broad role for Lef/Tcf family members in coordinating the response to multiple signaling pathways.

Opposing gradients of Tcf3-mediated repression and caudalizing activity in the neurectoderm

Our results are consistent with the emerging view that a gradient of Wnt/β-catenin activity helps define discrete domains of gene expression in the neural plate. As mentioned above, we propose that Tcf3 represses targets of caudalizing factors, and β-catenin prevents Tcf3 from being effective as a repressor, resulting in a rostral-caudal gradient of effective Tcf3 repression (Fig. 7A, broken lines). This effect of the Tcf3 repression gradient could be represented by lowering the rostral end of a caudalizing gradient (Fig. 7B, broken purple line). Loss of basal Tcf3 function would thus raise the low end of the caudalizing gradient, decreasing its slope (Fig. 7C,D). In the context of this gradient, specific thresholds of caudalizing activity define discrete windows of gene expression along the rostral-caudal axis (Fig. 7B-D). Progressive loss of Tcf3 function would cause loss of rostral and expansion of caudal gene expression domains (Fig. 7C,D).

Multiple factors shape the caudalizing gradient

A one-dimensional representation of the caudalizing gradient helps illustrate the caudal to rostral shift in gene expression when Tcf3 function is reduced. However, it does not provide an adequate explanation for actual changes in the size and shape of gene expression domains in the neural plate, in particular why caudal genes such as *gbx1* eventually expand rostrally around a *pax2.1* expression domain.

The *gbx1* gene is consistently expressed just caudal to *pax2.1*, defining a compartment expected to depend on a slightly higher window of caudalizing activity. Expansion of the *gbx1* domain around the *pax2.1* domain in severely caudalized embryos suggests that *pax2.1* represents the low end of the caudalizing gradient in these embryos and that the surrounding *gbx1* expression reflects a slightly higher caudalizing activity. Indeed, the arc-like rostral expansion of *gbx1* in caudalized embryos resembles the arc-like expression of *pax6* in wild-type embryos. These observations imply that the low end of the caudalizing gradient is not at the rostral edge of the neural plate, but rather in a slightly caudal domain that is surrounded by *pax6* in wild-type embryos and surrounded by *gbx1* in severely caudalized embryos.

We have shown here and in previous analysis of *hdl* mutants (Kim et al., 2000) that loss of Tcf3 function leads to changes in patterning that become evident by early gastrulation. Embryos treated with lithium chloride soon after the shield stage are caudalized in a manner similar to *hdl* mutants (van de Water et al., 2001; Kim et al., 2002). At shield stage the ventrolateral blastoderm margin in zebrafish is the source of caudalizing factors and corresponds with the highest β-catenin activity (Woo and Fraser, 1997; Dorsky et al., 2002). If caudalizing factors at the ventrolateral margin help establish the gradient of caudalizing activity, the low end of the gradient should be located at the furthest distance from the source, slightly dorsal to the animal pole (Fig. 7E). At the same time, BMP antagonists secreted at the dorsal margin define the prospective neurectoderm (Grinblat et al., 1998) (Fig. 7F). Different levels of caudalizing activity in the prospective neurectoderm are expected to define discrete domains of gene expression (Fig. 7G,H). The yellow, green and blue compartments defined by different thresholds of caudalizing activity illustrate how expression of *pax6*, *pax2.1* and *gbx1*, respectively, might be determined in wild-type embryos. As described above, loss of Tcf3-mediated repression is expected to alter the shape of the caudalizing gradient (Fig. 7I,K) and thus alter *pax6*, *pax2.1* and *gbx1* expression domains (Fig. 7J,L). This model provides a potential explanation for the arc-shaped early expression of *pax6* in wild-type embryos and illustrates why *gbx1* expression would expand rostrally around an oval *pax2.1* expression domain in severely caudalized embryos.

The shape of the caudalizing gradient can also be influenced by factors that inhibit function of Wnts. As gastrulation proceeds, the prechordal plate, which is the source of at least one secreted Wnt antagonist, Dkk1 (Hashimoto et al., 2000; Shinya et al., 2000), might help define the low point of the Wnt activity gradient in the overlying rostral neurectoderm. Furthermore, during gastrulation the anterior neural ridge also becomes a source of a Wnt antagonist, Tlc (Houart et al., 2002), and it probably contributes to the pattern of Wnt-mediated derepression as gastrulation is completed. Clearly, other factors such as cell and tissue movements contribute to patterning of the neurectoderm throughout this process. However, for simplicity their contribution is not emphasized in our model, which represents a static view at the beginning of gastrulation.

tcf3b is uniquely required for rhombomere boundary formation

Following injection of the *tcf3b* MO, we observed a marked defect in morphogenesis of the MHB and hindbrain rhombomeres. Although we were unable to rescue this phenotype by overexpressing either gene, we believe it is specific to *tcf3b* because we never observed hindbrain defects in other MO-injected embryos. Our data predict that Tcf3b might affect the expression of genes involved in hindbrain morphogenesis. One obvious target for further investigation would be the Ephrin/Eph family of receptor tyrosine kinases and ligands, which have been demonstrated to play a role in cell sorting and boundary formation in the hindbrain (Cooke et al., 2001; Lumsden, 1999).

Redundant and unique functions of *hdl* and *tcf3b*

The *hdl* gene plays a unique role in forebrain patterning during development. Likewise, injection of the *tcf3b* MO produced unique phenotypes in hindbrain and MHB morphogenesis. Because our rescue experiments indicate that *hdl* and *tcf3b* encode proteins that can function identically, some of these unique roles can be explained by nonoverlapping expression patterns of the two genes. This may be true in the hindbrain and MHB as well, where we observed subtle differences in the expression patterns of *hdl* and *tcf3b* (Fig. 2K,L). Alternatively, the two genes may encode proteins with different DNA targets or transcriptional cofactors in the hindbrain and MHB, and the function encoded by *hdl* may be dispensable. Our inability to rescue the *tcf3b* MO phenotype with either gene leaves these possibilities open.

In some tissues in which either one or both genes are expressed, we observed no phenotype in our MO injections.

For example, both *hdl* and *tcf3b* are expressed in the notochord, but no obvious notochord defects were seen in MO-injected embryos. The function of *hdl* in the tailbud and paraxial mesoderm is unclear as well, as neither MO-injected embryos nor *hdl* mutants exhibit patterning defects in these tissues. Loss of *hdl* and *tcf3b* function prior to gastrulation resulted in minimal effects on initial dorsal-ventral patterning. The most probable explanation for these results is that in zebrafish, other genes are able to compensate for *hdl* and *tcf3b* in these regions.

In this study, we have demonstrated specific and overlapping developmental roles for two zebrafish *tcf3* genes. Our results suggest regions in the embryo where Tcf3 function may be important for patterning and morphogenesis. It will now be important to identify the transcriptional targets of Hdl and Tcf3b in these regions so the cellular responses to this pathway become clear. In addition, the biochemical differences between Lef/Tcf proteins must be further investigated, so that both their redundancies and distinct functions can be better understood.

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