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RESEARCH ARTICLE

β-catenin regulates Pax3 and Cdx2 for caudal neural tube closure and elongation

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ABSTRACT

Non-canonical Wnt/planar cell polarity (PCP) signaling plays a primary role in the convergent extension that drives neural tube closure and body axis elongation. PCP signaling gene mutations cause severe neural tube defects (NTDs). However, the role of canonical Wnt/β-catenin signaling in neural tube closure and NTDs remains poorly understood. This study shows that conditional gene targeting of β-catenin in the dorsal neural folds of mouse embryos represses the expression of the homeobox-containing genes *Pax3* and *Cdx2* at the dorsal posterior neuropore (PNP), and subsequently diminishes the expression of the Wnt/β-catenin signaling target genes *T*, *Tbx6* and *Fgf8* at the tail bud, leading to spina bifida aperta, caudal axis bending and tail truncation. We demonstrate that *Pax3* and *Cdx2* are novel downstream targets of Wnt/β-catenin signaling. Transgenic activation of *Pax3* cDNA can rescue the closure defect in the β-catenin mutants, suggesting that Pax3 is a key downstream effector of β-catenin signaling in the PNP closure process. *Cdx2* is known to be crucial in posterior axis elongation and in neural tube closure. We found that *Cdx2* expression is also repressed in the dorsal PNPs of *Pax3*-null embryos. However, the ectopically activated Pax3 in the β-catenin mutants cannot restore *Cdx2* mRNA in the dorsal PNP, suggesting that the presence of both β-catenin and Pax3 is required for regional *Cdx2* expression. Thus, β-catenin signaling is required for caudal neural tube closure and elongation, acting through the transcriptional regulation of key target genes in the PNP.

KEY WORDS: Wnt/β-catenin signaling, Posterior neuropore (PNP), Spina bifida

INTRODUCTION

Mammalian neural tubes exhibit an initial closure at the future hindbrain and cervical boundary, subsequent closures at several different brain regions, and a final closure at the posterior neuropore (PNP) (Bassuk and Kibar, 2009; Copp and Greene, 2010). Failure of closure at these anatomically distinct zones may lead to a spectrum of neural tube defects (NTDs). The cause and prevention of NTDs remain poorly understood (Copp et al., 2013; Wallingford et al., 2013). NTDs are common birth defects with inheritable risks, but only a small number of gene mutations have thus far been linked with human NTDs (De Marco et al., 2011; Kibar et al., 2007). From mutant mouse studies, more than 200 genes have been linked with NTDs (Harris and Juriloff, 2007; Harris and Juriloff, 2010), reflecting the complex genetic basis of neural tube closure.

β-catenin-independent non-canonical Wnt/planar cell polarity (PCP) signaling regulates cytoskeleton dynamics and is a core signaling program for oriented tissue movements such as convergent extension, which is a potential driving force directing neural tube closure and body axis elongation (Copp et al., 2003; Wallingford, 2006; Ybot-Gonzalez et al., 2007). Craniorachischisis, a severe type of NTD associated with defective convergent extension, has been observed in mutant mice of the Celsr, Dvl, Fzd and Vangl PCP signaling gene families, suggesting that mutations in these genes might also cause NTDs in humans (De Marco et al., 2011; Juriloff and Harris, 2012). However, several of these PCP signaling molecules, such as Fzds and Dvls, are also essential components of the canonical Wnt/β-catenin signaling pathway (MacDonald et al., 2009). In addition, Lrp6 is a key co-receptor in the canonical Wnt pathway and is required for a wide range of organogenetic events, including neural tube closure in mice (Carter et al., 2005; Kokubu et al., 2004; Mao et al., 2001; Pinson et al., 2000; Song et al., 2009; Song et al., 2010; Tamai et al., 2000; Wehrli et al., 2000; Zhou et al., 2008; Zhou et al., 2004; Zhou et al., 2010). However, Lrp6 is also implicated in convergent extension during *Xenopus* gastrulation (Tahinci et al., 2007) and may mediate non-canonical Wnt signaling for neural tube closure (Gray et al., 2013). Thus, the role of canonical Wnt signaling, and especially of β-catenin, in neural tube closure and NTDs remains unknown.

The canonical Wnt/β-catenin pathway has vital roles in development and disease (Clevers and Nusse, 2012). When Wnt proteins bind to the Fzd and Lrp receptors, the degradation function of the Axin1-APC-Gsk3 complex is repressed, resulting in elevated levels of free β-catenin (Li et al., 2012). The accumulated cytoplasmic β-catenin translocates to the nucleus where it binds the Tcf/Lef transcription complex to regulate the promoter activity of downstream target genes for various biological functions (Cadigan, 2012). We hypothesize that canonical Wnt/β-catenin signaling is required for neural tube closure through transcriptional regulation of crucial downstream target genes. β-catenin-null mouse embryos arrest during gastrulation (Haegel et al., 1995; Huelsken et al., 2000). β-catenin conditional gene-targeting analyses have been performed widely in various developmental processes, although not specifically with respect to neural tube closure (Grigoryan et al., 2008). This study focuses on the signaling function of β-catenin, and presents evidence that β-catenin is required for transcriptional activation of the paired-box gene *Pax3* and the caudal-type homeobox gene *Cdx2*, which are crucial effectors for caudal neural tube closure and/or elongation processes (Epstein et al., 1991; Savory et al., 2011; Young et al., 2009). We further demonstrate that ectopic activation of a *Pax3* transgene can rescue spina bifida in β-

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catenin mutant mice and that *Pax3* is required, but insufficient, for *Cdx2* activation in the dorsal PNP. These results reveal a novel cascade of β-catenin/Pax3/Cdx2 signaling that is required for PNP closure and/or elongation processes during neurulation.

RESULTS

Conditional gene targeting of β-catenin in the dorsal neural tube leads to defective PNP closure and elongation

To define the role of β-catenin signaling in neural tube closure, we employed the Cre-loxP conditional gene-targeting approach using *β-catenin*$^{ex(2\text{-}6)flox}$ mice (Brault et al., 2001) crossed with *Pax3*^{Cre} knock-in mice (Lang et al., 2005). A genetic fate-mapping experiment with the Cre reporter *Rosa26-lacZ* demonstrates high activities of Cre recombinase driven by endogenous *Pax3* promoters in the dorsal neural tube, particularly around the closure sites of the PNP regions of E9.5 mouse embryos (Fig. 1A). Wholemount *in situ* hybridization and real-time PCR experiments demonstrate that β-catenin mRNA expression is effectively ablated in the dorsal neural tube/fold, whereas it is unaffected in other tissues including the paraxial mesoderm around the PNP region, during the closure process of E9.5 *β-catenin*$^{(ex2\text{-}6)flox/flox}$*;Pax3*$^{Cre/+}$ conditional knockout (abbreviated as β-catenin cKO) mutant embryos (Fig. 1B,C).

Because β-catenin is a key molecule in the canonical Wnt pathway, we analyzed Wnt signaling alterations in the β-catenin cKO embryos. The expression of *Axin2*, a general Wnt/β-catenin signaling downstream target and negative-feedback regulator in the canonical Wnt/β-catenin signaling pathway (Jho et al., 2002), is repressed in the dorsal neural tube, as shown at E9.5 (Fig. 2A,B). The activity of the Wnt/β-catenin signaling reporter BATgal (Maretto et al., 2003) is also dramatically diminished in the dorsal neural tube of β-catenin cKO embryos at E9.5 (Fig. 2C). Whereas the control neural tube closed completely by E10.5, all β-catenin cKO embryos exhibited persistently open PNPs at this age, which consequently developed as spina bifida aperta, as shown at E14.5 (Fig. 2D). The mutant embryos also exhibit dorsally bent and shorter PNPs at E10.5 and kinked and truncated tails at E14.5. These results demonstrate an essential role of β-catenin signaling in PNP closure and elongation processes in mice.

Expression of Pax3 and Cdx transcription factors is specifically diminished in the dorsal PNPs of the β-catenin cKO at E9.5

To address the β-catenin-regulated signaling mechanism in the PNP, we examined a panel of locally expressed and functionally important transcription factors and signaling molecules in the mutant embryos. The transcription factor Pax3 is expressed in the dorsal neural folds/tubes during the closure process, and *Pax3*-null embryos display spina bifida and/or exencephaly, two common types of NTDs (Epstein et al., 1991; Goulding et al., 1991). In addition, Pax3 has been suggested to mediate Fgf8 and/or Wnt signaling for neural crest development (Degenhardt et al., 2010; Monsoro-Burq et al., 2005). Therefore, we hypothesize that *Pax3* is a downstream effector of Wnt/β-catenin signaling for the caudal neural tube closure process.

Wholemount *in situ* hybridization demonstrates that *Pax3* expression is diminished markedly in the dorsal PNP of β-catenin cKOs at E9.5 (Fig. 3A). *Pax3* expression is diminished more dramatically in the early-born somites, and moderately in the late-born somites of the E9.5 mutants (Fig. 3A). The caudal-type homeobox genes *Cdx2* and *Cdx4* are also specifically diminished in the dorsal PNP of β-catenin cKOs at E9.5 (Fig. 3B,C). Cdx2 is a survival factor for early embryos around implantation and is also required for caudal body axis elongation during neurulation (Young et al., 2009). Cdx4 is dispensable in mice (van Nes et al., 2006). In addition, the homeobox gene *Msx1* is diminished throughout the dorsal neural tube, especially in the dorsal PNPs, and in the upper spinal and cranial regions of the β-catenin mutants (Fig. 3D). Although Msx1 does not play any substantial role in neural tube closure, it is a potential regulator of *Pax3* (Monsoro-Burq et al., 2005) and is also a known β-catenin downstream effector (Foerst-Potts and Sadler, 1997; Song et al., 2009). These results suggest that β-catenin is the common upstream regulator of crucial transcription factors required for PNP development.

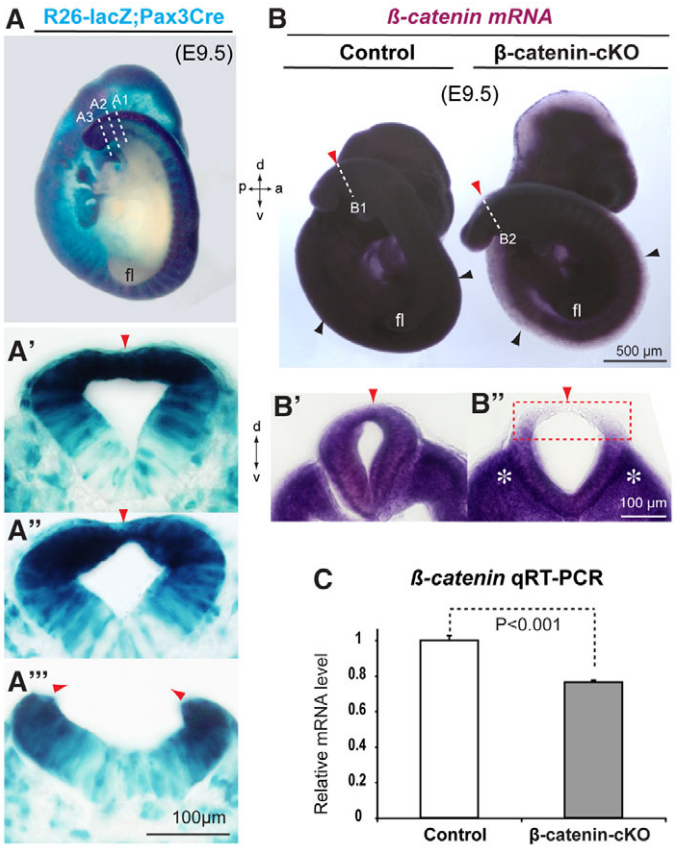

Fig. 1. Effective ablation of β-catenin in the mouse dorsal neural tube with Pax3-Cre. (A-A‴) X-gal staining (blue) for genetic fate mapping of *Rosa26-lacZ;Pax3*$^{Cre/+}$ demonstrates the Cre recombination pattern in the dorsal neural tube, including closed (section A1 in A′), closing (section A2 in A″) and pending closure (section A3 in A‴) posterior neuropore (PNP). (B-C) *In situ* hybridization and real-time PCR results for conditional inactivation of β-catenin mRNA in the dorsal neural tube of the β-catenin cKO [the abbreviation for *β-catenin*$^{(ex2\text{-}6)flox/flox}$*;Pax3*$^{Cre/+}$] at E9.5. Sections B1 and B2 are shown in B′ and B″, respectively. Note that β-catenin mRNAs were ablated in the dorsal PNP (boxed region in B″), but were still expressed in the majority of the cKO tissues including the ventral neural tube and the paraxial mesoderm (asterisks in B″) around the PNP closure site. Error bars indicate s.e.m. (*n*=3). Black arrowheads, dorsal neural tube midlines; red arrowheads, the dorsal midline around the PNP closure regions; dashed lines, planes of transverse PNP sections. a, anterior; d, dorsal; fl, forelimb bud; p, posterior; v, ventral.

Pax3 and *Cdx2* are transcriptionally activated by β-catenin signaling

To determine the molecular mechanisms by which β-catenin signaling regulates the key downstream factors involved in caudal neural tube formation, we searched the upstream promoter region of the mouse *Pax3* gene, which is specifically activated in the dorsal

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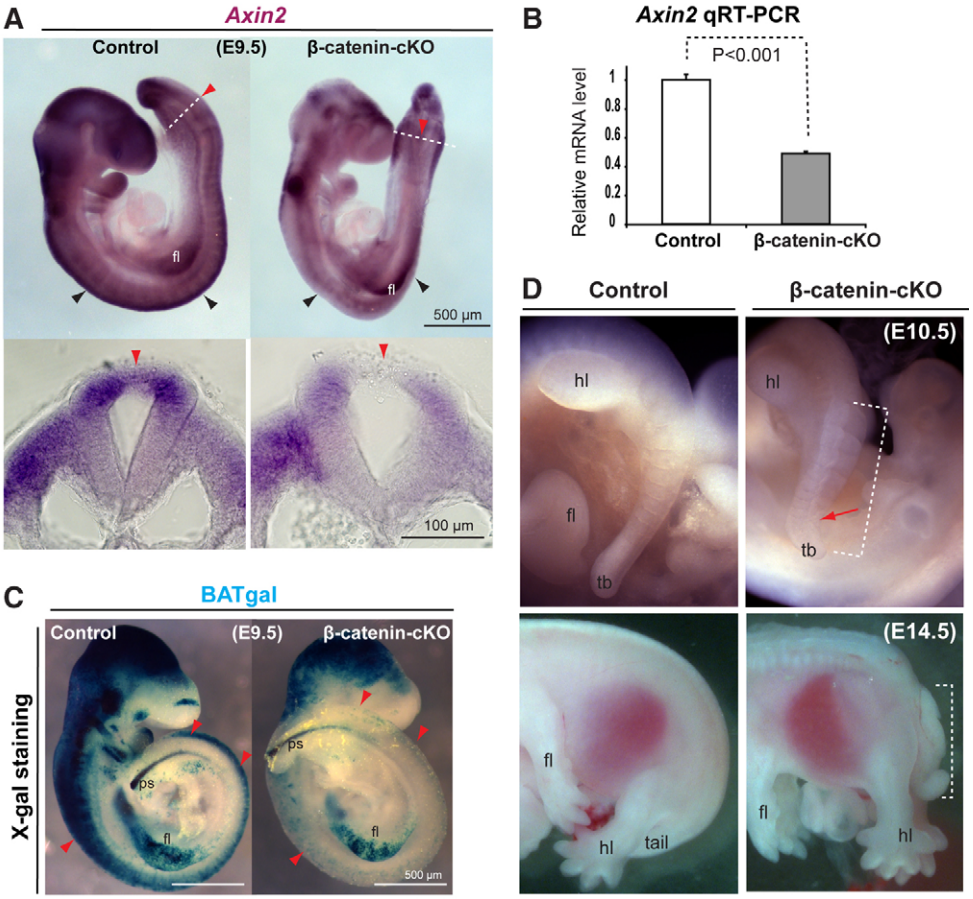

Fig. 2. Diminished canonical Wnt signaling and occurrence of spinal bifida aperta in β-catenin cKO embryos. (A,B) *In situ* hybridization and real-time PCR results showing the repressed mRNA level of the Wnt/β-catenin target and feedback gene *Axin2* in the β-catenin cKO neural tube. In A, dashed lines indicate planes of the sections shown beneath. Error bars indicate s.e.m. (*n*=3). (C) Diminished X-gal staining indicating reduced activity of the Wnt/β-catenin signaling reporter BATgal in the mutant neural tube. (D) Persistently open (dashed bracket) and dorsally bent (red arrow) caudal neural fold in the β-catenin cKO at E10.5, and spina bifida aperta (dashed bracket) and short tail at E14.5. fl/hl, forelimb/hindlimb bud; ps, primitive streak; tb, tail bud. Arrowheads (A,C) indicate dorsal neural tube midline regions.

neural tube (Natoli et al., 1997), and found three conserved putative Tcf/Lef1 binding activation sites (termed *AS1-3*, Fig. 4A; supplementary material Fig. S1). Among these binding sites, *AS1* is crucial for Wnt signaling responsiveness, as demonstrated in luciferase reporter assays (Fig. 4B-D). Chromatin immunoprecipitation assays demonstrate the specific binding of the β-catenin–Tcf complex to *AS1* in the *Pax3* promoter in tissue samples isolated from the caudal part of E9.5 mouse embryos (Fig. 4E). We found a conserved Tcf/Lef1 binding site in the putative *Cdx2* promoter region, which also demonstrates binding and responsiveness to the β-catenin transcriptional complex (Fig. 4E-G). These results suggest that both *Pax3* and *Cdx2* are direct transcriptional targets of β-catenin signaling in the dorsal PNP.

Candidate Wnt signaling target genes in the tail bud are relatively unaffected at E9.5 but altered at E10.5 in *Pax3^{Cre};β-catenin* cKOs

The tail truncation in the β-catenin cKO embryo suggests a defective tail bud. The tail bud is the most posterior signaling center, expressing various signaling molecules including T-box genes, Fgfs and Wnts (Gofflot et al., 1997), and is functionally important in posterior body axis development. Wholemount *in situ* hybridization demonstrates that the expression patterns of brachyury (*T*) (Wilkinson et al., 1990), *Tbx6* (Chapman and Papaioannou, 1998), *Wnt5a* (Yamaguchi et al., 1999a), *Fgf8* (Crossley and Martin, 1995) and *Fgf18* are apparently unaltered in the mutant tail buds at E9.5 (Fig. 5A-E). The expression patterns of *Mesp2* in the rostral presomite mesoderm (Takahashi et al., 2000) and of *Uncx4.1* (also known as *Uncx*) in the newly formed somites (Mansouri et al., 1997) are also not significantly altered in the caudal region of E9.5 β-catenin cKOs (Fig. 5F,G). In addition, the expression patterns of the roof plate marker genes *Lmx1a* and *Lmx1b* (Mishima et al., 2009) are intact in the mutant embryos at E9.5 (Fig. 5H,I). Moreover, neither representative PCP signaling gene expression patterns nor cell proliferation or apoptosis are significantly affected in the mutant PNPs at E9.5 (supplementary material Figs S2, S3).

Among the crucial tail bud patterning genes, *T*, *Tbx6* and *Fgf8* have been identified as the downstream targets of Wnt/β-catenin signaling in the tail bud or in the anterior neural ridge (Szeto and Kimelman, 2004; Wang et al., 2011; Yamaguchi et al., 1999b). Because these genes are not altered at E9.5 in the tail buds of the mutants, we then examined these genes at a later stage when neural tube closure is completed in wild-type or double-heterozygous embryos. Indeed, the *in situ* mRNA signals of these genes are markedly diminished in the mutant tail buds at E10.5 (Fig. 6) to varied degrees, with dramatically diminished *T* expression in the tail bud but relatively unaffected *T* expression in the primitive streak (Fig. 6A-B′), and nearly absent *Tbx6* (Fig. 6C-D′) and residual *Fgf8* (Fig. 6E-F′) tail bud expression. *Uncx4.1 in situ* results demonstrate conserved somite numbers, with disrupted somite organization and/or formation in the caudal axis of the E10.5 mutants. These results suggest that a delayed disruption of the tail bud patterning genes contributes to the defective posterior axis in the β-catenin cKO mutants at later ages.

Ectopic activation of a *Pax3* cDNA transgene can rescue spina bifida in β-catenin cKO embryos

To further demonstrate that Pax3 is a key effector of β-catenin signaling in the caudal neural tube closure process, we conducted a genetic rescue experiment using Cre-mediated gain-of-function

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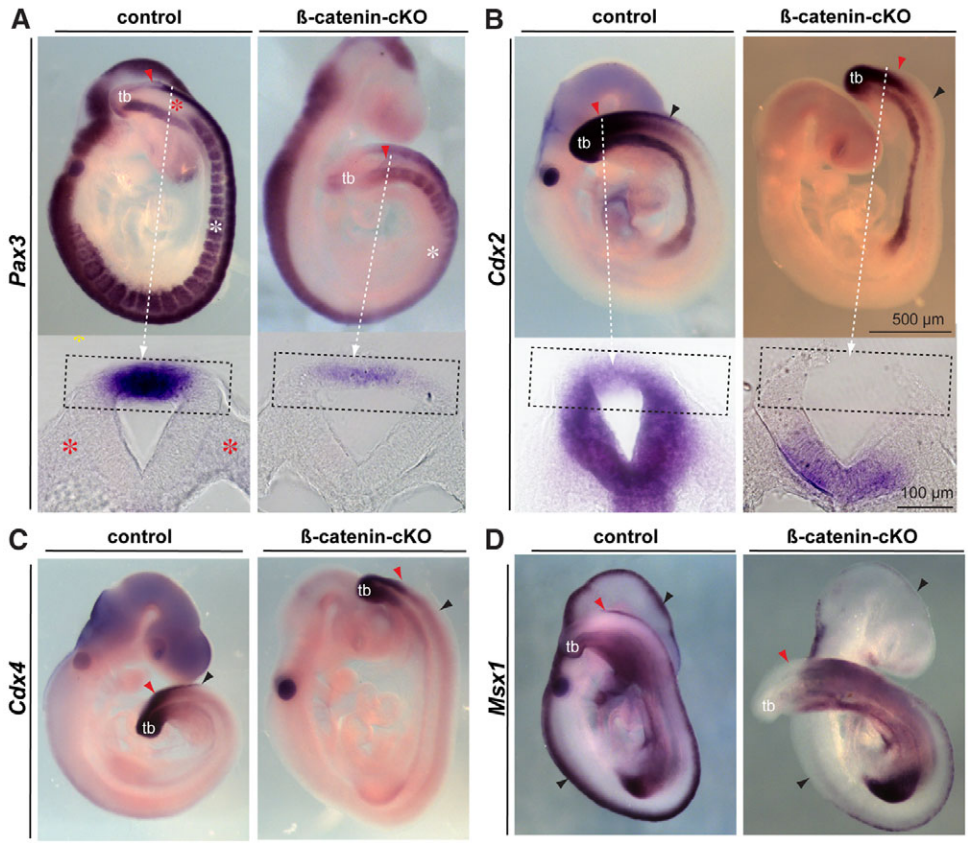

Fig. 3. Region-specific inactivation of transcription factors *Pax3* and *Cdx2* and related genes in the dorsal PNPs of β-catenin cKOs. (A,B) *Pax3* and *Cdx2* mRNA signals in wholemount embryos and transverse sections (dashed rectangles) in the closing PNP region (red arrowheads and dashed arrows) of heterozygous controls and β-catenin cKOs at E9.5. (C,D) *Cdx4* and *Msx1* mRNA signals in the dorsal PNPs of controls and β-catenin cKOs at E9.5. Note that *Pax3* mRNA signals in the cKO somites were absent in the anterior and middle (white asterisks in A) but remained in the posterior body axis; also note that *Pax3* mRNA signals were absent in the ventral PNP and paraxial mesoderm (red asterisks in A) around the closure site in normal control or cKO embryos. Black arrowheads, dorsal neural tube midline regions; red arrowheads, PNP closure site. tb, tail bud.

(GOF) of Pax3 (*Rosa26-loxP-stop-loxP-Pax3-cDNA*) (Wu et al., 2008) in the β-catenin cKO mutants. We found that six out of eight (75%) *β-catenin cKO;R26-Pax3-GOF* compound embryos [obtained by crossing *β-catenin*$^{(ex2\text{-}6)flox/flox}$*;Rosa26-loxP-stop-loxP-Pax3-cDNA*$^{+/-}$ with *β-catenin*$^{(ex2\text{-}6)flox/+}$*;Pax3*$^{Cre/+}$] had closed caudal neural tubes with restored Pax3 mRNA and protein in the dorsal PNP (Fig. 7A-D). Although *in situ* hybridization and immunolabeling for Pax3 demonstrate that the expression level of

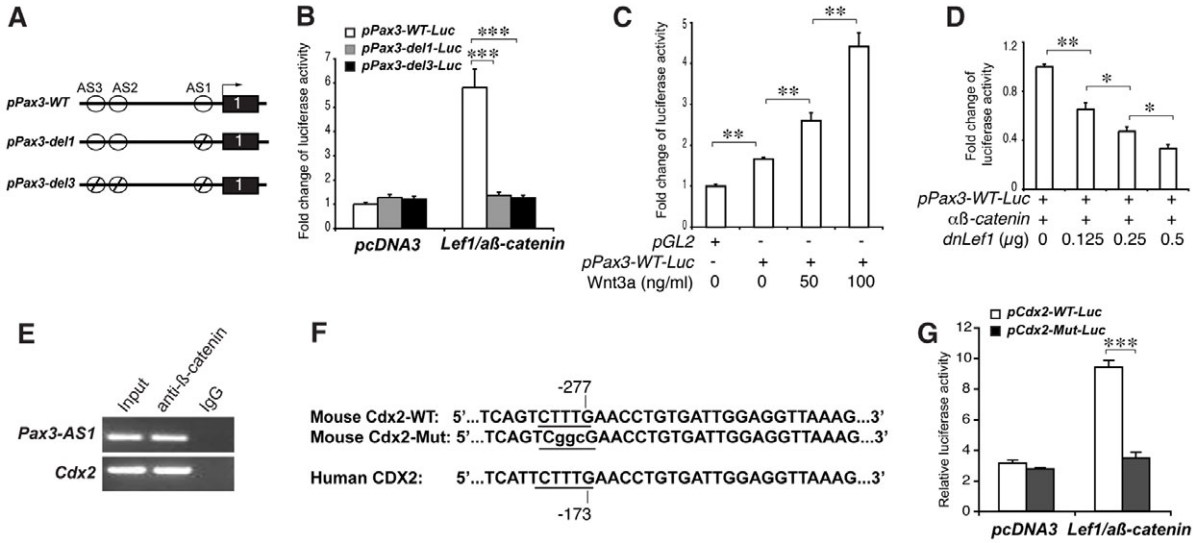

Fig. 4. Transcriptional activation of *Pax3* and *Cdx2* promoters by Wnt/β-catenin signaling. (A) Three putative Tcf/Lef1 binding activation sites (*AS1-AS3*) are present in the presumptive 5′ promoter region of the *Pax3* gene. The intact (WT) or deletions (∅) of the activation sites are indicated. (B) Luciferase reporter assays demonstrate the specific activation of the promoter with the wild-type, but not the deletion, of *AS1* or *AS1-AS3* after co-transfection with *Lef1* and active β-catenin (aß-catenin) cDNAs. (C) The dose-dependent activation of the *Pax3* promoter treated with various amounts of Wnt3a protein. (D) The dose-dependent repression of the intact *Pax3* promoter activity by dominant-negative (dn) Lef1. (E) Chromatin immunoprecipitation demonstrates the specific recruitment of the *Pax3 AS1* or the *Cdx2* promoter region by β-catenin antibodies, but not the non-specific IgG, from wild-type caudal neural tubes of E9.5 mouse embryos. (F) The wild-type and mutated Tcf/Lef1 binding sites in the mouse *Cdx2* promoter region, which is conserved in the human *CDX2* gene. (G) Luciferase reporter assays demonstrate the specific activation of the *Cdx2* promoter with the wild-type, but not the mutated, Tcf/Lef1 binding site by β-catenin signaling. **P*<0.05, ***P*<0.01, ****P*<0.001. Error bars indicate s.e.m.

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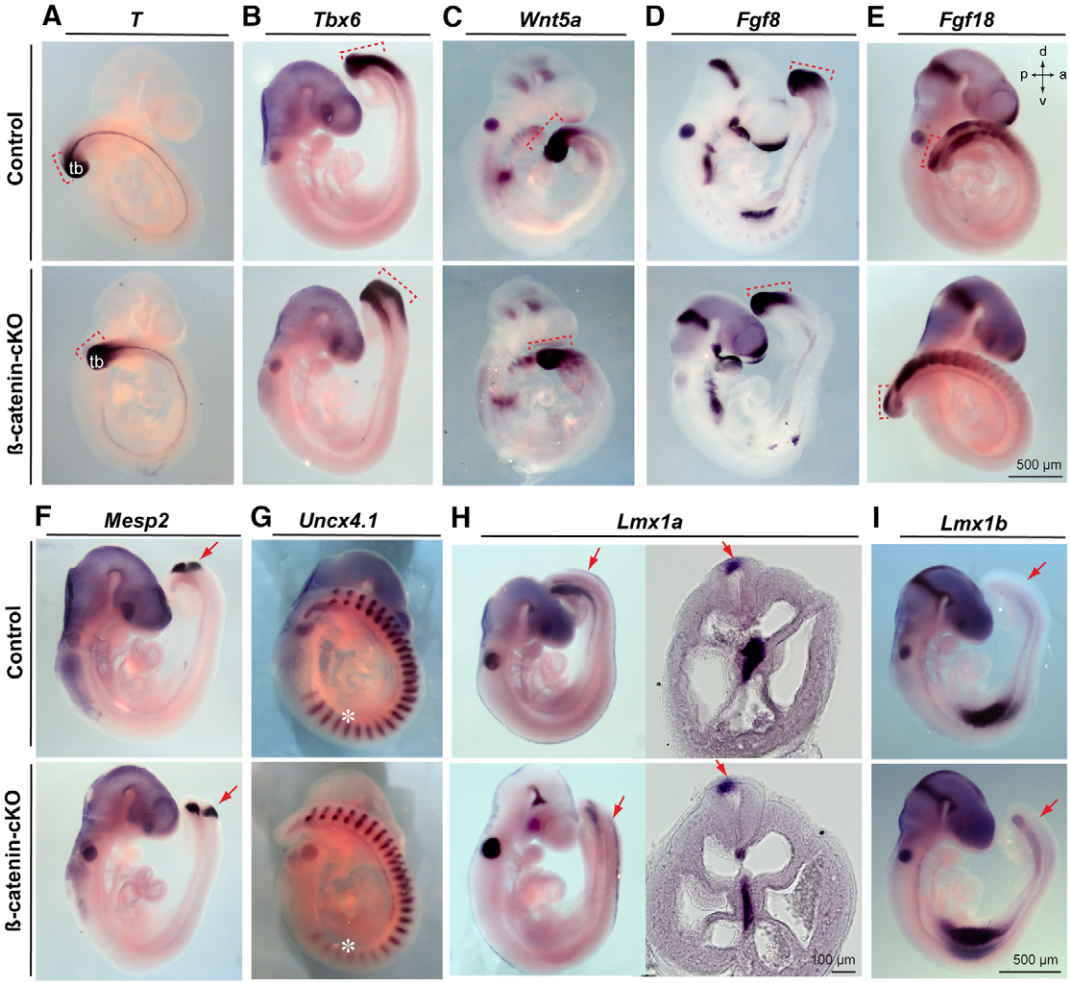

Fig. 5. Expression of patterning genes in the tail bud, somites or roof plate of β-catenin cKO embryos at E9.5 (~26 somite pairs). (A-E) Wholemount *in situ* hybridization reveals no obvious changes in *T*, *Tbx6*, *Wnt5a*, *Fgf8* and *Fgf18* mRNA signals in the mutant tail bud or adjacent PNP regions (dashed brackets). (F,G) *Mesp2* signals in the presomite mesoderm (arrows) and *Uncx4.1* signals in most late-born somites were apparently unchanged, whereas *Uncx4.1* signals were diminished in the early-born somites (asterisks) of the mutant embryos. (H) *Lmx1a* signals were unchanged throughout the roof plate of the whole embryo or in transverse section at the PNP region (arrows) of the mutant compared with the control. (I) *Lmx1b* signals in the cranial roof pate were unchanged in the mutant embryo and were not found at the PNP regions (arrows) of control and mutant embryos. tb, tail bud.

the *Pax3* cDNA single allele activated by Cre in the β-catenin cKO is lower than the normal level of endogenous Pax3 expression in control embryos (Fig. 7C,D and Fig. 3A), the ectopically activated Pax3 is able to direct PNP closure in the absence of β-catenin in the compound mutants. Interestingly, an abnormally thick roof plate is seen in the closed PNP of the *β-catenin cKO;R26-Pax3-GOF* embryos at E12.5 (Fig. 7D), which is likely to be related to the ectopic expression of *Pax3* cDNA in the mutant mice although the mechanism is as yet undetermined.

Pax3 might be required, but insufficient, for *Cdx2* expression in the dorsal PNPs

A recent study reports that Cdx2 is also involved in neural tube closure in addition to its established role in caudal body axis extension (Savory et al., 2011). We examined whether Cdx2 interacts with Pax3 in PNP closure. Taking advantage of the $Pax3^{Cre/+}$ knock-in mouse, we examined *Cdx2* expression in *Pax3*-null ($Pax3^{Cre/Cre}$) embryos that exhibit the known closure defects in the PNP. Significantly, *Cdx2* mRNA is lost in the dorsal PNP of *Pax3*-null embryos at E9.5 (Fig. 8A,B), indicating that *Cdx2* expression might also be regulated by Pax3. To address this possibility, we examined *Cdx2* expression in *β-catenin cKO;R26-Pax3-GOF* compound embryos. We detected no or little *Cdx2* mRNA signal in the dorsal PNP of the *β-catenin cKO;R26-Pax3-GOF* compound mutant (Fig. 8C), whereas we detected a mild expansion of the *Cdx2* expression domain in the dorsal PNP of the $Pax3^{Cre}$*;R26-Pax3-GOF* mutant (Fig. 8D) at E9.5. These results suggest that the ectopically activated Pax3 is unable to induce *Cdx2* expression in the absence of β-catenin in the dorsal PNP, and that the ectopically activated Pax3 can rescue the closure defects without Cdx2 activation in the β-catenin cKOs.

Taken together, this study suggests that β-catenin is required for caudal neural tube closure and elongation processes and may act through the transcriptional activation of key downstream target genes in the dorsal PNP as well as in the tail bud (Fig. 8E).

DISCUSSION

Pax3 mediates β-catenin signaling for caudal neural tube closure

With mouse genetic and molecular biological approaches, this study has revealed a novel function of β-catenin in neurulation. Conditional ablation of β-catenin in the Pax3-expressing dorsal

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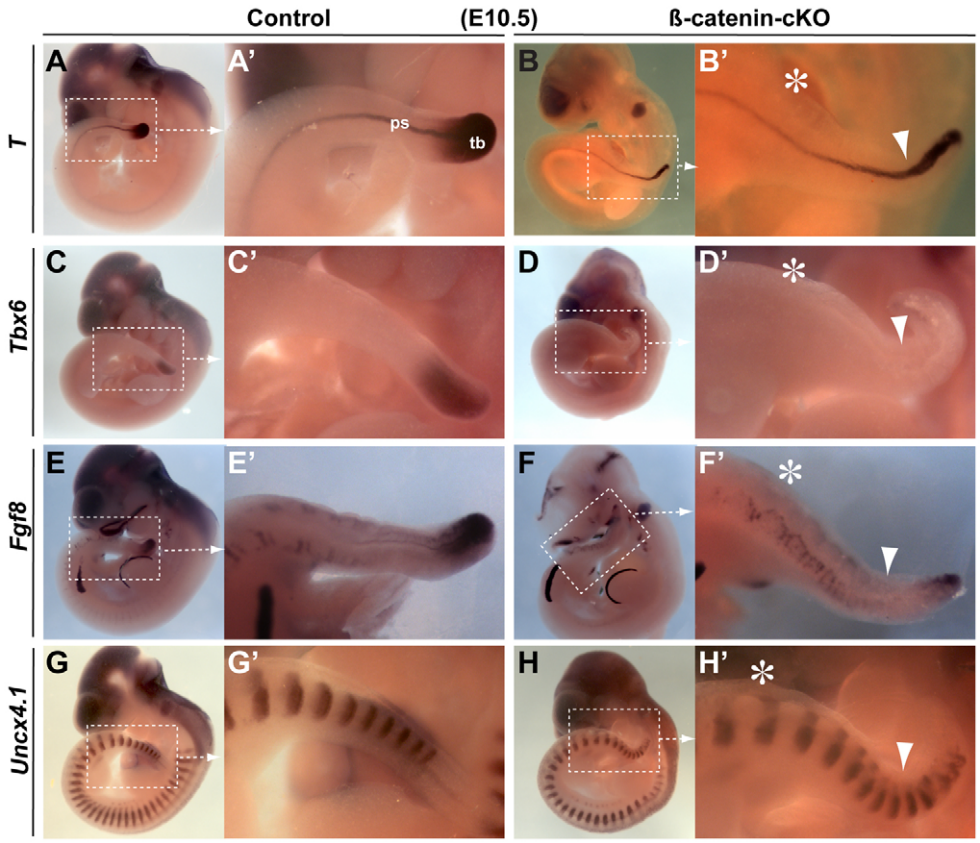

Fig. 6. Expression of known Wnt signaling target genes at the tail bud of E10.5 embryos (~37 somite pairs). (A-B′) Wholemount *in situ* hybridization reveals that, after neural tube closure is completed in the control embryo (A,A′), *T* mRNA signals were dramatically diminished at the tail bud (tb), but relatively unchanged in the primitive streak (ps), of the E10.5 β-catenin cKO embryo (B,B′). (C-D′) *Tbx6* signals were almost absent in the mutant tail bud. (E-F′) *Fgf8* signals were specifically diminished in the mutant tail bud. (G-H′) Somite marker gene *Uncx4.1* signals showed conserved numbers but apparently disrupted organization of mutant somites in the caudal body axis. Arrowheads, the regions of dorsally bent caudal body axis in the mutant embryos. Asterisks, the anterior-most opening sites of the caudal neural folds in the mutants.

neural tube/fold causes defective PNP closure and elongation and leads to spina bifida and tail truncation. These defects are associated with the diminished expression of *Pax3* and Cdx genes in the dorsal PNPs of β-catenin cKO embryos. Pax3 is expressed in the dorsal neuroepithelial cells along the entire anterior-posterior axis during the closure process (Goulding et al., 1991; Solloway and Robertson, 1999). A recent study suggests the association of *PAX3* mutations with spina bifida in humans (Agopian et al., 2013), which was not found in early studies (Greene et al., 2009; Lu et al., 2007). *Pax3* homozygous mutant mouse embryos exhibit both spina bifida and exencephaly (Engleka et al., 2005; Epstein et al., 1991), whereas the latter is not seen in the β-catenin cKO mutant. This difference might be related to the different mouse genetic approaches employed or to region-specific mechanisms. From the conditional gene-targeting experiment performed in this study, *Pax3* is expressed in the closing neural tube of the mutants prior to β-catenin ablation by $Pax3^{Cre/+}$. Hence, we observed a dramatic reduction, but not complete absence, of *Pax3* mRNAs in the dorsal PNPs of β-catenin cKOs at E9.5. The remaining Pax3 (expressed prior to β-catenin ablation) in the mutants is insufficient for PNP closure, but might be sufficient for cranial neural tube closure. Notably, when Pax3 protein is markedly reduced in heterozygous $Pax3^{Cre/+}$ embryos (Engleka et al., 2005), the single $Pax3^{Cre/+}$ or the double $\beta\text{-}catenin^{flox/+};Pax3^{Cre/+}$ heterozygotes do not exhibit NTDs, and are viable and fertile. Moreover, a single copy of the *Pax3* cDNA transgene induced by the same Cre is able to rescue the spina bifida in $\beta\text{-}catenin^{flox/flox};Pax3^{Cre/+}$ mutants. These results demonstrate that Pax3 is a key mediator of β-catenin signaling in the PNP closure process.

The cellular and molecular mechanisms of Pax3 function in neural tube closure remain largely unknown. Elevated apoptosis is found in the defective neural tube of Pax3-deficient *Splotch* mice (Phelan et al., 1997). Inhibition of p53-dependent apoptosis can reduce the NTD occurrence rate in these mutants (Pani et al., 2002), suggesting an anti-apoptotic role of Pax3 during neural tube closure. On the other hand, apoptosis occurs during normal neurulation and many other embryogenesis processes. Inhibition of caspase activity in chick embryos can prevent neural tube closure (Weil et al., 1997). By contrast, genetic and chemical inhibition of the caspase pathway in mouse embryos demonstrates that apoptosis is not required for the neural tube closure process (Massa et al., 2009). We did not detect any obvious change in apoptosis in association with the diminished *Pax3* expression in the mutant PNPs. However, future studies might determine whether inhibition of apoptosis can prevent NTDs in β-catenin cKO embryos. A chimeric mouse study has revealed that Pax3 acts cell-autonomously in the neural tube and may influence cell surface properties (which are as yet undefined) (Mansouri et al., 2001). An *in vitro* study has shown that Pax3 can induce cell aggregation and regulate phenotypic mesenchymal-epithelial interconversion (Wiggan et al., 2002). *Pax3* is also expressed in the secondary neural tube (Shum et al., 2010) that forms by secondary neurulation in the caudal axis at ~E10.5 (~31-somite stage) in mouse or at corresponding stages in chick embryos, just after primary neural tube closure has been completed (Cambray and Wilson, 2002; Gofflot et al., 1997; Shum and Copp, 1996). It is unclear whether β-catenin/Pax3 signaling is required for secondary neurulation and how this might contribute to spina bifida in the β-catenin cKO or related mutants. The cellular actions and underlying molecular mechanisms of β-catenin/Pax3 signaling in neural tube closure warrant further study.

Co-regulation of Cdx2 by β-catenin and Pax3 during neurulation

The current study shows that *Cdx2* expression is diminished in the dorsal PNPs of β-catenin cKOs or *Pax3*-null embryos.

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Significantly, ectopic activation of the *Pax3* transgene can rescue NTDs, but not tail truncations, in the β-catenin cKOs without *Cdx2* restoration in the dorsal PNPs. These results suggest that Cdx2 might be activated by the presence of both β-catenin and Pax3 at the closure site of the dorsal PNP, and that Cdx2 might play a less dominant role in PNP closure. Consistent with this, $Cdx2^{+/-};Cdx4^{-/-}$ mice exhibit truncated caudal axial skeletons, but no spina bifida (Young et al., 2009). Among Cdx genes, only *Cdx1* and *Cdx4* are known Wnt/β-catenin signaling targets (Béland et al., 2004; Lickert et al., 2000; Pilon et al., 2006). However, *Cdx1*-null and *Cdx4*-null mice are viable and fertile (Subramanian et al., 1995; van Nes et al., 2006). Intriguingly, *Cdx2*-null mice die at E3.5 (Chawengsaksophak et al., 1997), whereas *Cdx2;Cdx1* compound mutants exhibit the severe NTD craniorachischisis with reduced *Ptk7* expression (Savory et al., 2011). These results suggest that *Cdx2* has functions distinct from those of other Cdx genes and is crucial for initiating neural tube closure and also for PNP elongation. Our results show that *Cdx2* expression is regulated by β-catenin and possibly also by Pax3 during PNP development. Interestingly, a recent study reports that *Pax3* expression may be regulated by Cdx and Wnt during neural tube development (Sanchez-Ferras et al., 2012). These opposing observations suggest that Pax3 and Cdx2 might be interactively regulated by each other under the transcriptional control of β-catenin for the PNP closure and elongation processes (Fig. 8E). Future studies might determine whether genetic activation of *Cdx2* can rescue spina bifida in either *Pax3* or β-catenin mutants. Our results also demonstrate a dorsally bent and shorter PNP or posterior axis in the β-catenin mutant at E10.5, which may be caused by defective Pax3 and Cdx in the dorsal PNP, with relatively conserved signaling gene expression in the ventral axis and tail bud at E9.5. However, it remains unclear whether, or how, β-catenin/Pax3/Cdx signaling integrates the dorsal PNP closure and elongation processes. We also observed that tail bud signaling is relatively unaffected at E9.5 but diminished at E10.5 in the β-catenin mutant embryos, which furthers posterior axis disruptions.

Cell adhesion function of β-catenin in neurulation

β-catenin has dual roles in Wnt signaling and cell adhesion (Valenta et al., 2012), and the latter has recently been demonstrated to be important in cell integration in the caudal body axis. Conditional ablation of β-catenin with *Cdx1-Cre* in all three germ layers of the posterior embryo leads to a progressive disintegration and loss of posterior structures before caudal neural tube closure (Hierholzer and Kemler, 2010). The posterior disintegration in these mutant embryos is linked with the abnormal localization of N-cadherin (Hierholzer and Kemler, 2010). A more severe disintegration phenotype is observed in the N-cadherin-null embryo, which exhibits an abnormally weaving neural tube and undetermined phenotypes in PNP closure (Radice et al., 1997). However, our preliminary observations indicate that conditional ablation of N-cadherin in $Pax3^{Cre}$ knock-in mice (the same as used in this study) does not cause NTDs. These observations suggest that the cell adhesion function of β-catenin in the Pax3-expressing neuroepithelial cells might not be required for neural tube closure.

Wnt/β-catenin signaling versus PCP signaling during neural tube closure

β-catenin-independent PCP signaling acts through cytoskeletal dynamics to control cell polarity and oriented tissue movements. Single or combined mutations in mouse PCP signaling genes, such as *Celsr1* (Curtin et al., 2003), *Dvl1/2/3* (Etheridge et al., 2008; Hamblet et al., 2002; Wang et al., 2006a), *Fzd3/6* (Wang et al., 2006b), *Ptk7* (Lu et al., 2004), *Scrib* (Murdoch et al., 2003) and *Vangl2* (Kibar et al., 2001; Murdoch et al., 2001), will lead to the failure of initial neural tube closure and result in severe NTDs. The PCP-related Daam1-binding protein MIM is required for neural tube closure in *Xenopus* (Liu et al., 2011). Among these genes, Fzds and Dvls also act upstream of β-catenin in the canonical Wnt pathway. In addition, Lrp6, a Wnt co-receptor acting upstream of β-catenin

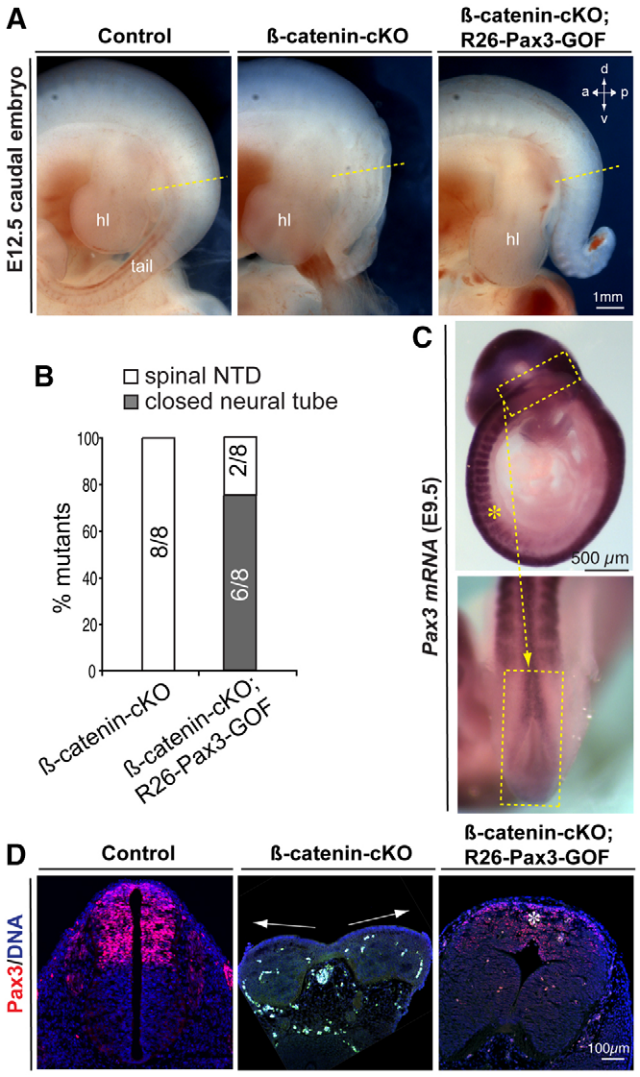

Fig. 7. Genetic activation of *Pax3* cDNA partly rescues spina bifida aperta in the β-catenin cKO. (A,B) The closure defects in 75% of mutant embryos (six out of eight β-catenin cKOs) were prevented by one-allele activation of *Rosa26-loxP-stop-loxP-Pax3-cDNA* (abbreviated as *R26-Pax3-GOF*) in compound mutants (*β-catenin cKO;R26-Pax3-GOF*), as shown at E12.5. (C) Sagittal and enlarged dorsal views of the restored *Pax3* mRNA signals in the dorsal PNP (dashed rectangles) of the *β-catenin cKO;R26-Pax3-GOF* embryo at E9.5. Note that the partially restored *Pax3* mRNA signals were also found in the somites around the middle neural tube region (asterisk). (D) Immunolabeling for Pax3 proteins on transverse PNP sections (approximate planes indicated by the dashed lines in A) at E12.5. Note that Pax3 immunolabeling was totally absent in the caudal neural tube section with defective closure (arrows in the middle panel in D) of the β-catenin cKO, but was detected in the rescued caudal neural tube section (although not as strong as in the control section). Also, note that the closure-rescued PNP has a thicker roof plate (asterisk in the right panel in D) than in the normal control. hl, hindlimb bud.

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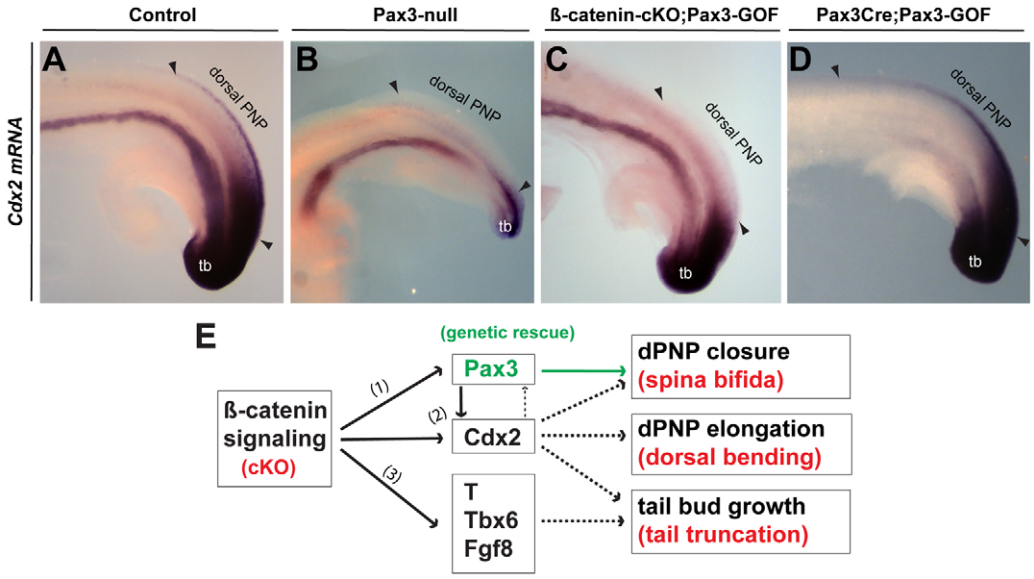

Fig. 8. Regulatory mechanisms among β-catenin, Pax3, Cdx2 and tail bud signaling genes during posterior neural tube closure and elongation processes. (A-D) *Cdx2* mRNA is also inactivated in the dorsal PNP domain (between the arrowheads) of the *Pax3*-null embryo (B) at E9.5; it cannot be restored by the conditional gain-of-function (GOF) of Pax3 in β-catenin cKOs (C) but is expanded in the dorsal PNP of the *Pax3*^{Cre}*;Pax3-GOF* embryo (D). (E) Summary of transcriptional regulation by β-catenin of *Pax3* expression (1) and possibly co-regulation by β-catenin and Pax3 of *Cdx2* expression (2) in the dorsal PNPs, and the regulation by Wnt/β-catenin signaling of *T*, *Tbx6* and *Fgf8* at the tail bud (3) during caudal neural tube closure and elongation processes. Solid arrows indicate interactions demonstrated in this study (1-3) and in the literature (3); dashed arrows indicate interactions demonstrated only in the literature. tb, tail bud; dPNP, dorsal posterior neuropore.

and also involved in convergent extension, is required for neural tube closure and caudal axis elongation (Carter et al., 2005; Kokubu et al., 2004; Pinson et al., 2000; Tahinci et al., 2007; Zhou et al., 2010). Although we did not detect significant alterations in representative PCP signaling gene expression patterns in the conditional β-catenin mutants, future studies are needed to examine whether PCP signaling functions are indirectly altered in β-catenin signaling mutants or whether β-catenin signaling is altered and contributes to NTDs in the PCP signaling mutants, including mutants of Fzds and Dvls.

In summary, this study revealed a novel function and underlying mechanism of β-catenin signaling in the transcriptional modulation of *Pax3* and *Cdx2* in the caudal neural tube closure and elongation processes, which might provide a basis for a better understanding of the cause and prevention of spina bifida and related disorders.

MATERIALS AND METHODS

Animals

The conditional *β-catenin*$^{(ex2\text{-}6)flox}$, the β-catenin signaling reporter *BATgal* and the *Pax3*^{Cre} knock-in mice were obtained through the Jackson Laboratory (Brault et al., 2001; Lang et al., 2005; Maretto et al., 2003). According to the Jackson Laboratory, these mouse strains were maintained on C57BL/6J or a mixed B6;129 background. The Cre-inducible *Rosa26-loxP-stop-loxP-Pax3-cDNA* (*Pax3-GOF*) mice obtained from the Epstein laboratory at the University of Pennsylvania were described previously (Wu et al., 2008). All research procedures using laboratory mice were approved by the UC Davis Animal Care and Use Committee and conform to NIH guidelines.

X-gal staining and wholemount *in situ* hybridization

Embryos were fixed in 1% paraformaldehyde (PFA) for ~30 minutes on ice and processed for X-gal staining as described previously (Song et al., 2009; Wang et al., 2011). Embryos fixed in 4% PFA overnight at 4°C were processed for wholemount *in situ* hybridization using digoxigenin-labeled antisense RNA probes (supplementary material Table S1). Wild-type (*β-catenin*^{flox}) or double-heterozygous (*β-catenin*$^{flox/+}$*;Pax3*$^{Cre/+}$) embryos were used for the normal controls, which showed no significant differences in X-gal staining or *in situ* mRNA signals. At least two mutants and two control embryos were used for each *in situ* experiment, which showed consistent results.

RNA isolation and real-time quantitative RT-PCR

Total RNAs were isolated from the caudal neural folds and pooled from three to five E9.5 embryos. Heterozygous *Pax3*$^{Cre/+}$ embryos were used as controls. After reverse transcription, real-time PCR was carried out as described (Song et al., 2009). The mRNA levels of β-catenin or *Axin2* were normalized to that of *Gapdh* to allow for comparisons among different experimental groups using the ΔCT method.

BrdU labeling and immunohistochemistry

Acute BrdU labeling was performed by intraperitoneal injection of BrdU at 100 mg/kg body weight of pregnant mice 1 hour prior to sampling. Immunohistochemistry was carried out on sections using primary antibodies against BrdU (1:100; Dako), active caspase 3 (1:200; Promega) and Pax3 (1:10; Developmental Studies Hybridoma Bank).

Luciferase reporter assay

The 1205 bp region from −1189 to +16 of the mouse *Pax3* gene promoter contains three presumptive Tcf/Lef activation sites (supplementary material Fig. S1). This 'wild-type' regulatory region was amplified by PCR and cloned into the pGL2-basic luciferase reporter vector. The mutated promoter constructs were generated by deleting one or three Pax3 activation sites (Fig. 4A). The 685 bp promoter-containing region (from −394 to +291) of the mouse *Cdx2* gene has one Tcf/Lef binding site that was also mutated for luciferase reporter assays (Fig. 4F). Similar to previously described studies (Song et al., 2009; Wang et al., 2011), L cells were transiently transfected with the wild-type or mutated promoter-driven luciferase reporters, together with the constitutively active β-catenin and wild-type *Lef1* expression constructs. A control expression vector (pcDNA) was introduced into L cells to monitor the baseline luciferase reporter activity. Varying amounts of the dominant-negative *Lef1* expression constructs or Wnt3a proteins were used for competitive repression or dose-dependent activation of the wild-type *Pax3* promoters. Twenty-four hours after transfection, luciferase activities

were measured using the Dual-Luciferase Assay Kit (Promega) as described previously.

Chromatin immunoprecipitation

Extracts were prepared from caudal tissue of five E9.5 wild-type mouse embryos. Chromatin extraction and immunoprecipitation were performed as previously described (Song et al., 2009; Wang et al., 2011). Anti-β-catenin antibody (Santa Cruz Biotech; sc-7199) was used to pull down the β-catenin–Tcf/Lef DNA-binding complex, and rabbit IgG (Invitrogen; 10500C) was used for the negative control. The activation sites were amplified with specific primers (supplementary material Table S1).

Statistical analyses

At least three controls and three mutant mice were used for each statistical evaluation. Significances were assessed by Student's *t*-test or pairwise (one-way ANOVA) when appropriate. In all cases, $P \leq 0.05$ were considered significant.

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Competing interests

The authors declare no competing financial interests.

Author contributions

T.Z. and Q.G. contributed to experimental design, collection and/or assembly of data, data analysis and interpretation and partial manuscript writing. A.S., R.N.T.L., Y.W., J.C. and J.X.H. contributed to the collection and/or assembly of data. D.E.P. edited the manuscript. J.A.E. provided a crucial mouse line. C.J.Z. conceived and supervised the study, collected and assembled data, analyzed and interpreted data and wrote the manuscript.

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Supplementary material

Supplementary material available online at
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