Available online at www.sciencedirect.com

SCIENCE DIRECT®

Developmental Biology 267 (2004) 165–180

DEVELOPMENTAL BIOLOGY

www.elsevier.com/locate/ydbio

Sequential antagonism of early and late Wnt-signaling by zebrafish *colgate* promotes dorsal and anterior fates

Roopa M. Nambiar[a] and Paul D. Henion[b,*]

[a] *Molecular, Cellular and Developmental Biology Program, Center for Molecular Neurobiology, Ohio State University, Columbus, OH 43210, USA*
[b] *Department of Neuroscience, Center for Molecular Neurobiology, Ohio State University, Columbus, OH 43210, USA*

Received for publication 3 October 2003, revised 10 November 2003, accepted 11 November 2003

Abstract

The establishment of the vertebrate body plan involves patterning of the ectoderm, mesoderm, and endoderm along the dorsoventral and antero-posterior axes. Interactions among numerous signaling molecules from several multigene families, including Wnts, have been implicated in regulating these processes. Here we provide evidence that the zebrafish *colgate*^{b382} (*col*) mutation results in increased Wnt signaling that leads to defects in dorsal and anterior development. *col* mutants display early defects in dorsoventral patterning manifested by a decrease in the expression of dorsal shield-specific markers and ectopic expression of ventrolaterally expressed genes during gastrulation. In addition to these early patterning defects, *col* mutants display a striking regional posteriorization within the neuroectoderm, resulting in a reduction in anterior fates and an expansion of posterior fates within the forebrain and midbrain–hindbrain regions. We are able to correlate these phenotypes to the overactivation of Wnt signaling in *col* mutants. The early dorsal and anterior patterning phenotypes of the *col* mutant embryos are selectively rescued by inactivation of Wnt8 function by morpholino translational interference. In contrast, the regionalized neuroectoderm posteriorior ization phenotype is selectively rescued by morpholino-mediated inactivation of Wnt8b. These results suggest that *col*-mediated antagonism of early and late Wnt-signaling activity during gastrulation is normally required sequentially for both early dorsoventral patterning and the specification and patterning of regional fates within the anterior neuroectoderm.

Keywords: Organizer; Shield; Mesoderm; Neuroectoderm; Patterning; Wnt

Introduction

The vertebrate body plan is determined during gastrulation as the germ layers are induced and patterned along the dorsoventral (DV) and antero-posterior (AP) axes. These induction and patterning events involve a complex and incompletely understood series of signaling events dependent on interactions among numerous signaling molecules (Harland and Gerhart, 1997; Lemaire and Kodjabachian, 1996).

The process of dorsal specification is initiated soon after fertilization by an inductive signaling center called the gastrula organizer that was first recognized in amphibians (Nieuwkoop, 1973; Spemann, 1938; Spemann and Mangold, 1924). The embryonic shield in zebrafish is a signaling center analogous to the gastrula organizer in amphibians (Schneider et al., 1996). In *Xenopus* and zebrafish, the Wnt signaling pathway is involved in early DV patterning of the embryo before and after the mid-blastula transition (MBT; Kane and Kimmel, 1993; Moon et al., 1997). The pre-MBT Wnt pathway acts as an early dorsalizing signal that is involved in inducing organizer formation (Heasman et al., 1994; Moon et al., 1997; Sokol, 1999; Wylie et al., 1996). Once the organizer is formed, it secretes factors that can dorsalize lateral mesoderm and induce and pattern neural fates in the ectoderm (Gerhart et al., 1989). This activity can, at least in part, be attributed to its ability to inhibit the effects of the ventralizing bone morphogenetic proteins (BMP) and the post-MBT Wnt signaling pathways (reviewed in Harland and Gerhart, 1997; Moon et al., 1998).

Following induction, the neural plate is subdivided along the AP axis. Studies have shown that a Wnt/β-catenin

* Corresponding author. Department of Neuroscience, Center for Molecular Neurobiology, Ohio State University, 105 Rightmire Hall, 1060 Carmack Road, Columbus, OH 43210. Fax: +1-614-292-5379.
E-mail address: henion.1@osu.edu (P.D. Henion).

0012-1606/$ - see front matter © 2003 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2003.11.019

signaling gradient regulates AP patterning of the neuroectoderm in *Xenopus* as well as in chick gastrulae (Kiecker and Niehrs, 2001; Nordstrom et al., 2002). This gradient is characterized by high Wnt activity in posterior regions and lower activity in the anterior regions of the embryo, a likely consequence of Wnt inhibitors being predominantly expressed anteriorly. The anterior endomesoderm acts as a source of Wnt antagonists such as Cerberus (Bouwmeester et al., 1996), secreted Frizzled Related Proteins (Leyns et al., 1997), and Dickkopf 1 (Dkk1; Glinka et al., 1998). The importance of Wnt antagonists in neural patterning is evident in experiments where the overexpression of Wnt inhibitors in *Xenopus* and zebrafish embryos resulted in embryos with enlarged heads (Deardorff et al., 1998; Fekany-Lee et al., 2000; Glinka et al., 1997, 1998; Hashimoto et al., 2000; Heasman et al., 2000; Hoppler et al., 1996; Leyns et al., 1997; Pierce and Kimelman, 1995). Loss of function and inhibition of Wnt antagonists have also provided support for their role in anterior neural development. For example, inhibition of Dkk1 function in *Xenopus* embryos results in embryos with truncated anterior structures (Glinka et al., 1998). Likewise, mouse embryos lacking *dkk1* function lack head structures anterior to the midbrain (Mukhopadhyay et al., 2001) and the absence of *six3* function, which is a repressor of Wnt signaling, results in truncated forebrain development and posteriorization of the head (Lagutin et al., 2003).

Genetic evidence for the functions of regulated Wnt signaling comes in part from the study of zebrafish mutants. Zebrafish *wnt8* mutant embryos display an enlargement of the forebrain and significant loss of midbrain (Lekven et al., 2001). A mutation in the transcriptional repressor Tcf3, the product of the *headless* (*hdl*) gene, causes loss of eyes, forebrain, and part of the midbrain (Kim et al., 2000). *masterblind* (*mbl*) mutants, in which the function of Axin is abolished, exhibit fate changes within the forebrain with the transformation of telencephalon and eyes to diencephalon (Heisenberg et al., 2001). The mutant *bozozok* (*boz*) displays defects in the formation of the organizer and in the development of dorso-anterior structures due to a disruption in the function of the homeobox gene *nieuwkoid/dharma* that negatively regulates both the Wnt and BMP pathways (Fekany-Lee et al., 2000). These studies further support an important role for Wnts as neural posteriorizing factors.

The current model of the role for Wnt-signaling in posteriorizing neural tissue suggests that Wnt8 activity establishes the initial subdivisions along the neuraxis (Erter et al., 2001). Although Wnt8 is involved in the early AP patterning of the neural plate, it appears unlikely to play a later role in refining these initial AP positional values since altering early Wnt activity by manipulating the expression of the Wnt inhibitors *dkk1*, *cerberus*, and *frzb1* increases or decreases the overall size of the forebrain without affecting the patterning of regional fates within the forebrain (Hashimoto et al., 2000; Leyns et al., 1997; Piccolo et al., 1999). Instead, local interactions between organizing centers such as the anterior neural plate border, caudal diencephalon, and the midbrain–hindbrain boundary appear likely to regulate further regionalization within the brain anlage (Houart et al., 2002; Kim et al., 2002). Recent studies indicate that Wnt8b is a likely candidate for mediating some later neural patterning events within the anterior neuroectoderm (Houart et al., 2002; Kim et al., 2002). In addition, Wnt1 has been shown to play an important role in establishing the midbrain–hindbrain boundary (Kelly and Moon, 1995). These studies suggest that regional patterning of the neural plate involves a continuous refinement of positional values influenced by both early and late acting Wnt signals.

Although the importance of Wnt-signaling in embryonic patterning is established, it remains unclear to what extent individual positive and negative regulators of Wnt signaling contribute to the patterning events mediated by early and later acting Wnt signaling. In this study, we characterize the phenotype of zebrafish *col* mutant embryos. *col* mutants display defects in early DV patterning and later regional AP patterning within the anterior neural plate. We provide evidence that the *col* locus normally functions as a negative regulator of Wnt signaling involved in maintenance of the gastrula organizer and the promotion of anterior fates within the forebrain and midbrain–hindbrain regions of the neural plate. Specifically, our results indicate that sequential *col*-mediated inhibition of early and late Wnt-signaling during gastrulation is normally required for proper DV and AP patterning of the zebrafish embryo.

Materials and methods

Zebrafish

Adult zebrafish and embryos were maintained in the Ohio State University zebrafish facility. Adults and embryos were raised at 28.5°C and were staged by anatomical criteria (e.g. %epiboly; numbers of somite pairs) or hours (hpf) or days post fertilization (dpf). Mutant embryos (*AB and WIK backgrounds) were collected from pairwise matings of heterozygous adults.

In situ hybridization and immunohistochemistry

Staged embryos were fixed overnight at 4°C in phosphate-buffered saline solution containing 4% paraformaldehyde. In situ hybridization was carried out as described by Thisse et al. (1999) with minor modifications. A detailed protocol is available on request. The following probes were used: *anf* (Kazanskaya et al., 1997), *bmp2* (Kishimoto et al., 1997; Nguyen et al., 1998; Nikaido et al., 1997), *bmp4* (Chin et al., 1997; Nikaido et al., 1997), *chordin* (Miller-Bertoglio et al., 1997), *dHAND* (Yelon et al., 2000), *dickkopf1* (Hashimoto et al., 2000), *dlx2* (Akimenko et al., 1994), *fgf8* (Reifers et al., 1998), *gata1* (Detrich et al.,

1995), *gata2* (Detrich et al., 1995), *goosecoid* (Stachel et al., 1993), *islet1* (Korzh et al., 1993), *nieuwkoid* (Koos and Ho, 1998), *nkx2.5* (Lee et al., 1996), *no tail* (Schulte-Merker et al., 1992), *otx2* (Li et al., 1994), *pax 2.1* (Krauss et al., 1991), *sonic hedgehog* (Krauss et al., 1993), *tyrosine hydroxylase* (provided by T. Look, Dana Farber Cancer Institute, Boston, MA), *wnt1* (Kelly and Moon, 1995), *wnt8* (Kelly et al., 1995), and *wnt8b* (Kelly et al., 1995).

Probes were synthesized using T7, T3, or SP6 RNA polymerases and DIG-labeled rNTPs as appropriate. For in situ hybridizations on embryos older than 24 hpf, the embryos were raised in 0.03g/l 1-phenyl-2-thiourea (PTU) to prevent melanin synthesis, which allowed clear analysis of gene expression patterns and antibody staining without interference from pigmented melanocytes.

Immunohistochemistry was done on embryos fixed in 4% paraformaldehyde for 2 h at room temperature and performed according to Henion et al. (1996). The following antibodies were used: zn-12 (Trevarrow et al., 1990), zn-5 (Trevarrow et al., 1990), and acetylated tubulin (Sigma).

mRNA and morpholino injections

mRNA was synthesized using Ambion's mMessage mMachine kit. Following transcription, the mRNA was extracted using phenol/chloroform and concentrated in Microcon YM-50 (Amicon) microconcentrator filter devices. RNA quality was assayed using gel electrophoresis. mRNA was diluted in 1% phenol red and pressure injected into the YSL of one- to eight-cell-stage embryos.

The following constructs were used for injections: *X glycogen synthase kinase 3β* (*Xgsk3β)* in pCS2 (Pierce and Kimelman, 1995), dominant negative *Xgsk3β* in pCS2 (Pierce and Kimelman, 1995), *Xaxin* GID2 fragment in pCS2MT (Hedgepeth et al., 1999), *tcf3* in pCS2 (Kim et al., 2000), *VP16-tcf3* in pCS2 (Kim et al., 2000), *dickkopf1* in pCS2 (Hashimoto et al., 2000), *chordin* in pCS2 (Miller-Bertoglio et al., 1997), *antivin* in pCS2 (Thisse et al., 1999), and *XFD* (Amaya et al., 1991). The amount of RNA injected in each embryo varied from 10 to 600 pg depending on the particular RNA.

The antisense *wnt8* morpholino (Erter et al., 2001) was diluted with phenol red/1× Danieau Medium (1:6) and approximately 6–8 ng was injected into each embryo. Coinjections of a modified and functional version of *wnt8* mRNA that is not recognized by the morpholino (Erter et al., 2001) were performed as specificity controls. Antisense *wnt8b* morpholino (Houart et al., 2002) was similarly diluted and approximately 8–10 ng was injected into each embryo.

Linkage analysis and genotyping

For linkage analysis, AB background heterozygous *col* carriers were crossed to a polymorphic WIK strain and gynogentic half-tetrad F2 embryos from the ABXWIK females were used. PCR was performed using Simple Sequence Length Polymorphic markers (Knapik et al., 1996). We have mapped the col^{b382} mutation to chromosome 19. To determine the genotype of embryos used in experiments before a readily apparent visible phenotype is seen, DNA from individual embryos was obtained and PCR was performed on genomic DNA using chromosome 19 linked polymorphic SSLP markers.

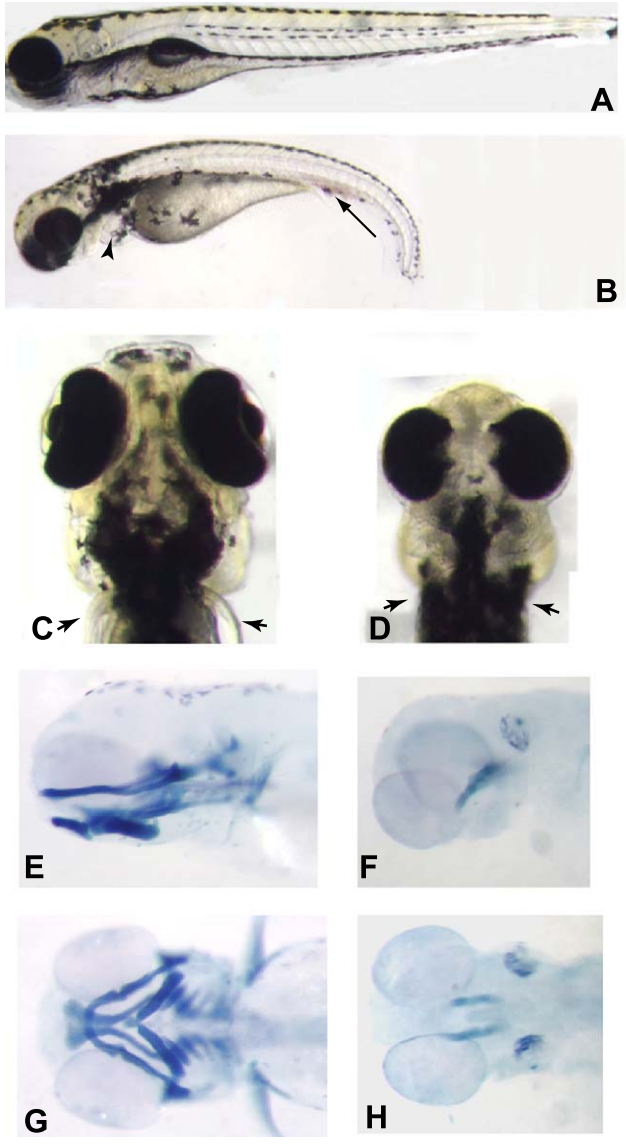

Fig. 1. (A,B) Wild-type (A) and *col* mutant (B) embryos at 3.5 dpf. *col* mutant embryos have a shorter and curved body axis and also display an accumulation of blood cells (arrow in B) and edema of the heart (arrowhead) .(C,D) Ventral views of the head of a wild-type (C) and *col* (D) mutant embryo at 3.5 dpf. *col* embryos have a smaller head with reduced, partially cyclopic eyes. Arrowheads mark the pectoral fins in wild-type (C) embryos. *col* mutants lack pectoral fins (arrowheads in D). (E–H) Alcian blue preparations of 3.5 dpf embryos to reveal craniofacial cartilages. Lateral (E,F) and ventral (G,H) views of wild-type (E,G) and *col* mutant (F,H) embryos illustrate the dramatic reduction of cartilages in mutant embryos.

Results

Isolation of the colgate^{b382} mutant

colgate^{b382} (*col*) is an ENU-induced recessive, embryonic lethal mutation isolated in an early pressure screen (Henion et al., 1996). Homozygous mutant embryos die at 7 dpf. Mutant embryos were identified based on their abnormal melanocyte distribution pattern (Figs. 1A,B). In addition to the pigment defect, *col* mutant embryos display a gross morphological loss of anterior structures. They have smaller heads (Figs. 1A–D) and partially cyclopic eyes (Figs. 1C,D), and craniofacial cartilages are grossly abnormal (Figs. 1E–H). The body axis of mutant embryos is also shorter compared to their wild-type siblings (Figs. 1A,B). Heart development is abnormal in *col* embryos evidenced by edema in mutants at 2 dpf (Fig. 1B) and *col* mutants completely lack pectoral fins (Figs. 1C,D). In this study, we have focused on the early patterning phenotypes of *col* mutant embryos and investigated potential causative mechanisms to explain these developmental abnormalities.

Disruption of early dorsoventral patterning in col mutant embryos

col mutant embryos display truncations in anterior neural structures. Since factors from the organizer derived prechordal plate promote anterior neural development (Ang and Rossant, 1993; Ang et al., 1994; Dale et al., 1997; Foley et al., 1997; Gerhart et al., 1989; Glinka et al., 1998; Shinya et al., 2000), we tested whether abnormal organizer development contributes to the development of the *col* mutant phenotype.

In the zebrafish early gastrula, *goosecoid* (*gsc*) is expressed by the developing shield (Stachel et al., 1993). At 6 hpf, *col* embryos show a decrease in shield expression

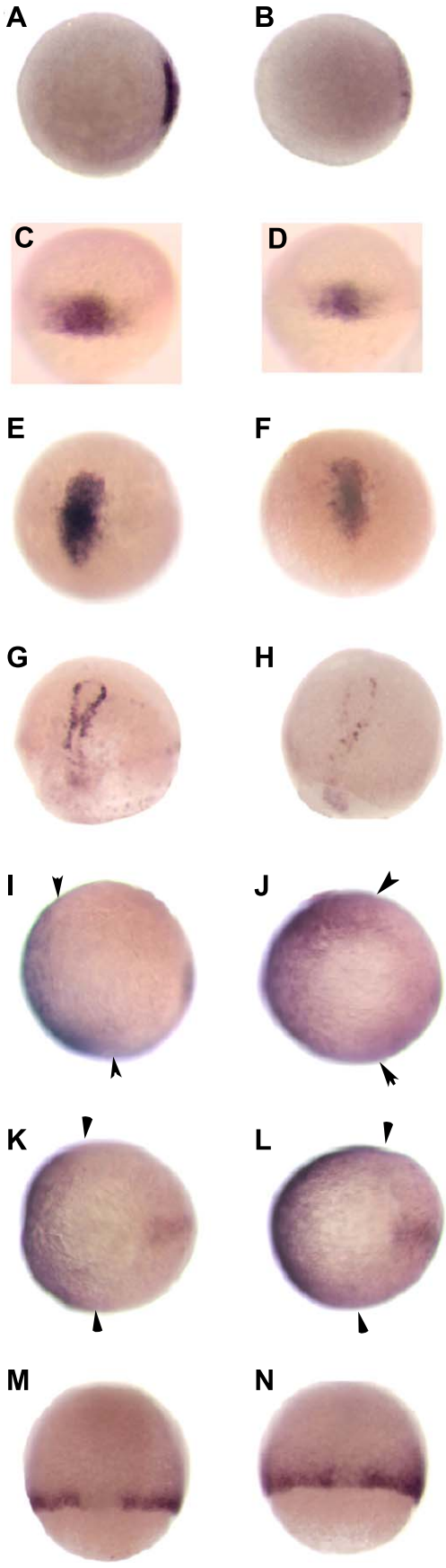

Fig. 2. *col* mutants display a reduction in the expression of shield-specific markers (A–D). Animal pole views of 6 hpf (shield stage) wild-type (A, C) and *col* mutant (B,D) embryos. *gsc* is normally expressed in the shield of wild-type embryos (A) and *gsc* expression is down-regulated in *col* mutant embryos (B). A mild reduction is also seen in *chd* expression in the shield at 6 hpf in *col* mutants (D) as compared to wild-type embryos (C). A decrease in expression of prechordal plate markers is also seen in *col* mutant embryos (E–H). At 80% epiboly (8.5 hpf), *gsc* is expressed in the developing prechordal plate of wild-type embryos (E). Prechordal plate *gsc* expression is reduced in *col* mutants (F). A similar decrease in expression is seen in dorsal views of *col* mutant embryos at 90% epiboly (H), when *dkk1* expression is observed in the prechordal plate in wild-type embryos (G). *col* embryos display an expansion in the expression of ventralizing signals. (I–L) Animal pole views of *col* mutant embryos at 80% epiboly show an expansion of *bmp2* (J) and *bmp4* expression (L) as compared to wild-type embryos (I,K). Arrowheads indicate approximate limits of expression domains. (M,N) In dorsal views, *wnt8* expression is apparent in the ventrolateral mesoderm and is excluded from the dorsal midline in wild-type embryos (M) at 60% epiboly (7 hpf). In *col* mutant embryos (N) at the same stage of development, *wnt8* expression expands into the dorsal midline.

of *gsc* as compared to wild-type siblings (Figs. 2A,B). A similar decrease in expression of *dickkopf1* (*dkk1*), a Wnt antagonist (Shinya et al., 2000), is also seen in *col* mutants (not shown). A mild reduction is also observed in *chordin* (*chd*) expression in *col* mutants at shield stage (Figs. 2C,D). However, the expression of *bozozok/niewkoid* (*boz*), a gene involved in the induction of the organizer, was unaffected at 4 and at 6 hpf (data not shown). These defects in shield development occur during mid- to late gastrulation since the early expression of *gsc* in *col* embryos at 4 hpf is indistinguishable from wild-type siblings (not shown). At 80% epiboly, *gsc* is expressed by the prechordal plate (Stachel et al., 1993) and *col* mutants display a reduction in *gsc*-expressing presumptive prechordal plate (Figs. 2E,F). A reduction is also seen in *dkk1* expression at 80% epiboly (Figs. 2G,H). Notably though, the development of the notochord, the posterior axial derivative, does not appear to be affected in these embryos since there is little change in the expression of *ntl* in the notochord anlage in *col* mutants (not shown). These results suggest that *col* function is essential for the maintenance of gastrula organizer tissue but is unlikely to play a role in the initial induction of the organizer. Since the notochord is unaffected in *col* mutant embryos, these results indicate a more specific role for *col* in organizer development such as promoting the development of the head organizer.

To test if the loss of dorso-anterior fates is accompanied by defects in the development of ventral fates, we examined the expression of *bmp2* and *bmp4* whose gene products act as morphogens that specify ventral fates in the ectoderm and mesoderm (Kishimoto et al., 1997; Neave et al., 1997; Nguyen et al., 1998). *col* embryos

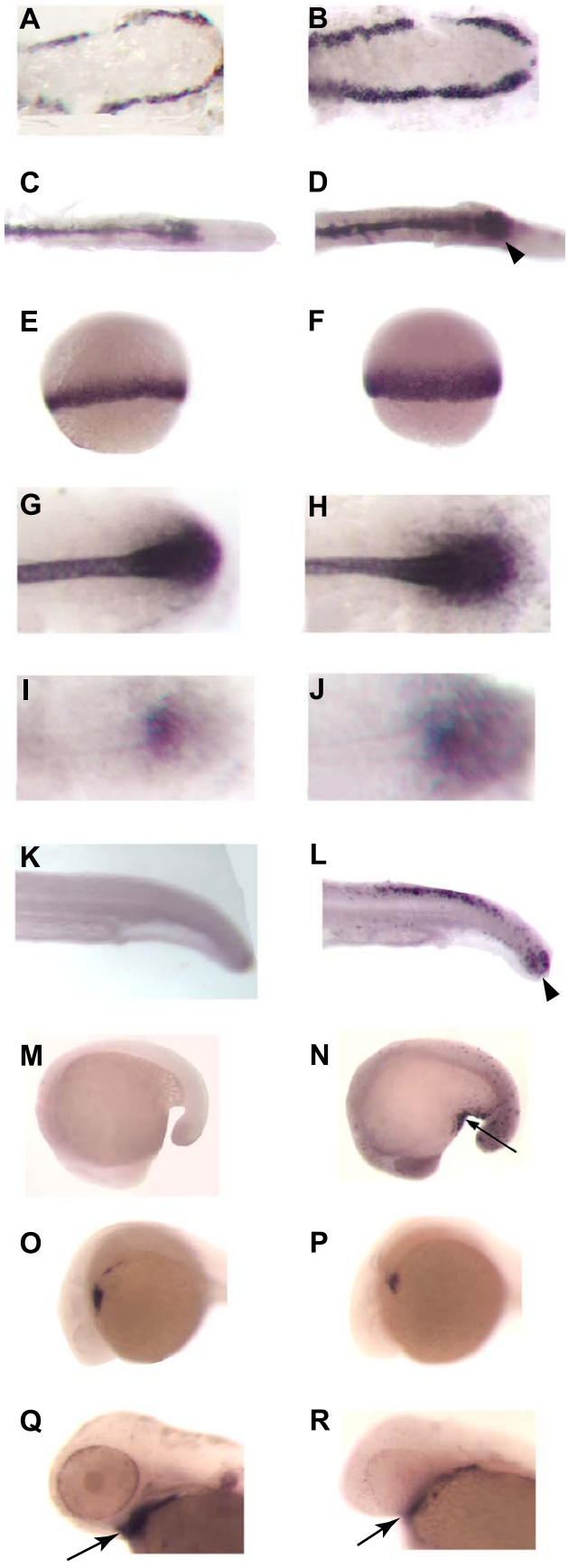

Fig. 3. *col* mutant embryos display an increase ventral and posterior mesoderm-derived cells including blood and tail bud cells as well as a decrease in dorso-anterior mesoderm-derived cardiac mesoderm. (A–D) *gata1* is expressed by hematopoietic precursor cells of 10- (A) and 26- (C) somite-stage wild-type embryos. At the same developmental stages, there are increased numbers of *gata1*-expressing cells in *col* mutants (B,D). The arrowhead in D marks the large number of blood cells in the region of the anus in *col* embryos. (E,F) At 60% epiboly (7 hpf), *ntl* is expressed in the ventrolateral mesoderm along the blastoderm margin of wild-type embryos (E). *ntl* expression is expanded in *col* mutant embryos (F). *ntl* at the 10-somite stage (G,H) and *wnt8* at the 3-somite stage (I,J) are expressed by mesodermal cells of the tail bud. Increased expression of both *ntl* (H) and *wnt8* (J) in the tail bud mesoderm is observed in *col* mutant embryos. Twenty-six-somite- (K) and 16-somite- (M) stage, wild-type embryos showing apoptotic cells revealed by TUNEL. The increase in the number of tail bud cells in *col* mutants is accompanied by an increase in the number of apoptotic cells in the tail (L). The arrow marks the TUNEL -positive cells in the tail of *col* mutants. (M,N) Lateral views of wild-type (M) and *col* mutant (N) embryos processed for TUNEL with arrow (N) indicating apoptotic cells ventral to the forming yolk extension in mutant embryos. (O,P) Lateral views of cardiac mesoderm *nkx2.5* expression in 24 hpf wild-type (O) and *col* mutant (P) embryos reveal a reduction in the expression domain in *col* mutant embryos. (Q,R) *dHAND* expression in the developing heart (arrows) in 48 hpf wild-type (Q) and *col* mutant embryos (R) reveals decreased expression in *col* mutant embryos.

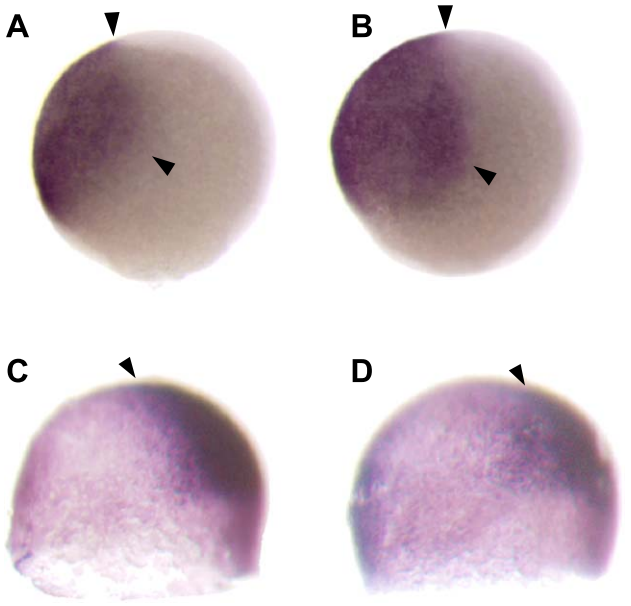

Fig. 4. *col* mutants display an expansion of non-neural ectoderm and a reduction in neural tissue during late gastrulation. (A,B) At 80% epiboly (8.5 hpf; A; lateral view), *gata2* is expressed by non-neural ectoderm in wild-type embryos. The domain of *gata2* expression is expanded dorsally and anteriorly in *col* mutants (B). Arrowheads in A and B denote limits of the *gata2* expression domains. (C,D) At 75% epiboly (8 hpf; C; lateral view), *otx2* is expressed by the forebrain and midbrain anlage of the neuroectoderm in wild-type embryos (C). In *col* mutants (D), *otx2* expression is reduced and the anterior border of the expression domain (arrowheads) does not reach the animal pole.

display a dorsal expansion of *bmp2* expression (Figs. 2I,J). Similarly, *bmp4* expression is also dorsally expanded in *col* mutants (Figs. 2K,L). The expression of *wnt8*, which is restricted to the ventrolateral blastoderm margin and is excluded from the dorsal midline at mid-gastrulation in wild-type embryos (Kelly et al., 1995), expands into the dorsal midline in *col* mutants. (Figs. 2M,N). At 60% epiboly, *ntl* is expressed by the ventrolateral mesoderm in addition to the dorsal notochord. We also observe an expansion of *ntl* expression along the ventrolateral margin in *col* mutants (Figs. 3E,F). Hence, in *col* mutants, the reduction in the expression of shield-specific genes at the dorsal midline is accompanied by an expansion of ventrolaterally expressed genes.

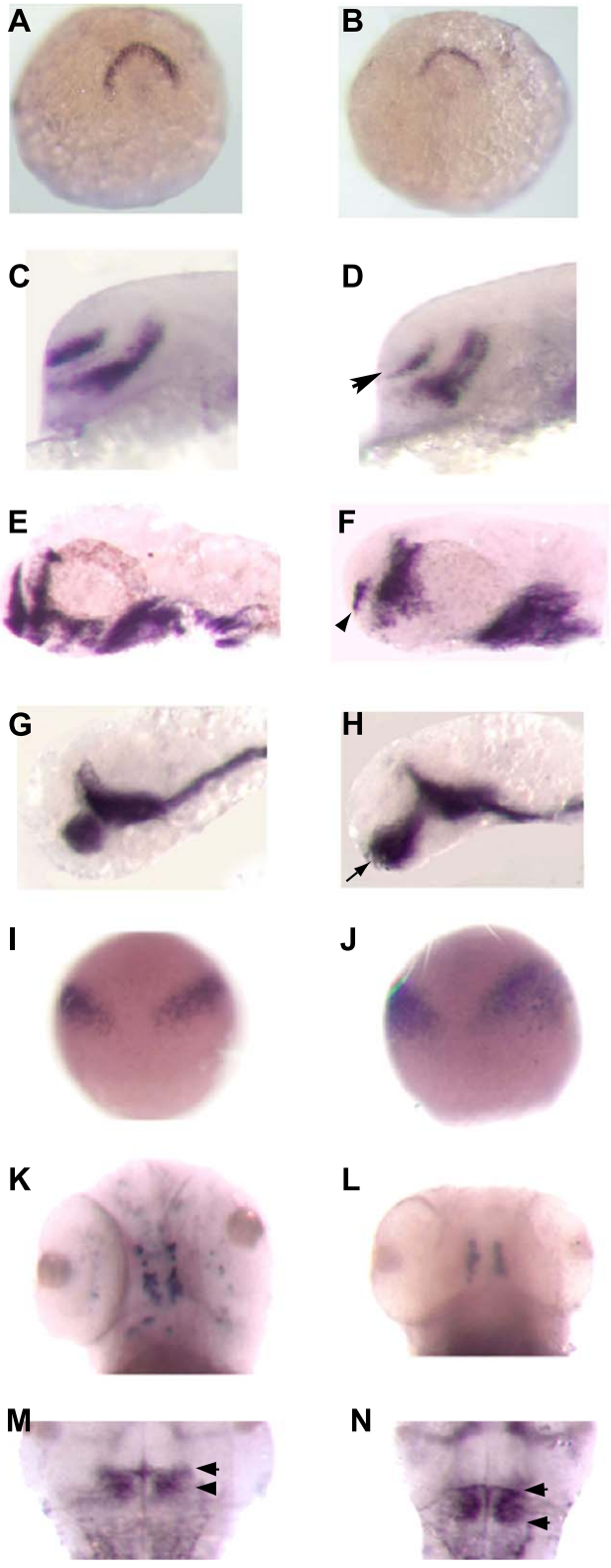

Fig. 5. *col* mutants display regionalized posteriorization within the anterior neuroectoderm. (A,B) *anf* is expressed along the anterior edge of the neuroectoderm in wild-type embryos (A; dorsal view) at tailbud stage and *anf* expression is decreased in *col* mutant embryos (B). (C–F) The telencephalic stripe of *dlx2* expression in a lateral view of a (C,D) 24 hpf and (E,F) 40 hpf *col* mutant (D,F arrowhead) is reduced compared to wild-type (C,E). (G,H) Lateral views of ventral diencephalic expression of *shh* in 32 hpf wild-type (G) and *col* mutant (H) embryos illustrates the anterior expansion of *shh* expression (arrowhead in H) in *col* mutant embryos. (I,J) At 90% epiboly, *wnt8b* is expressed at the MHB in wild-type embryos (I). *wnt8b* expression is expanded in *col* mutants (J). (K,L) The number of *tyrosine hydroxylase*-expressing dopaminergic neurons of the midbrain is reduced in *col* mutant (L) embryos as compared to wild-type (K) embryos at 3 dpf. (M,N) *pax2.1* expression at the MHB in 48 hpf wild-type (M) and *col* mutant (N) embryos. Arrowheads indicate the expanded anterior–posterior extent of *pax2.1* expression in *col* mutant embryos.

In addition to the increase in the expression of ventral markers, we observed an excessive development of ventral and posterior structures in *col* mutants. For example, the blood islands appear to contain many more cells as compared to wild-type embryos. The expression of *gata1*, which is expressed in hematopoietic precursor cells (Detrich et al., 1995), is increased in *col* mutant embryos at the 10-somite and 22-hpf stages (Figs. 3A–D). We also observed an increase in the number of cells in the tailbud. The tailbud expression of *wnt8* at the 3-somite stage and *ntl* expression at the 10-somite stage are increased in *col* mutant embryos (Figs. 3G–J). Interestingly, results using the TUNEL assay for apoptotic cell death revealed a large number of cells undergoing apoptosis in this region of *col* mutants (Figs. 3K,L). Also, at the 16-somite stage, the TUNEL assay revealed a high degree of cell death in a population of cells anterior to the anus on the ventral side of the yolk extension in *col* mutants (Figs. 3M,N). A similar phenotype has been observed in the ventralized mutants *chordino* and *mercedes* (Hammerschmidt et al., 1996). Therefore, *col* mutants exhibit an expansion of ventral and posterior structures and this ventralized phenotype is likely due to a reduction in dorsalizing signals and a concomitant expansion of ventral signals. Consistent with this possibility, we also observed a reduction in dorso-anterior-derived cardiac mesoderm. Specifically, we observed a reduction in the expression domain of *nkx2.5* (Lee et al., 1996) at 24 hpf (Figs. 3O,P) and a more drastic reduction at 48 hpf of *dHAND* expression (Figs. 3Q,R; Angelo et al., 2000; Yelon et al., 2000).

Non-neural ectoderm fates are expanded in col mutant embryos

BMPs are ventralizing signals that have been shown to promote non-neural epidermis in the ectoderm (Kishimoto et al., 1997; Neave et al., 1997; Nguyen et al., 1998; Wilson and Hemmati-Brivanlou, 1995). The observed increases in the expression of *bmp2* and *bmp4* in *col* mutants could result in a decrease in neural induction and an expansion of the epidermis. To determine whether this occurs, we examined the development of the prospective non-neural ectoderm by determining the expression of *gata-2*. The expression of *gata-2* is normally restricted to the ventrolateral half of the ectoderm of wild-type embryos (Detrich et al., 1995) but expands into more dorsal and anterior parts of the ectoderm in *col* mutant embryos (Figs. 4A,B). To determine if neural development is abnormal, we examined the expression of *otx2*. *otx2* is expressed in a triangular domain that extends to the animal pole during the early gastrula stages in wild-type embryos (Li et al., 1994). The anterior edge of *otx2* expression in *col* embryos shifts posteriorly and does not extend to the animal pole, indicating a reduction in the anterior forebrain anlage (Figs. 4C,D). These results suggest that *col* normally functions to limit the ventralization

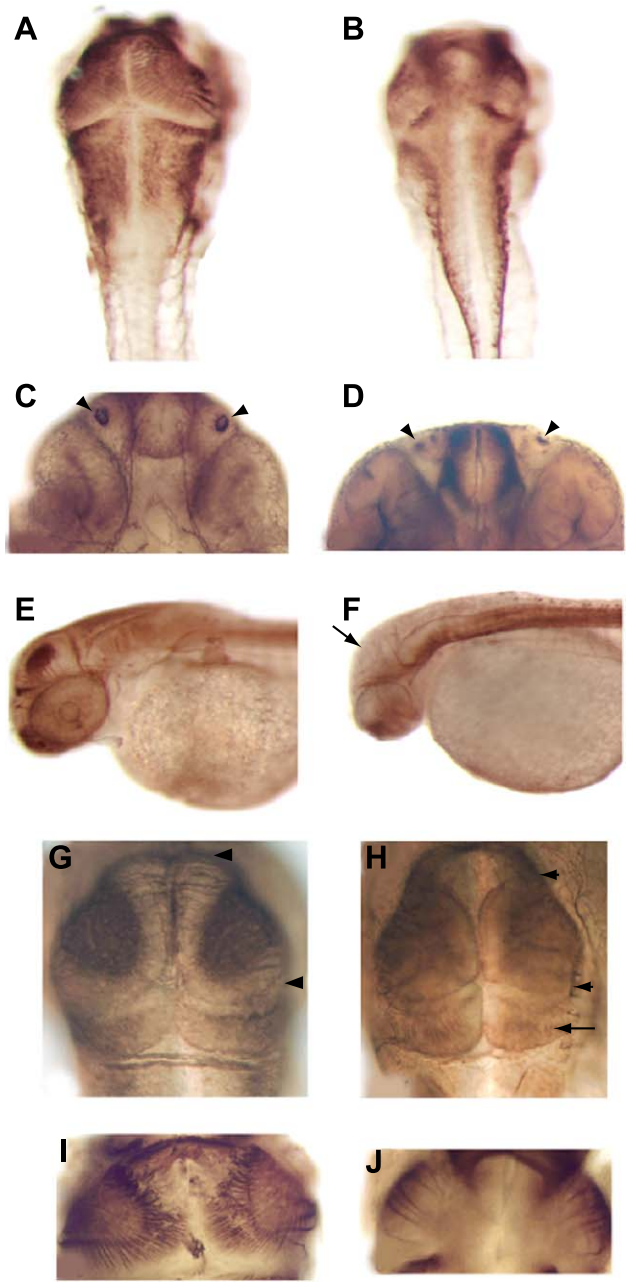

Fig. 6. *col* mutant embryos display a reduction of neural tissue and regional posteriorization of the neuroectoderm. (A,B) Dorsal views of wild-type (A) and *col* mutant (B) embryos at 2 dpf labeled with zn5 antibody. The developing brain in *col* mutants appears laterally reduced (B). (C,D) Ventral views of the heads of wild-type (C) and *col* mutant (D) embryos at 2 dpf labeled with anti-acetylated tubulin antibody with arrowheads indicating the olfactory organs that are reduced in size in *col* mutants. (E,F) Lateral views of wild type (E) and *col* mutants (F) labeled with anti-acetylated tubulin antibody at 2 dpf. Arrow in (E) marks staining in the tectum of wild-type embryos. A dramatic reduction in staining in seen in *col* mutants (F). (G,H) Dorsal views of wild-type (G) and *col* mutant (H) embryos at 48 hpf labeled with zn12 antibody with arrowheads marking the anterior–posterior extent of the tectum. The size of the tectum is reduced in *col* mutants whereas the cerebellum is larger (arrow in H). (I,J) A drastic reduction in tectal projections is evident in 2 dpf *col* mutants (J) labeled with zn5 antibody as compared to wild type (I).

of the ectoderm and to permit the specification of neural fates.

col mutants display regional posteriorization of the neural plate

We next sought to understand the nature of the patterning defects within the neural tissue of *col* mutants. We examined the general morphology of the brain by staining embryos at 2 dpf with antibodies to acetylated tubulin. The neural tissue in *col* mutant embryos appeared to be laterally reduced, giving an overall appearance of being narrower as compared to wild-type embryos (Figs. 6A,B). More strikingly, staining in the telencephalic region of the forebrain and the tectal lobes of the midbrain was reduced (Figs. 6E,F). These observations lead us to speculate that there might be regional patterning disruptions within the developing brain of *col* mutants. To assess this, we examined genes expressed in distinct domains within the forebrain including the anterior telencephalon and posterior diencephalon. The expression of an early marker, *anf* (Kazanskaya et al., 1997), which is expressed along the anterior edge of the neuroectoderm, is reduced in *col* mutant embryos as compared to wild-type siblings (Figs. 5A,B). The loss of anterior fates is more pronounced later in development. We observed a severe decrease in the telencephalic expression of *dlx2* (Figs. 5C–F; Akimenko et al., 1994) and *islet-1* (not shown; Inoue et al., 1994; Korzh et al., 1993) at later stages. This was accompanied by a reduction in anterior structures such as the olfactory organs (Figs. 6C,D) and neural retinal tissue (not shown). In contrast, a subtle anterior expansion of *shh* expression in the diencephalon was evident at the 10-somite stage (not shown) that becomes more pronounced at later stages (Figs. 5G,H). Therefore, in *col* mutant embryos, the loss of anterior telencephalic fate is accompanied by the rostral expansion of posterior diencephalon.

In addition to the patterning defects in the forebrain, abnormalities within the midbrain region were also observed. *col* embryos labeled with zn-12 antibody (Trevarrow et al., 1990) showed a clear decrease in the size of the tectal lobes (Figs. 6G,H). A decrease in staining in the midbrain region is seen in *col* embryos stained with acetylated tubulin antibody at 2 dpf (Figs. 6E,F). Examination of tectal axon projections using the zn-5 antibody (Trevarrow et al., 1990) revealed a drastic reduction in *col* mutants as compared to wild-type siblings (Figs. 6I,J). Neuronal populations found in the midbrain such as dopaminergic neurons (defined by *tyrosine hydroxylase* expression; Figs. 5K,L) are significantly reduced. Staining with zn-12 and acetylated tubulin antibodies also revealed that while the tectum appeared reduced, the midbrain–hindbrain boundary (MHB), including the cerebellum, was expanded along the AP axis (Figs. 6G,H). These morphological changes are accompanied by changes in the expression of genes at the MHB. At 90% epiboly *wnt8b* (Kelly et al., 1995) expression in the cells of the prospective caudal diencephalon and MHB region is expanded in mutant embryos (Figs. 5I,J). In contrast, the expression of *wnt1*, another gene involved in MHB development (Kelly and Moon, 1995), is not significantly altered in *col* mutants as compared to wild-type siblings (data not shown). *col* embryos also display an expansion in *pax2* expression at the MHB (Krauss et al., 1991) at 48 hpf (Figs. 5M,N). A similar expansion in *fgf8* expression at the MHB (not shown; Reifers et al., 1998) was also observed. Examination of *pax2* expression at earlier stages revealed a subtle expansion in expression at the MHB in *col* mutant embryos (not shown) that becomes more obvious later in development (Figs. 5M,N). These results are similar to those observed in the forebrain, in that the posterior tissue,

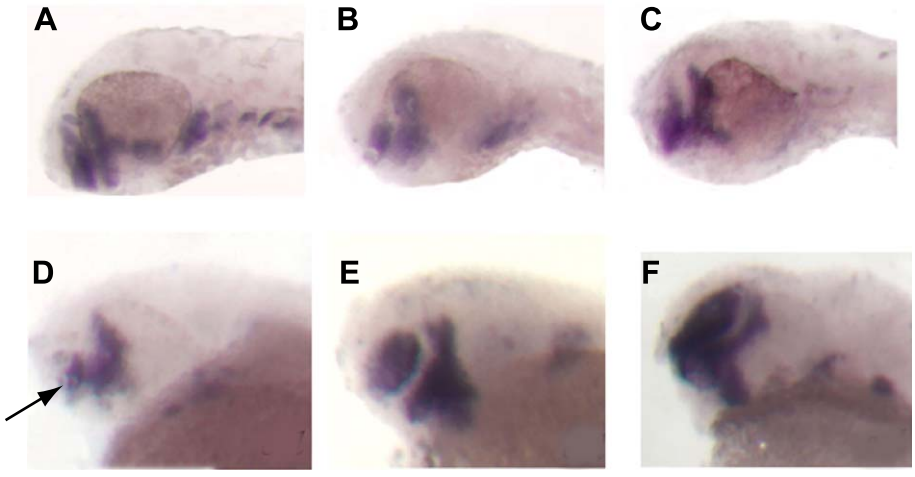

Fig. 7. Expression of Wnt pathway antagonists partially rescue the *col* mutant phenotype. (A–C) The telencephalic stripe of *dlx2* expression is reduced in *col* mutants (B) as compared to wild-type embryos (A) at 48 hpf. *dlx2* expression is restored in *col* mutants injected with *gsk-3β* mRNA (C). Telencephalic expression of *dlx2* is restored in *col* embryos injected with *dkk1* mRNA (E,F) compared to uninjected *col* mutants (D) at 40 hpf.

the cerebellum, expands at the apparent expense of anterior tectal tissue (see Figs. 6G,H). In addition to this, we measured the distance between the anterior tip of the forebrain and the MHB in *col* embryos at 40 hpf and compared it to the same measurement of wild-type embryos. We found that this distance is not altered in *col* embryos, suggesting that both the reduction in the telencephalon and expansion of the diencephalon in the forebrain region and the decrease in tectal tissue and expansion of the cerebellum in the midbrain region occur reciprocally. Importantly, the levels of cell death in neural tissue throughout development in *col* mutant and wild-type embryos as assayed by the TUNEL method were indistinguishable (not shown). Based on these data, we hypothesized that *col* function is required to establish regional fates along the AP axis of the developing zebrafish brain and that the disruption of *col* function results in a regionalized posteriorization of the neuroectoderm.

Antagonists of Wnt signaling suppress the col mutant phenotype

Studies have implicated the Wnt signaling pathway as well as FGFs and retinoids in patterning neural tissue along the AP axis (Erter et al., 2001; Lumsden and Krumlauf, 1996; McGrew et al., 1995, 1997; Yamaguchi, 2001). Consistent with this, zebrafish mutants such as *hdl*, *mbl*, and *boz*, all of which show defects in anterior neural development, are mutations in genes that antagonize Wnt signaling (Fekany et al., 1999; Heisenberg et al., 2001; Kim et al., 2000). *col* mutants also show a similar loss of anterior neural structures (Figs. 5A–F and 6C–F), an expansion of *wnt8* expression (Figs. 2M,N) and a downregulation of the Wnt antagonist, *dkk1* (Figs. 2G,H and not shown). These data suggest that excess Wnt signaling may contribute to the manifestation of the *col* mutant phenotype and led us to test whether the inhibition of this signaling pathway might then suppress the *col* mutant phenotype.

Shinya et al. (2000) have shown that injection of *dkk-1* RNA into wild-type embryos results in the formation of larger telencephalon and eyes. We injected *dkk-1* RNA into embryos at one- to eight-cell-stage and allowed them to develop until 40 hpf. These embryos were then tested with appropriate molecular markers to analyze patterning within the brain. Subsequent to each experiment, embryos were genotyped to distinguish mutant and wild-type embryos. Forebrain expression of *dlx2* was analyzed, and of the *col* mutant embryos examined, 90% ($n = 32$) showed a dramatic increase in the telencephalic expression of *dlx2*, to levels comparable to that of uninjected wild type embryos (Figs. 7D–F). Thus, *dkk-1* overexpression is able to rescue the loss of anterior telencephalic fates in *col* mutants. To test if other downstream negative regulators of Wnt signaling can ameliorate the mutant phenotype, we injected either *gsk-3β* (Figs. 7A–C; Pierce and Kimelman, 1995), a protein kinase that phosphorylates β-catenin and targets it for degradation, or *tcf3* RNA (not shown; Kim et al., 2000), a transcriptional repressor into one- to eight-cell-stage embryos. In situ hybridization with *dlx2* revealed that normal telencephalic development was restored in 75% ($n = 25$) of injected *col* mutant embryos (Figs. 7A–C).

Other signaling molecules such as Nodals and FGFs have also been shown to play an important role in neural posteriorization (Donaich, 1995; Gamse and Sive, 2000).

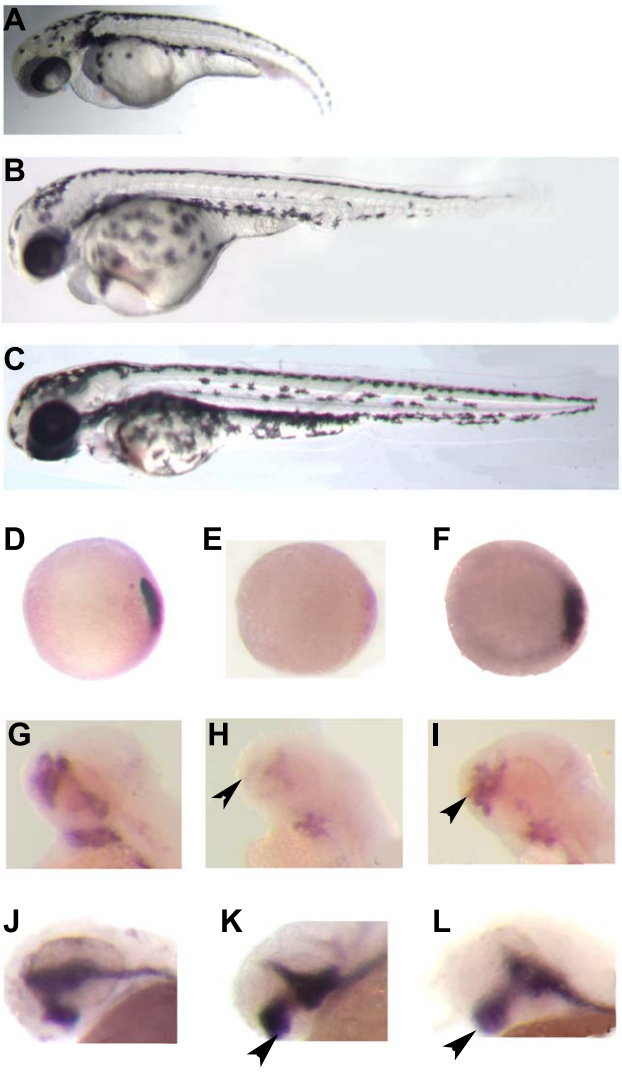

Fig. 8. Antisense morpholino-mediated knockdown of *wnt8* rescues the shield and forebrain phenotypes of *col* mutant phenotype. (A–I) An uninjected *col* mutant embryo at 3 dpf (A), a *col* mutant embryo injected with *wnt8* MO (B) and an uninjected wild-type embryo (C) at 3 dpf. *gsc* expression in wild-type embryos (D) at shield stage (6 hpf) is reduced in *col* mutants (E, see Figs. 3A,B). *gsc* expression is restored in *col* mutants injected with *wnt8* MO (F). *dlx2* expression in the telencephalon (arrowhead) of injected *col* embryos at 48 hpf is rescued (I) as compared to uninjected mutants (H) and uninjected wild-type embryos (G). Expanded diencephalon revealed by *shh* expression (arrows) still persists in injected *col* mutants embryos at 40 hpf (L) as in uninjected mutant embryos (K) and unlike uninjected wild-type embryos (J).

We tested their role in the development of the *col* mutant phenotype by overexpressing *XFD* (Amaya et al., 1991), a dominant-negative FGF receptor and the nodal antagonist *antivin* (Thisse et al., 1999). Neither of these genes exhibited phenotype rescue activity when expressed in *col* mutant embryos (not shown). Also, a decrease in BMP activity in zebrafish embryos has been shown to cause an expansion of anterior neural fates (Barth et al., 1999; Nguyen et al., 1998). Therefore, we tested if *chordin* (Miller-Bertoglio et al., 1997), a BMP antagonist, is able to rescue *col* mutants. We did not see a significant rescue although *chd* injected *col* embryos did not show the expanded blood islands and dorsal expansion of *gata2* expression seen in uninjected *col* mutant embryos (not shown). Hence, inhibition of Wnt signaling is able to suppress the *col* mutant phenotype, indicating that *col* function selectively and negatively regulates Wnt signaling.

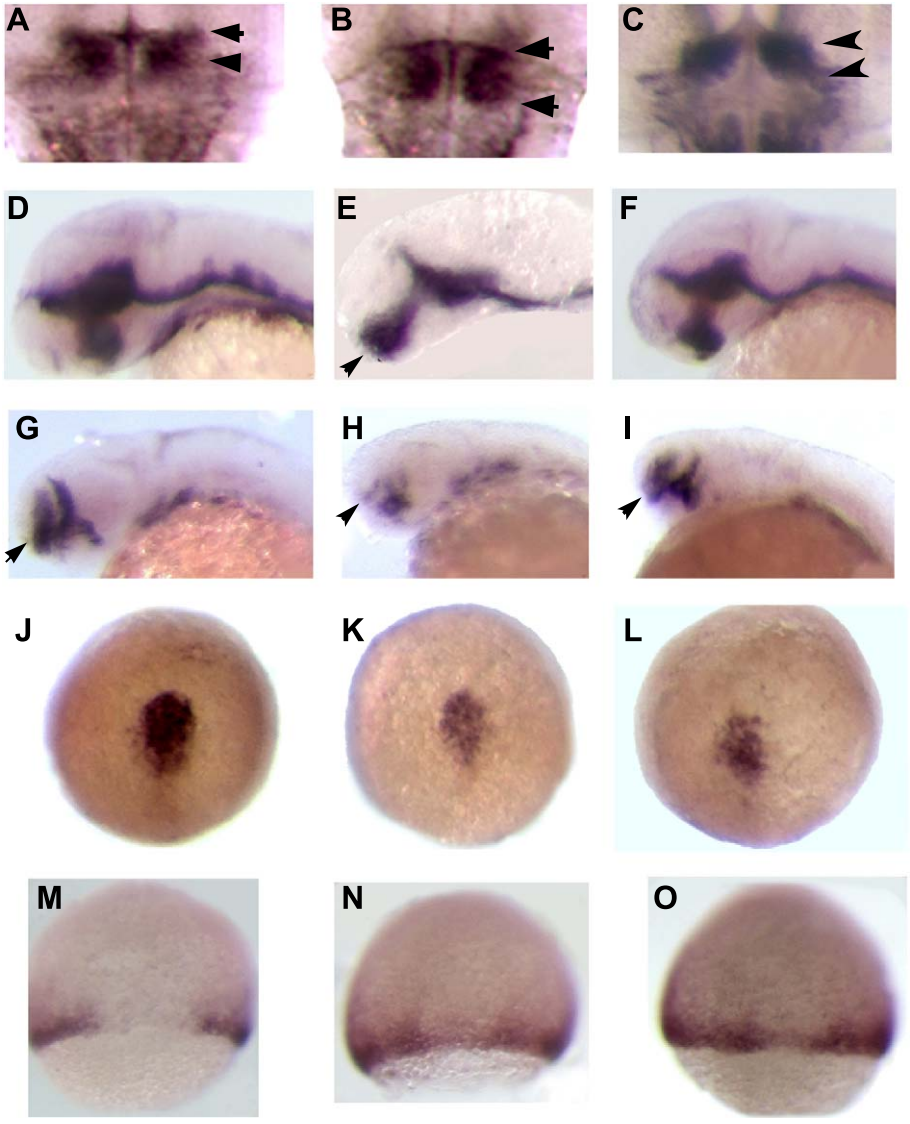

Fig. 9. Antisense morpholino-mediated knockdown of *wnt8b* rescues regional patterning defects in the brain of *col* embryos. (A–I) The midbrain–hindbrain boundary region marked by *pax2* expression is expanded in *col* mutants (B) as compared to wild-type embryos at 40 dpf (A). Injection of *wnt8b* MO results in a reduction in MHB tissue in mutant embryos (C). The extent of the MHB region is shown with arrowheads. *wnt8b* MO injection of mutant embryos abolishes ectopic diencephalic *shh* expression (F) observed in uninjected *col* embryos at 32 hpf (E, arrow; see Figs. 8G,H). Injected mutant embryos (F) strongly resemble uninjected wild-type embryos (D). The reduced telencephalic *dlx2* expression in uninjected *col* embryos (H; see Figs. 8E,F) is rescued in injected embryos (I, compare to wild type, G). The telencephalic stripe of *dlx2* expression is marked with arrows. Early patterning events during gastrulation are unaffected in *wnt8b* morpholino-injected *col* mutants (J–O). *gsc* expression is down-regulated in the prechordal plate in uninjected (K) and injected (L) *col* embryos as compared to wild-type embryos at 70% epiboly (J). *wnt8* expression is ectopically expressed in the dorsal midline in uninjected (N) and injected (O) *col* mutant embryos unlike in wild-type embryos (M) at 70% epiboly.

Abrogation of Wnt8 and Wnt8b function rescues distinct aspects of the col mutant phenotype

Because antagonists of Wnt signaling were found to suppress aspects of the *col* mutant phenotype and because Wnt8 function has been implicated in the development of tissues affected by the *col* mutation (Erter et al., 2001), we specifically inhibited Wnt8 function by injecting *wnt8* morpholino (MO) directed to ORF1. Injection of this *wnt8* MO has been previously shown to cause a loss of posterior neural fates with an expansion of anterior fates in zebrafish embryos (Erter et al., 2001). Embryos were injected at the one- to four-cell stage and were genotyped to identify mutants. Some injected embryos were allowed to develop until 3 dpf and approximately 70% (n = 36) of *col* mutant embryos injected with *wnt8* MO displayed a significant rescue of the mutant phenotype including grossly normal head development (Figs. 8A–C). We also performed RNA in situ hybridization to look at the expression of *dlx2* in the telencephalon, *gsc* in the shield, and *gata2* in the ectoderm. Shield expression of *gsc* was restored to wild-type levels in 80% (n = 26) of injected *col* mutants (Figs. 8D–F). Seventy-five percent (n = 30) of injected *col* mutant embryos displayed rescue of the telencephalon based on *dlx2* expression (Figs. 8G–I). Also, dorsal expansion of *gata2* in the ectoderm was not observed in the injected mutants (not shown). However, although the injected *col* mutant embryos displayed a significant rescue of anterior neural tissue development, regional patterning defects within the anterior brain persisted. For example, the expanded ventral diencephalon, based on *shh* expression, still remained in the injected *col* embryos (Figs. 8J–L).

Recent studies in zebrafish have implicated Wnt8b in playing an important role in regional patterning within the anterior neuroectoderm (Houart et al., 2002; Kim et al., 2002). To test the idea that the increased *wnt8b* expression observed in *col* mutant embryos (Figs. 5G,H) may contribute to the development of some aspects of the *col* mutant phenotype, we injected *col* mutants with antisense MOs directed against *wnt8b*. In situ hybridization was performed on injected embryos at 70% epiboly using *gsc* and *wnt8* and at 40 hpf using *dlx2* and *shh*. We did not observe a rescue of the shield based on *gsc* expression in injected *col* mutants (Figs. 9J–L). Likewise, injected *col* embryos also retained ectopic *wnt8* expression in the dorsal midline (Figs. 9M–O). However, a striking rescue of the telencephalon (*dlx2* expression; 60% n = 25) as well as amelioration of expanded diencephalon (*shh* expression; 50% n = 26; Figs. 9D–I)

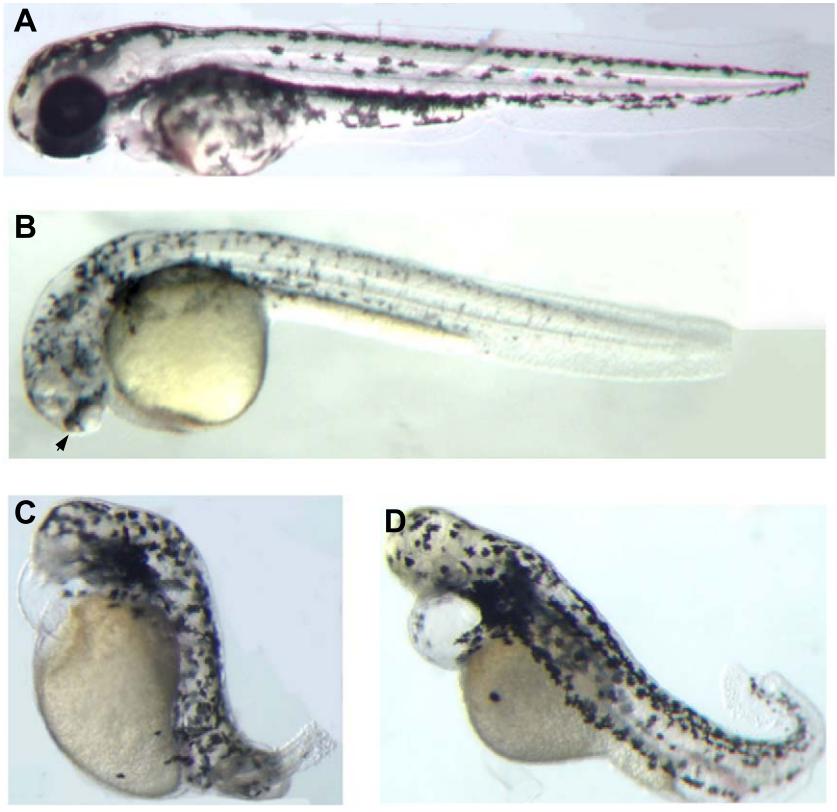

Fig. 10. Expression of dominant-negative forms of negative regulators of Wnt signalling enhances the *col* mutant phenotype. (A–D) Injection of dominant-negative *Xgsk-3β* results in a partial loss of anterior tissue and reduced eyes in wild-type embryos (B) compared to uninjected wild-type embryos (A). The arrowhead marks a reduced, cyclopic eye in an injected wild-type embryo. Expression of dominant-negative *axin* (GID-2 fragment; C) and dominant-negative *Xgsk-3β* (D) in *col* mutant embryos results in a complete loss of eyes and anterior tissue. All embryos are 48 hpf.

was observed in the injected *col* mutants. Also, examining live, injected *col* embryos at 3 dpf revealed a clear reduction in tissue in the region of the posterior MHB (not shown). Consistent with this, we do not observe the expanded posterior *pax2* expression in *wnt8b* MO-injected *col* mutants (60% $n = 20$; Figs. 9A–C). Taken together, these results suggest that while reducing excess Wnt8 activity in *col* mutants is able to rescue early dorsoventral patterning defects and permit normal anterior forebrain tissue development, the regional patterning defects in the brain but not earlier patterning events are selectively rescued by reducing excess Wnt8b activity.

Activation of the Wnt pathway enhances the col mutant phenotype

Since the overexpression of negative regulators of the Wnt signaling pathway as well as inhibition of Wnt8 and Wnt8b activities was able to rescue the *col* mutant phenotype, we tested if the converse also holds true. We injected a dominant-negative form of *Xgsk-3β* (Pierce and Kimelman, 1995) and a form of *Xaxin* (GID2) that is able to bind Gsk-3β and inhibit it (Hedgepeth et al., 1999). Both of these RNAs caused phenotypes such as reduction in the size of the eyes, loss of one eye or cyclopia in wild-type embryos (Fig. 10B). In *col* mutant embryos, however, both caused more dramatic defects compared to uninjected mutant embryos and wild-type embryos injected with the same concentration of each construct. Injected *col* mutant embryos were characterized by the complete loss of both eyes as well as the anterior region of the head (Figs. 10C,D). Hence, activation of the Wnt pathway in *col* mutants greatly enhances the loss of anterior neural structures like the forebrain and eyes. These results further lend support to the idea that there is an overactivation of Wnt signaling in *col* mutants.

Discussion

col function is required to inhibit Wnt signaling

The precise regulation of the Wnt signaling pathway is essential for normal development. This is evidenced by the catastrophic consequences in development that occur in response to abnormal Wnt-signaling and the presence of numerous levels at which this pathway is controlled, including inhibition. For example, Axin2 has been implicated in feedback repression of the Wnt signal (Leung et al., 2002). Also, extracellular molecules such as secreted Frizzled Related Proteins, Dickkopf-1, and Cerberus have been shown to antagonize Wnt signaling (Glinka et al., 1998; Leyns et al., 1997; Piccolo et al., 1999). Intracellular signaling proteins such as Gsk-3β, Axin, and Adenomatous Polyposis Coli (APC), all of which are involved in forming a complex with β-catenin and targeting it for degradation, form another set of molecules implicated in negatively regulating Wnt signaling (Barker and Clevers, 2000; Kim et al., 1999). These and other regulators together provide very precise control over how Wnt signaling is deployed. In addition to the essential roles of the inhibition of Wnt-signaling in development, when the Wnt pathway is deregulated in humans the consequences are severe and often lead to mortality. For example, mutations in the human orthologue of APC leads to colorectal cancer (Bienz and Clevers, 2000; Polakis, 2000), while the AXIN1 gene has been reported to be mutated in hepatocellular carcinomas and medulloblastomas (Dahmen et al., 2001; Satoh et al., 2000).

Our studies suggest that *col* is required to specifically negatively regulate Wnt signaling. Many aspects of the *col* mutant phenotype such as defects in early dorsoventral patterning of the embryo and anterior–posterior patterning of the neuroectoderm are consistent with previously reported defects resulting from excess Wnt signaling. Accordingly, the injection of negative regulators of Wnt signaling are able to partially rescue the *col* mutant phenotype. Further, by reducing early and later Wnt signaling events using *wnt8* and *wnt8b* morpholinos, we are able to rescue distinct aspects of the *col* mutant phenotype. Thus, although it is also possible that *col* defines the expression territories of other genes such as *wnt8* and *bmp4*, the phenotype rescue activity of *wnt8* and *wnt8b* morpholinos is consistent with *col* regulating the Wnt signaling pathway itself.

In addition to the upregulation of Wnt activity, *col* mutants also exhibit ectopic expression of *wnt8* in the dorsal midline where it is normally not expressed. Expression of *wnt8b* is also increased in *col* mutants. How the *col* mutation results in the increased expression of both *wnt8* and *wnt8b* is not yet clear, but an interesting possibility is that *col* might function as a transcriptional repressor of *wnt8* and *wnt8b*. However, we cannot rule out a myriad of potential indirect influences of the *col* mutation on *wnt* expression and activity ranging from disruption of shield development to alterations in the expression of other genes, including *dkk1* (Hashimoto et al., 2000). It is also possible that *col* directly inhibits Wnt activity by disrupting ligand–receptor interactions or intracellular signaling. Identification and analysis of the gene whose function is disrupted by the *col* mutation will be critical to evaluating these and other potential mechanisms of *col* function.

col plays a role in the specification of dorsoventral fates

col mutants display a mild ventralized phenotype. They possess reduced neural tissue, an accumulation of cells in the blood island and in the tailbud region and also show excess cell death in the ventral yolk region. Ventralized mutants have enlarged tailbuds due to the inability of these cells to engage in normal convergence and extension movements (Myers et al., 2002). Conversely, dorso-anterior-

derived cardiac mesoderm is reduced. Similar phenotypes are observed in other ventralized mutants such as *chordino* and *mercedes* (Hammerschmidt et al., 1996). These defects are apparent at gastrulation with reductions in the expression of dorsally expressed genes in the ectoderm and shield-specific genes. In contrast, there is an expansion of the expression of ventralizing signals. Ventralization of the ectoderm results in a loss of neural tissue and an expansion of epidermal cell fates. This raises the possibility that *col* is involved in limiting the effects of ventralizing factors, such as BMPs, in the ectoderm. Our results, however, suggest that the effect on BMPs is likely to be indirect since the overexpression of *chordin* does not result in a significant rescue shield or neural patterning phenotypes of *col* mutant embryos, although a partial rescue of mesodermally derived tissues was observed. Therefore, we support the hypothesis that the effect on BMPs is a secondary consequence of the disruption of organizer development in *col* mutants since the shield is a source of BMP antagonists (reviewed in Graff, 1997).

Although very early in development the Wnt pathway is involved in inducing organizer formation, at late blastula stages Wnt signaling inhibits organizer development (Hoppler et al., 1996). This is supported by studies with *boz* mutants, where it has been demonstrated that inhibition of Wnt signaling by *boz* is required for maintaining induced organizer tissue (Fekany-Lee et al., 2000). Organizer induction in *col* mutants was found to be normal whereas maintenance of organizer tissue was defective. The overactivation of Wnt signaling at late blastula stages in *col* mutants might then explain the disruption of organizer development, resulting in decreased levels of organizer-specific genes such as *gsc*. This explanation is supported by our analysis where we have demonstrated that blocking excess Wnt8 activity in *col* mutants using *wnt8* MO is able to rescue the organizer phenotype. Hence, these results indicate that *col* functions to limit the activity of Wnt8 and maintain organizer fate either directly or through interactions with other molecules. It is also important to note here that our results suggest that *boz* acts upstream of *col* since overexpression of *boz* is unable to rescue the *col* mutant phenotype and *boz* expression is unaffected in *col* mutants.

col inhibition of Wnt signaling limits global and regional posteriorization of the neuroectoderm

col mutants display truncated development of anterior neural structures. This phenotype has been consistently observed in most mutants for components of the Wnt pathway. *boz* mutants show defects in the development of forebrain and midbrain (Fekany-Lee et al., 2000); *hdl* mutants completely lack eyes, the forebrain, and part of the midbrain (Kim et al., 2000); and *mbl* mutants have reduced or absent eyes and telencephalon (Heisenberg et al., 2001). However, a striking aspect of the posteriorized phenotype in the brain of *col* mutants is that the expansion of posterior cell fates occurs within regional subdivisions of the prospective brain. So, within the forebrain, we observe a reduction in telencephalic fates and an expansion of the more caudal diencephalon; in the midbrain–hindbrain region, there is a drastic reduction in the midbrain and an accompanying expansion of the cerebellum. Although a similar phenotype has been noted in the case of *mbl*, the regionalized posteriorization in this mutant is restricted to the forebrain region (Heisenberg et al., 2001).

Transplantation experiments done by Woo and Fraser (1997) suggest that planar signals from lateral mesendodermal cells are responsible for posteriorization of the neuroectoderm and data from studies done by Erter et al. (2001) indicate that this signal is or requires Wnt8. They also propose that specification of cell fates along the neuraxis is controlled by graded activity of Wnt8. Hence, in the more anterior regions of the brain anlage, there is high activity of Wnt antagonists that block Wnt8 activity and therefore allows specification of forebrain fates, while caudally along the neuraxis Wnt8 activity progressively increases and cells take on more posterior neural fates. This then divides the neuraxis broadly into forebrain, midbrain, and hindbrain regions (see Nieuwkoop et al., 1952).

Subsequent to the initial patterning of the neural plate, these territories are further subdivided by local patterning events. In a recent study, Houart et al. (2002) identified *tlc*, a Wnt antagonist expressed at the anterior neural border. *tlc* promotes the development of the telencephalon by antagonizing the effects of a later acting Wnt ligand,Wnt8b, expressed in the caudal diencephalon and at the MHB (Kelly et al., 1995). They propose that the local activation of genes by the early Wnt8 signal within the already specified territories regulate regional patterning. Thus, Wnt8 is responsible for specifying the initial positional values along the neuroectoderm and regional patterning would involve a further refinement of these AP positional values by the activation of later acting Wnt ligands, such as Wnt8b, Wnt antagonists, and interactions between these genes. Our characterization of *col* is consistent with these results since we are able to restore anterior forebrain development with *wnt8* MO, and rescue the regional patterning defects by blocking excess Wnt8b activity.

Taken together, our results suggest a dual role for *col*. First, *col* plays an early role in promoting the specification of dorso-anterior cell fates by antagonizing Wnt8 activity. Second, *col* plays a later role in regional patterning of the neuroectoderm by interfering with the posteriorizing activity of late acting Wnts such as Wnt8b. These studies, therefore, suggest that *col* function may provide a critical link integrating early and late Wnt signaling activity during the establishment of the vertebrate body plan. In addition, given the pleiotropic nature of the *col* mutant phenotype and the involvement of other Wnt family members in early embry-

onic patterning, *col* likely antagonizes signaling by other Wnts during development.

Acknowledgments

The authors thank our many colleagues for critical reagents used in this study and for helpful discussions. We also thank Myron S. Ignatius for alcian blue staining. This work was supported by grants from NIH (NS38115) and NSF (IBN0315765).

References

Akimenko, M.A., Ekker, M., Wegner, J., Lin, W., Westerfield, M., 1994. Combinatorial expression of three zebrafish genes related to *distal-less*: part of a homeobox gene code for the head. J. Neurosci. 14, 3475–3486.

Amaya, E., Musci, T.J., Kirschner, M.W., 1991. Expression of a dominant negative mutant of the FGF receptor disrupts mesoderm formation in the *Xenopus* embryos. Cell 66, 257–270.

Ang, S.L., Rossant, J., 1993. Anterior mesendoderm induces mouse Engrailed genes in explant cultures. Development 118, 139–149.

Ang, S.L., Conlon, R.A., Jin, O., Rossant, J., 1994. Positive and negative signals from mesoderm regulate the expression of mouse Otx2 in ectoderm explants. Development 120, 2979–2989.

Angelo, S., Lohr, J., Lee, K.H., Ticho, B.S., Breitbart, R.E., Hill, S., Yost, H.J., Srivastava, D., 2000. Conservation of sequence and expression of *Xenopus* and zebrafish *dHAND* during cardiac, branchial arch and lateral mesoderm development. Mech. Dev. 95, 231–237.

Barker, N., Clevers, H., 2000. Catenins, Wnt signaling and cancer. BioEssays 22, 961–965.

Barth, K.A., Kishimoto, Y., Rohr, K., Seydler, C., Schulte-Merker, S., Wilson, S.W., 1999. BMP activity establishes a gradient of positional information throughout the entire neural plate. Development 126, 4977–4987.

Bienz, M., Clevers, H., 2000. Linking colorectal cancer to Wnt signaling. Cell 103, 311–320.

Bouwmeester, T., Kim, S., Sasai, Y., Lu, B., De Robertis, E.M., 1996. Cerberus is a head-inducing secreted factor expressed in the anterior endoderm of Spemann's organizer. Nature 382, 595–601.

Chin, A.J., Chen, J., Weinberg, E.S., 1997. Bone Morphogenetic Protein-4 characterizes inductive boundaries in organs of the developing zebrafish. Dev. Genes Evol. 207, 107–114.

Dahmen, R.P., Koch, A., Denkhaus, D., Tonn, J.C., Sorensen, N., Berthold, F., Behrens, J., Birchmeier, W., Wiestler, O.D., Pietsch, T., 2001. Deletions of AXIN1, a component of the WNT/wingless pathway, in sporadic medulloblastomas. Cancer Res. 61, 7039–7043.

Dale, J.K., Vesque, C., Lints, T., Sampath, T.K., Furley, A., Dodd, J., Placzek, M., 1997. Cooperation of BMP7 and SHH in the induction of forebrain ventral midline cells by prechordal mesoderm. Cell 90, 257–269.

Deardorff, M.A., Tan, C., Conrad, L.J., Klein, P.S., 1998. Frizzled-8 is expressed in the Spemann organizer and plays a role in early morphogenesis. Development 125, 2687–2700.

Detrich III, H.W., Kieran, M.W., Chan, F.Y., Barone, L.M., Yee, K., Rundstadler, J.A., Pratt, S., Ransom, D., Zon, L.I., 1995. Intraembryonic hematopoietic cell migration during vertebrate development. Proc. Natl. Acad. Sci. U. S. A. 92, 10713–10717.

Donaich, T., 1995. Basic FGF as an inducer of anteroposterior neural pattern. Cell 83, 1067–1070.

Erter, C.E., Wilm, T.P., Basler, N., Wright, C.V., Solnica-Krezel, L., 2001. Wnt8 is required in lateral mesendodermal precursors for neural posteriorization in vivo. Development 128, 3571–3583.

Fekany, K., Yamanaka, Y., Leung, T., Sirotkin, H.I., Topczewski, J., Gates, M.A., Hibi, M., Renucci, A., Stemple, D., Radbill, A., Schier, A.F., Driever, W., Hirano, T., Talbot, W.S., Solnica-Krezel, L., 1999. The zebrafish *bozozok* locus encodes Dharma, a homeodomain protein essential for induction of gastrula organizer and dorsoanterior embryonic structures. Development 126, 1427–1438.

Fekany-Lee, K., Gonzalez, E., Miller-Bertoglio, V., Solnica-Krezel, L., 2000. The homeobox gene *bozozok* promotes anterior neuroectoderm formation in zebrafish through negative regulation of BMP2/4 and Wnt pathways. Development 127, 2333–2345.

Foley, A.C., Storey, K.G., Stern, C.D., 1997. The prechordal region lacks neural inducing ability, but can confer anterior character to more posterior neuroepithelium. Development 124, 2983–2996.

Gamse, J., Sive, H., 2000. Vertebrate anteroposterior patterning: the *Xenopus* neurectoderm as a paradigm. BioEssays 22, 976–986.

Gerhart, J., Danilchik, M., Doniach, T., Roberts, S., Rowning, B., Stewart, R., 1989. Cortical rotation of the *Xenopus* egg: consequences for the anteroposterior pattern of embryonic dorsal development. Development 107, 37–51.

Glinka, A., Wu, W., Onichtchouk, D., Blumenstock, C., Niehrs, C., 1997. Head induction by simultaneous repression of Bmp and Wnt signalling in *Xenopus*. Nature 389, 517–519.

Glinka, A., Wu, W., Delius, H., Monaghan, A.P., Blumenstock, C., Niehrs, C., 1998. Dickkopf-1 is a member of a new family of secreted proteins and functions in head induction. Nature 391, 357–362.

Graff, J.M., 1997. Embryonic patterning: to BMP or not to BMP, that is the question. Cell 89, 171–174.

Hammerschmidt, M., Pelegri, F., Mullins, M.C., Kane, D.A., van Eeden, F.J., Granato, M., Brand, M., Furutani-Seiki, M., Haffter, P., Heisenberg, C.P., Jiang, Y.-J., Kelsh, R., Odenthal, J., Warga, R.M., Nusslein-Volhard, C., 1996. *Dino* and *mercedes*, two genes regulating dorsal development in the zebrafish embryo. Development 123, 95–102.

Harland, R., Gerhart, J., 1997. Formation and function of Spemann's organizer. Annu. Rev. Cell Dev. Biol. 13, 611–667.

Hashimoto, H., Itoh, M., Yamanaka, Y., Yamashita, S., Shimizu, T., Solnica-Krezel, L., Hibi, M., Hirano, T., 2000. Zebrafish Dkk1 functions in forebrain specification and axial mesendoderm formation. Dev. Biol. 217, 138–152.

Heasman, J., Crawford, A., Goldstone, K., Garner-Hamrick, P., Gumbiner, B., McCrea, P., Kintner, C., Noro, C.Y., Wylie, C., 1994. Overexpression of *cadherins* and underexpression of *beta-catenin* inhibit dorsal mesoderm induction in early *Xenopus* embryos. Cell 79, 791–803.

Heasman, J., Kofron, M., Wylie, C., 2000. Beta-catenin signaling activity dissected in the early *Xenopus* embryo: a novel antisense approach. Dev. Biol. 222, 124–134.

Hedgepeth, C.M., Deardorff, M.A., Rankin, K., Klein, P.S., 1999. Regulation of glycogen synthase kinase 3beta and downstream Wnt signaling by axin. Mol. Cell Biol. 19, 7147–7157.

Heisenberg, C.P., Houart, C., Take-Uchi, M., Rauch, G.J., Young, N., Coutinho, P., Masai, I., Caneparo, L., Concha, M.L., Geisler, R., Dale, T.C., Wilson, S.W., Stemple, D.L., 2001. A mutation in the Gsk3-binding domain of zebrafish Masterblind/Axin1 leads to a fate transformation of telencephalon and eyes to diencephalon. Genes Dev. 15, 1427–1434.

Henion, P.D., Raible, D.W., Beattie, C.E., Stoesser, K.L., Weston, J.A., Eisen, J.S., 1996. Screen for mutations affecting development of Zebrafish neural crest. Dev. Genet. 18, 11–17.

Hoppler, S., Brown, J.D., Moon, R.T., 1996. Expression of a dominant-negative Wnt blocks induction of MyoD in *Xenopus* embryos. Genes Dev. 10, 2805–2817.

Houart, C., Caneparo, L., Heisenberg, C., Barth, K., Take-Uchi, M., Wilson, S., 2002. Establishment of the telencephalon during gastrulation by local antagonism of Wnt signaling. Neuron 35, 255.

Inoue, A., Takahashi, M., Hatta, K., Hotta, Y., Okamoto, H., 1994. Developmental regulation of *islet-1* mRNA expression during neuronal differentiation in embryonic zebrafish. Dev. Dyn. 199, 1–11.

Kane, D.A., Kimmel, C.B., 1993. The zebrafish midblastula transition. Development 119, 447–456.

Kazanskaya, O.V., Severtzova, E.A., Barth, K.A., Ermakova, G.V., Lukyanov, S.A., Benyumov, A.O., Pannese, M., Boncinelli, E., Wilson, S.W., Zaraisky, A.G., 1997. Anf: a novel class of vertebrate homeobox genes expressed at the anterior end of the main embryonic axis. Gene 200, 25–34.

Kelly, G.M., Moon, R.T., 1995. Involvement of *wnt1* and *pax2* in the formation of the midbrain-hindbrain boundary in the zebrafish gastrula. Dev. Genet. 17 (2), 129–140.

Kelly, G.M., Greenstein, P., Erezyilmaz, D.F., Moon, R.T., 1995. Zebrafish *wnt8* and *wnt8b* share a common activity but are involved in distinct developmental pathways. Development 121, 1787–1799.

Kiecker, C., Niehrs, C., 2001. A morphogen gradient of Wnt/β-catenin signaling regulates anteroposterior neural patterning in *Xenopus*. Development 128, 4189–4201.

Kim, L., Liu, J., Kimmel, A.R., 1999. The novel tyrosine kinase ZAK1 activates GSK3 to direct cell fate specification. Cell 99, 399–408.

Kim, C.H., Oda, T., Itoh, M., Jiang, D., Artinger, K.B., Chandrasekharappa, S.C., Driever, W., Chitnis, A.B., 2000. Repressor activity of Headless/Tcf3 is essential for vertebrate head formation. Nature 407, 913–916.

Kim, S.H., Shin, J., Park, H.C., Yeo, S.Y., Hong, S.K., Han, S., Rhee, M., Kim, C.H., Chitnis, A.B., Huh, T.L., 2002. Specification of an anterior neuroectoderm patterning by Frizzled8a-mediated Wnt8b signalling during late gastrulation in zebrafish. Development 129, 4443–4455.

Kishimoto, Y., Lee, K.H., Zon, L., Hammerschmidt, M., Schulte-Merker, S., 1997. The molecular nature of zebrafish *swirl*: BMP2 function is essential during early dorsoventral patterning. Development 124, 4457–4466.

Knapik, E.W., Goodman, A., Atkinson, O.S., Roberts, C.T., Shiozawa, M., Sim, C.U., Weksler-Zangen, S., Trolliet, M.R., Futrell, C., Innes, B.A., et al., 1996. A reference cross DNA panel for zebrafish (*Danio rerio*) anchored with simple sequence length polymorphisms. Development 123, 451–460.

Koos, D.S., Ho, R.K., 1998. The *nieuwkoid* gene characterizes and mediates a Nieuwkoop-center-like activity in the zebrafish. Curr. Biol. 8, 1199–1206.

Korzh, V., Edlund, T., Thor, S., 1993. Zebrafish primary neurons initiate expression of the LIM homeodomain protein Isl-1 at the end of gastrulation. Development 118, 417–425.

Krauss, S., Johansen, T., Korzh, V., Fjose, A., 1991. Expression pattern of zebrafish pax genes suggests a role in early brain regionalization. Nature 353, 267–270.

Krauss, S., Concordet, J.P., Ingham, P.W., 1993. A functionally conserved homolog of the *Drosophila* segment polarity gene hh is expressed in tissues with polarizing activity in zebrafish embryos. Cell 75, 1431–1444.

Lagutin, O.V., Zhu, C.C., Kobayashi, D., Topczewski, J., Shimamura, K., Puelles, L., Russell, H.R.C., McKinnon, P.J., Solnica-Krezel, L., Oliver, G., 2003. Six3 repression of Wnt signaling in the anterior neuroectoderm is essential for vertebrate forebrain development. Genes Dev. 17, 368–379.

Lee, K.H., Xu, Q., Breitbart, R.E., 1996. A new tinman-related gene, nkx2.7, anticipates the expression of nkx2.5 and nkx2.3 in zebrafish heart and pharyngeal endoderm. Dev. Biol. 180, 722–731.

Lekven, A.C., Thorpe, C.J., Waxman, J.S., Moon, R.T., 2001. Zebrafish wnt8 encodes two wnt8 proteins on a bicistronic transcript and is required for mesoderm and neurectoderm patterning. Dev. Cell 1, 103–114.

Lemaire, P., Kodjabachian, L., 1996. The vertebrate organizer: structure and molecules. Trends Genet. 12, 525–531.

Leung, J.Y., Kolligs, F.T., Wu, R., Zhai, Y., Kuick, R., Hanash, S., Cho, K.R., Fearon, E.R., 2002. Activation of AXIN2 expression by beta-catenin-T cell factor. A feedback repressor pathway regulating Wnt signaling. J. Biol. Chem. 277, 21657–21665.

Leyns, L., Bouwmeester, T., Kim, S.H., Piccolo, S., De Robertis, E.M., 1997. Frzb-1 is a secreted antagonist of Wnt signaling expressed in the Spemann organizer. Cell 88, 747–756.

Li, Y., Allende, M.L., Finkelstein, R., Weinberg, E.S., 1994. Expression of two zebrafish orthodenticle-related genes in the embryonic brain. Mech. Dev. 48, 229–244.

Lumsden, A., Krumlauf, R., 1996. Patterning the vertebrate neuraxis. Science 274, 1109–1115.

McGrew, L.L., Lai, C.J., Moon, R.T., 1995. Specification of the anteroposterior neural axis through synergistic interaction of the Wnt signaling cascade with noggin and follistatin. Dev. Biol. 172, 337–342.

McGrew, L.L., Hoppler, S., Moon, R.T., 1997. Wnt and FGF pathways cooperatively pattern anteroposterior neural ectoderm in *Xenopus*. Mech. Dev. 69, 105–114.

Miller-Bertoglio, V.E., Fisher, S., Sanchez, A., Mullins, M.C., Halpern, M.E., 1997. Differential regulation of *chordin* expression domains in mutant zebrafish. Dev. Biol. 192, 537–550.

Moon, R.T., Brown, J.D., Torres, M., 1997. WNTs modulate cell fate and behavior during vertebrate development. Trends Genet. 3, 157–162.

Moon, R.T., Brown, J.D., Yang-Snyder, J.A., Miller, J.R., 1998. Structurally related receptors and antagonists compete for secreted Wnt ligands. Cell 88, 725–728.

Mukhopadhyay, M., Shtrom, S., Rodriguez-Esteban, C., Chen, L., Tsukui, T., Gomer, L., Dorward, D.W., Glinka, A., Grinberg, A., Huang, S.-P., et al., 2001. *Dickkopf1* is required for head induction and limb morphogenesis in the mouse. Dev. Cell 1, 423–434.

Myers, D.C., Sepich, D.S., Solnica-Krezel, L., 2002. BMP activity gradient regulates convergent extension during zebrafish gastrulation. Dev. Biol. 243, 81–98.

Neave, B., Holder, N., Patient, R., 1997. A graded response to BMP-4 spatially coordinates patterning of the mesoderm and ectoderm in the zebrafish. Mech. Dev. 62, 183–195.

Nguyen, V.H., Schmid, B., Trout, J., Connors, S.A., Ekker, M., Mullins, M.C., 1998. Ventral and lateral regions of the zebrafish gastrula, including the neural crest progenitors, are established by a bmp2b/swirl pathway of genes. Dev. Biol. 199, 93–110.

Nieuwkoop, P.D., 1973. The organization center of the amphibian embryo: its origin, spatial organization, and morphogenetic action. Adv. Morphog. 10, 1–39.

Nieuwkoop, P.D., Boterenbrood, E.C., Kremer, A., Bloesma, F.F.S.N., Hoessels, E.L.M.J., Meyer, G., Verheyen, F.J., 1952. Activation and organization of the central nervous system in amphibians. J. Exp. Zool. 120, 1–108.

Nikaido, M., Tada, M., Saji, T., Ueno, N., 1997. Conservation of BMP signaling in zebrafish mesoderm patterning. Mech. Dev. 1–2, 75–88 (Jan.).

Nordstrom, U., Jessel, T.M., Edlund, T., 2002. Progressive induction of caudal neural character by graded Wnt signaling. Nat. Neurosci. 5, 525–532.

Piccolo, S., Agius, E., Leyns, L., Bhattacharyya, S., Grunz, H., Bouwmeester, T., De Robertis, E.M., 1999. The head inducer Cerberus is a multifunctional antagonist of Nodal, BMP and Wnt signals. Nature 397, 707–710.

Pierce, S.B., Kimelman, D., 1995. Regulation of Spemann organizer formation by the intracellular kinase *Xgsk-3*. Development 121, 755–765.

Polakis, P., 2000. Wnt signaling and cancer. Genes Dev. 14, 1837–1851.

Reifers, F., Bohli, H., Walsh, E.C., Crossley, P.H., Stainier, D.Y., Brand, M., 1998. Fgf8 is mutated in zebrafish acerebellar (ace) mutants and is required for maintenance of midbrain-hindbrain boundary development and somitogenesis. Development 125, 2381–2395.

Satoh, S., Daigo, Y., Furukawa, Y., Kato, T., Miwa, N., Nishiwaki, T., Kawasoe, T., Ishiguro, H., Fujita, M., Tokino, T., Sasaki, Y., Imaoka, S., Murata, M., Shimano, T., Yamaoka, Y., Nakamura, Y., 2000. AXIN1 mutations in hepatocellular carcinomas, and growth suppression in cancer cells by virus-mediated transfer of AXIN1. Nat. Genet. 24, 245–250.

Schneider, S., Steinbeisser, H., Warga, R.M., Hausen, P., 1996. Beta-catenin translocation into nuclei demarcates the dorsalizing centers in frog and fish embryos. Mech. Dev. 57, 191–198.

Schulte-Merker, S., Ho, R.K., Herrmann, B.G., Nusslein-Volhard, C., 1992. The protein product of the zebrafish homologue of the mouse T gene is expressed in nuclei of the germ ring and the notochord of the early embryo. Development 116, 1021–1032.

Shinya, M., Eschbach, C., Clark, M., Lehrach, H., Furutani-Seiki, M., 2000. Zebrafish Dkk1, induced by the pre-MBT Wnt signaling, is secreted from the prechordal plate and patterns the anterior neural plate. Mech. Dev. 98, 3–17.

Sokol, S.Y., 1999. Wnt signaling and dorso-ventral axis specification in vertebrates. Curr. Opin. Genet. Dev. 9, 405–410.

Spemann, H., 1938. Embryonic Development and Induction. Yale Univ. Press, New Haven, CT.

Spemann, H., Mangold, H., 1924. Uber Induktion von Embryoanalgen durch Implantation artfremder Organisatoren. Wilhelm Roux' Arch. Entwicklungsmech. Org. 100, 599–638.

Stachel, S.E., Grunwald, D.J., Myers, P.Z., 1993. Lithium perturbation and *goosecoid* expression identify a dorsal specification pathway in the pregastrula zebrafish. Development 117, 1261–1274.

Thisse, B., Wright, C.V., Thisse, C., 1999. Antivin, a novel and divergent member of the TGFβ superfamily regulates mesoderm induction. Development 126, 229–240.

Trevarrow, B., Marks, D.L., Kimmel, C.B., 1990. Organization of hindbrain segments in the zebrafish embryo. Neuron 4, 669–679.

Wilson, P.A., Hemmati-Brivanlou, A., 1995. Induction of epidermis and inhibition of neural fate by Bmp-4. Nature 376, 331–333.

Woo, K., Fraser, S.E., 1997. Specification of the zebrafish nervous system by nonaxial signals. Science 277, 254–257.

Wylie, C., Kofron, M., Payne, C., Anderson, R., Hosobuchi, M., Joseph, E., Heasman, J., 1996. Maternal beta-catenin establishes a 'dorsal signal' in early *Xenopus* embryos. Development 122, 2987–2996.

Yamaguchi, T.P., 2001. Heads or tails: Wnts and anterior–posterior patterning. Curr. Biol. 11, 713–724.

Yelon, D., Ticho, B., Halpern, M.E., Ruvinsky, I., Ho, R.K., Silver, L.M., Stainier, D.Y.R., 2000. The bHLH transcription factor Hand2 plays parallel roles in zebrafish heart and pectoral fin development. Development 127, 2573–2582.