Cdx mediates neural tube closure through transcriptional regulation of the planar cell polarity gene Ptk7

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SUMMARY
The vertebrate Cdx genes (Cdx1, Cdx2 and Cdx4) encode homeodomain transcription factors with well-established roles in anteroposterior patterning. To circumvent the peri-implantation lethality inherent to Cdx2 loss of function, we previously used the Cre-loxP system to ablate Cdx2 at post-implantation stages and confirmed a crucial role for Cdx2 function in events related to axial extension. As considerable data suggest that the Cdx family members functionally overlap, we extended this analysis to assess the consequence of concomitant loss of both Cdx1 and Cdx2. Here, we report that Cdx1-Cdx2 double mutants exhibit a severely truncated anteroposterior axis. In addition, these double mutants exhibit fused somites, a widened mediolateral axis and craniorachischisis, a severe form of neural tube defect in which early neurulation fails and the neural tube remains open. These defects are typically associated with deficits in planar cell polarity (PCP) signaling in vertebrates. Consistent with this, we found that expression of Ptk7, which encodes a gene involved in PCP, is markedly reduced in Cdx1-Cdx2 double mutants, and is a candidate Cdx target. Genetic interaction between Cdx mutants and a mutant allele of Scrib, a gene involved in PCP signaling, is suggestive of a role for Cdx signaling in the PCP pathway. These findings illustrate a novel and pivotal role for Cdx function upstream of Ptk7 and neural tube closure in vertebrates.

KEY WORDS: Cdx, Craniorachischisis, Neural tube, Ptk7, Mouse

INTRODUCTION
The Cdx gene family (Cdx1, Cdx2 and Cdx4) encodes homeodomain transcription factors related to the Drosophila gene cad (cad). In the mouse embryo proper, Cdx genes are sequentially activated beginning at late streak stage (E7.5). Cdx1 (Meyer and Gruss, 1993) and Cdx2 (Beck et al., 1995) expression begins in the primitive streak region at E7.5, followed by Cdx4 expression (Gamer and Wright, 1993) at E8.5, with Cdx1 exhibiting the rostral-most limit of transcript distribution, followed by Cdx2 and Cdx4. This yields a nested, caudal-high, pattern of Cdx transcript distribution that is maintained in the caudal embryo until extinction of expression in the tail bud, with the loss first of Cdx1, followed by Cdx4 and Cdx2. Cdx1 and Cdx2 are also expressed in the intestinal hindgut epithelium in the embryo, with expression persisting throughout the life of the animal (Duprey et al., 1988; Meyer and Gruss, 1993; Beck et al., 1995; Chawengsaksophak et al., 1997; Silberg et al., 2000).

In Drosophila, cad is involved in the specification of the caudal embryo, as well as antero-posterior (AP) patterning (Mlodzik et al., 1985; Macdonald and Struhl, 1986; Mlodzik and Gehring, 1987; Kispert et al., 1994). Functional studies in the mouse have revealed that vertebrate Cdx genes are similarly involved in AP patterning, and that they mediate these effects via direct regulation of Hox gene expression (Shashikant et al., 1995; Subramanian et al., 1995; Epstein et al., 1997; Isaacs et al., 1998; van den Akker et al., 2002; Houle et al., 2003; Chawengsaksophak et al., 2004; Tabaries et al., 2005; Pilon et al., 2007; Savory et al., 2009; Young et al., 2009).

More recent work has also demonstrated Hox-independent mechanisms for Cdx function. In this regard, we and others have used conditional mutagenesis approaches to circumvent the peri-implantation lethality associated with the Cdx2 null mutation. This has led to the finding that Cdx2 controls crucial aspects of intestinal endoderm patterning and posterior development, and that both of these programs appear to be governed, at least in part, via Hox-independent pathways (Gao et al., 2009; Savory et al., 2009a; Grainger et al., 2010).

Other than the homeodomain, and limited residues involved in protein processing, Cdx members exhibit little sequence similarity, suggesting functional specificity (Marom et al., 1997). However, analysis of compound Cdx mutants (van den Akker et al., 2002; van Nes et al., 2006; Young et al., 2009) as well as gene substitution experiments (Savory et al., 2009b), argue that Cdx members are functionally similar. Given this, and the extensive overlap of expression in the caudal embryo between all three Cdx members, we generated and assessed Cdx1-Cdx2 double null mutants (referred to hereafter as Cdx1/2 DKO for simplicity). The phenotype of these mutants suggests previously unsuspected roles for Cdx function in events related to somitogenesis and neural tube closure.

The central nervous system (CNS) develops from the neural tube through the process of neurulation. The neural tube results from the folding and midline fusion of the neural plate. In mammals, this folding occurs sequentially and is nucleated at different levels of the body axis (Copp et al., 2003; Copp and Greene, 2010). Midline fusion is first initiated at the base of the future hindbrain, called closure 1, at around E8.5. Two subsequent de novo closure sites then initiate, with closure 2 at the forebrain/midbrain boundary and closure 3 at the rostral extremity of the forebrain. Continuation of closure between these latter two sites completes cranial closure, whereas the caudal spread of fusion from closure 1, which continues as the embryo elongates, culminates with closure at the posterior neural pore (Copp et al., 2003).
Neural tube defects (NTDs) comprise a group of congenital malformation resulting from a failure in neurulation. NTDs affect around 1 in 1000 pregnancies and are the second most common malformation after congenital heart defects (Mitchell, 2005). The phenotypes of NTDs vary depending on the region of the neural tube affected. For example, incomplete closure of the cranial neural tube leads to anencephaly, whereas disruption of posterior neuropore closure produces spina bifida (Copp et al., 2003; Copp and Greene, 2010). Failure of closure 1 leads to the most severe NTD, craniorachischisis (CRS), in which the neural tube remains open along its entire length. CRS accounts for 10% of all NTDs (Moore et al., 1997), and of the more than 200 mouse mutants with NTDs, 14 genes have been implicated in CRS (Harris and Juriloff, 2007; Harris and Juriloff, 2010). Of these, all but one is implicated in the planar cell polarity (PCP) signaling pathway.

PCP, which dictates the organization of cells within the plane of the epithelium, was first identified in Drosophila (Montcouquiol et al., 2006; Jones and Chen, 2007; Simons and Mlodzik, 2008) and is governed by a highly conserved core group of genes, including frizzled (fz), flamingo/starry night (ftn); stan – FlyBase), disheveled (dsh), strabismus (stb)/Van Gogh (vang) and prickle (pk). The PCP pathway also includes components of the Wnt signaling pathway (Montcouquiol et al., 2006) but is independent of β-catenin and is therefore considered a non-canonical arm of the Wnt pathway. Orthologs of the Drosophila PCP genes are present in vertebrates, and genetic analyses have confirmed that an intact PCP signaling pathway is required for shaping the post-gastrulation embryo, including neural tube closure (Wallfingard et al., 2000; Darken et al., 2002; Jessen et al., 2002; Keller, 2002).

Here, we report that Cdx1/2 DKO mutants exhibit CRS, along with additional defects consistent with a role for Cdx signaling upstream of PCP. In agreement with this, Cdx1/2 mutants interacted genetically with a Scrib mutant allele known to impact PCP. Finally, Cdx1/2 DKO mutants appear to regulate the expression of Ptk7 directly, a gene known to be involved in PCP signaling, as evidenced by expression analysis, chromatin immunoprecipitation and transcription assays, suggesting a mechanism by which Cdx impacts on PCP signaling. Together, these findings provide evidence indicative of a novel and crucial role for Cdx members in neural tube closure.

MATERIALS AND METHODS

Generation of mutant mice

Cdx2f/f, Cdx1–+/– and CMV-β-actin-Cre-ERT2 (Cre-ERT2) mice have been previously described (Subramanian et al., 1995; Santagati et al., 2005; Savory et al., 2009a). Cdx1/2 DKO mutants were generated by crossing Cdx2f/f and Cdx2f/f;Cre-ERT2 lines into the Cdx1–/+ background. Cdx1–/+ Cdx2f/f; Cre-ERT2 male embryos were mated with Cdx1–/–Cdx2+/f females, pregnant females were administered a single 2 mg dose of tamoxifen at E5.5 as described previously (Savory et al., 2009a) and embryos harvested at E8.5-E10.5 for analysis. Heterozygous ScribCRC males (Murdoch et al., 2001b; Murdoch et al., 2003), obtained from the Jackson Laboratories, were crossed with Cdx1–/+Cdx2f/f;Cre-ERT2 females to generate Cdx1–/+ Cdx2f/f; Cre-ERT2 Scrib–/+ males, which were mated with Cdx2f/f or Cdx1–/–Cdx2f/f females. Pregnant females were treated either as above, or with a single 3 mg dose of tamoxifen at E8.5 and embryos collected for analysis at E15.5.

Embryo collection and analysis

Animals were mated overnight and noon of the day of detection of a vaginal plug was considered as E0.5. Pregnant females were treated with tamoxifen by oral gavage as above, embryos dissected in PBS, yolk sacs collected for genotyping and embryos fixed overnight in 4% paraformaldehyde (PFA) at 4°C. Whole-mount in situ hybridization was performed as described previously (Houle et al., 2000; Pilon et al., 2007; Savory et al., 2009a) using probes generated from previously described plasmids: Wnt3a (Takada et al., 1994), T (Wilkinson et al., 1990), Cyp26a1 (Abu-Abed et al., 2003), Uncx4.1 (Uncx – Mouse Genome Informatics) (Mansouri et al., 1997), Sox2 (Kamachi et al., 1998), Pax3 (Goulding et al., 1991), Moxl (Mox1 – Mouse Genome Informatics; ATCC EST, IMAGE 3984366), Paraxis (Tef15 – Mouse Genome Informatics; ATCC EST, IMAGE 5143248), Vangl2 (ATCC EST, IMAGE 6509008), Dvl1 (ATCC EST, IMAGE 3583393), Dvl2 (ATCC EST, IMAGE 6402000), Scrib (ATCC EST, IMAGE 5721250) and Ptk7 (ATCC EST, IMAGE 30619280). In all cases, embryos to be compared were processed in parallel with control for variations in signal intensity.

Morphometric analysis

Embryos were dissected in PBS, fixed in 4% PFA overnight and then washed and photographed in PBS. Stage-matched embryos were measured at identical magnification, using a minimum of three embryos of each genotype.

Chromatin immunoprecipitation (ChIP) analysis

ChIP assays from embryonic material were performed as described previously (Pilon et al., 2006; Savory et al., 2009a) using polyclonal antisera to Cdx2 (Savory et al., 2009b). Rabbit pre-immune serum and an anti-Gal4 antibody (Santa Cruz) were employed as negative controls. PCR was performed using the primers: S’-TGACCGCGCTCTCCA-CACCCC-3’ and 5’-GCCGCCCTCTCTCCGCTGC-3’.

Transfection analysis

An 883 bp fragment S’ to the Ptk7 translation start site was PCR amplified from mouse genomic DNA, verified by sequencing and subcloned into pXP2 (Nordeen, 1988; Jung et al., 2002) to generate a luciferase-based reporter construct. The effect of Cdx on the activity of this promoter was assessed by transfection analyses in P19 embryo carcinoma cells as previously described (Pilon et al., 2006; Savory et al., 2009a).

Semi quantitative RT-PCR

Semi-quantitative RT-PCR was performed as previously described (Savory et al., 2009a). Primers for Cdx1, Cdx2, Cdx4 and β-actin have been described previously (Savory et al., 2009b). The Ptk7 fragment was amplified using the primers: S’-GGAGATCTCAAAACAGTTCCC-3’ and 5’-GCCGGTCCTTGGGGTTGC-3’.

RESULTS

Ablation of Cdx1 and Cdx2 leads to loss of Cdx4 expression

To generate embryos lacking both Cdx1 and Cdx2, Cdx2f/f; Cre-ERT2 lines (Santagati et al., 2005; Savory et al., 2009a) were crossed into the Cdx1–/+ background (Subramanian et al., 1995). Pregnant females from Cdx1–/+ Cdx2f/f; Cre-ERT2 × Cdx1–/–Cdx2f/f matings were administered a single 2 mg dose of tamoxifen at E5.5 by oral gavage; this treatment results in complete loss of Cdx2 protein (Savory et al., 2009a). Cdx1/2 double mutants (Cdx1/2 DKO) were recovered at E8.5-E10.5 at the expected ratio.

We, and others, have reported reduction of Cdx4 expression in Cdx2-null mutants (Chawengsaksophak et al., 2004; Savory et al., 2009a). As cross- and autoregulation have been reported among Cdx family members (Lorentz et al., 1997; Trinh et al., 1999; Prinos et al., 2001; Beland et al., 2004; Chawengsaksophak et al., 2004), we assessed the expression of Cdx4 by whole-mount immunohistochemistry in Cdx1/2 DKO, and found that Cdx4 was lost in the Cdx1/2 DKO mutants at E8.5 (Savory et al., 2011). This suggests that the Cdx1/2 DKO phenotype described below may be due to loss of all Cdx function.
**Cdx1/2 DKO mutant embryos exhibit severe axial elongation defects**

Cdx1/2 DKO mutants died at midgestation (E10.5), and exhibited a foreshortened AP axis and CRS, a severe NTD in which the entire neural tube remains open (Fig. 1B,C). Relative to Cdx2 conditional null mutants, Cdx1/2 DKO offspring exhibited a more severe truncation with axis elongation ending rostral to the forelimb bud region (Fig. 1B) (Savory et al., 2009a). This is consistent with the co-expression of Cdx family members in the posterior embryo, and their previously demonstrated functional overlap (van den Akker et al., 2002; Davidson and Zon, 2006; van Nes et al., 2006; Faas and Isaacs, 2009). However, none of the previously described Cdx mutants reported to date exhibit neural tube defects (Subramanian et al., 1995; Chawengsaksophak et al., 2004; Savory et al., 2009a; van den Akker et al., 2002; van Nes et al., 2006; Young et al., 2009), suggesting previously unsuspected roles for Cdx function (see below).

Axis truncation in Cdx2−/− conditional null embryos is associated with reduced expression of several Cdx target genes required for presomitic mesoderm (PSM) ontogenesis, including Wnt3a, T and Cyp26a1 (Savory et al., 2009a). We therefore assessed the expression of these targets to determine whether the increased severity of the axial defect in Cdx1/2 DKO manifested through convergent transcriptional regulation of these pathways. Wnt3a is expressed in the tailbud and PSM, and functions to maintain a pool of paraxial mesoderm precursor cells and to specify paraxial mesoderm in conjunction with T and Tbx6 (Chapman et al., 1996; Dunty et al., 2008; Galceran et al., 1999; Greco et al., 1996; Herrmann et al., 1990; Takada et al., 1994; Yamaguchi et al., 1999; Yoshikawa et al., 1997; Aulehla et al., 2007). Cyp26a1 mediates retinoic acid catabolism in the tailbud, establishing a region deplete of retinoid signaling believe to be necessary to prevent precocious differentiation (Abu-Abed et al., 2003; Sakai et al., 2001). Both Wnt3a and T were essentially completely extinguished in the tailbud of E8.5 Cdx1/2 DKO (Fig. 2B,D), in contrast to the Cdx2-null mutant background, where they were only modestly affected at this stage (Savory et al., 2009a). T expression was also maintained in the notochord of the mutants (Fig. 2D), consistent with Cdx1/2 function exclusively in the tailbud. Cyp26a1 expression was also severely compromised at E8.5 in the Cdx1/2 DKO background (Fig. 2F), although it is also significantly impacted by loss of Cdx2 alone at this stage (Savory et al., 2009a).

**Somite defects in Cdx1/2 DKO mutants**

Somites are derived from segmentation of the PSM in the caudal embryo. Once formed, somites differentiate into different compartments along the dorsoventral and mediolateral axes; dermal precursor cells and skeletal muscles are derived from the dorsally located dermamyotome, whereas the ventrally located sclerotome gives rise to bone and cartilage. Each somite is further subdivided into rostral and caudal compartments (Dubrulle and Pourquie, 2004). In the Cdx1/2 DKO mutants, somites appeared to be poorly condensed and irregularly shaped, as well as wider and...
paraxial mesoderm is specified in mutants embryos (Fig. 3J). Taken together, these data indicate that contrast, dermamyotome of condensed somites at E8.5 (Fig. 3I). By contrast, expression was markedly reduced in Pax3 mutants (Fig. 3B,D).

We next examined whether somite patterning was affected by loss of Cdx. In control embryos, Uncx4.1 was expressed as regular reiterations in the caudal somite compartment at E9.5 and E8.5 (Fig. 3E,G). By contrast, expression was markedly reduced in Cdx1/2 DKO at E9.5 (Fig. 3F). Moreover, although Uncx4.1 was detected in the double mutants at E8.5, its expression was lost in anterior somites, whereas transcripts in posterior somites exhibited an irregular and often fused appearance (Fig. 3H, arrow). Finally, Pax3 was expressed in the dorsal neural tube and in the dermamyotome of condensed somites at E8.5 (Fig. 3I). By contrast, Pax3 was expressed as a single, bilateral, stripe in the mutants embryos (Fig. 3J). Taken together, these data indicate that paraxial mesoderm is specified in Cdx1/2 DKO mutants, although somite segmentation and rostral-caudal somite patterning is defective.

**Cdx function is essential for neural tube closure**

Neural tube closure normally initiates at three sites along the rostrocaudal axis, with failure of the initial event at the midbrain-hindbrain boundary resulting in CRS (Copp et al., 2003). Neural tube closure is preceded by the extension of the AP axis with concomitant narrowing of the mediolateral axis (convergence). These morphogenetic movements are essential for neural tube closure, and are believed to rely, at least in part, on PCP signaling. During this process, the reshaping of the neural plate brings the neural folds in close juxtaposition to facilitate fusion. Impairment of these movements leads to an embryo that is typically shorter and wider, with the consequence being that the neural folds are spaced more widely apart, impinging on events necessary for neural tube closure (see Copp and Greene, 2010; Copp, 2003).

To determine whether Cdx1/2 DKO mutants exhibited defects characteristic of lesions in PCP-dependent morphogenetic movements, we measured the length and width of embryos over the window (four to eight somites) immediately preceding neural tube closure (Fig. 4A-C). Consistent with other CRS mutants (Garcia-Garcia et al., 2008; Wang et al., 2006a; Ybot-Gonzalez et al., 2007; Wallingford et al., 2002), we found a significant reduction in the length-to-width ratio (LWR) in Cdx1/2 DKO mutants relative to controls. Although control embryos from four to seven somites exhibited a continuous increase in LWR, no such increase was observed in the mutants (Fig. 4A,C). Whereas a foreshortened AP axis in the mutants may be ascribed, in part, to the premature termination of axis elongation, as seen in Cdx2−/− embryos, neither a concomitant widening of the mediolateral axis nor an open neural tube is seen in these single null mutants (Savory et al., 2009a).

CRS mouse mutants typically exhibit an abnormal broadening of the midline, which has been proposed to be due to defects in convergence-extension movements in some instances (see Ybot-Gonzalez et al., 2007; Yen et al., 2009). We performed a histological examination of transverse sections of Cdx1/2 DKO at E8.5 to assess midline morphology. In control embryos, a sharp bend in the midline of the neural plate at the median hinge point is evident in the caudal embryo (Fig. 4D, arrow). By contrast, in Cdx1/2 DKO mutants two apparent hinge points were evident on either side of a broadened midline (Fig. 4E arrows). In addition, the mutant neuroepithelium appeared thicker and both the neuroectoderm and mesoderm appeared disorganized, with an absence of the dorsal aorta and hindgut diverticulum (Fig. 4E).

The domain of genes expressed in the floor plate, such as Shh and T are broadened in a number of mutants exhibiting CRS, such as Dvl1/2 double mutants and Vangl2 mutants (Greene et al., 1998; Yen et al., 2009). Although the neural plate appeared wider in the Cdx1/2 DKO, both Shh and T were expressed in the midline

![Fig. 3. Cdx function is required for somite patterning. (A-J) Expression of Mox1, Paraxis (Tcf15), Uncx4.1 (Uncx) and Pax3 in control (A,C,E,G,I) and Cdx1/2 DKO mutant (B,D,F,H,J) embryos at E9.5 (A-F) and E8.5 (G-J). Clear somite boundaries are lacking in the mutants (B,D,F,H,J).](image)

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of mutants in a manner similar to controls (Fig. 2C,D; Fig. 5A,B) at E8.5 and E9.5. However, the expression domain of Shh was markedly broader in the peri-nodal region in Cdx1/2 DKO mutants relative to controls (Fig. 4F-H). The expression of the neural markers, Sox2 and Hes5, were also mediially expanded (Fig. 5C-F), consistent with the broadened neural plate in the mutants, and confirming that neuroectoderm was specified.

**Cdx1/2 DKO mutants and PCP signaling**

Of the 14 known genes that cause CRS in mouse, all but one encode members involved in PCP signaling (see Harris and Juriloff, 2007; Harris and Juriloff, 2010). Such PCP mutants also typically exhibit broadened and foreshortened somites (Lu et al., 2004; Curtin et al., 2003; Murdoch et al., 2001a; Murdoch et al., 2003; Wang et al., 2006a; Wang et al., 2006b), wider neural plates with a broadened midline (Greene et al., 1998; Lu et al., 2004; Wang et al., 2006a; Ybot-Gonzalez et al., 2007), a shortened and widened body axis as well as disruption of the stereociliary bundle orientation in the organ of Corti (Lu et al., 2004; Montcouquiol et al., 2003; Wang et al., 2006b), and in the hindgut diverticulum (Kibar et al., 2001). Scrib and other PCP components exhibit a broad and dynamic expression pattern during neurulation with the highest levels of expression in the posterior embryo (Lu et al., 2004).

Although the early lethality of Cdx1/2 DKO offspring precluded examination of the stereociliary bundle orientation in the organ of Corti, which is likely to be unaffected given the expression domain of Cdx members, the phenotypic commonalities between Cdx1/2 DKO mutants and known PCP mouse mutants is consistent with a crucial role for Cdx genes upstream of PCP signaling. In agreement with this, Cdx genes and many PCP components exhibit overlapping expression domains in the caudal embryo (Kibar et al., 2001; Klingensmith et al., 1996; Lohnes, 2003; Lu et al., 2004; Murdoch et al., 2003; Sussman et al., 1994). To begin to determine whether Cdx function impacted on transcription of PCP members, we compared the expression of several PCP genes between Cdx1/2 DKO and control embryos. Core PCP components, defined as those that are required for PCP in multiple tissues, include Vangl2, Dvl1/2, Ptk7 and Celsr1; several lines of evidence also indicate that Scrib plays key roles in PCP signaling (Cobard et al., 2009; Kallay et al., 2006; Montcouquiol et al., 2003; Montcouquiol et al., 2006; Murdoch et al., 2001) as does Ptk7 (Lu et al., 2004; Paudyal et al., 2010; Shnitsar and Borchers, 2008).

**Vangl2 is a Cdx target gene**

In Vangl2-null mutants, axis elongation is normal prior to neural tube closure (Gerrelli and Copp, 1997), while Dvl1/2 double mutants have a foreshortened body axis but relatively normal somite morphology (Wang et al., 2006a). By contrast, Ptk7-null mutants have defects in both axis elongation and in somite morphology by the five- to seven-somite stage (Lu et al., 2004),...
and is therefore the PCP mutant which most closely phenocopies the Cdx1/2 DKO phenotype. Moreover, unlike Dvl1/2 and Vangl2 mutants, neither Cdx1/2 DKO nor Ptk7-null embryos exhibit broadened floor plate gene expression patterns (Lu et al., 2004). In addition, Ptk7 expression was downregulated at E7.5 in Cdx1/2 DKO mutants, preceding initiation of neural tube closure (see Fig. S1 in the supplementary material), and consistent with in situ hybridization analysis at E8.5 (Fig. 6J).

Cdx and Ptk7 interact genetically with Vangl2 (see Lu et al., 2004; Paudyal et al., 2010) and is an essential regulator of neural tube closure in the mouse (Yen et al., 2009). Binding site algorithms identified several Cdx response elements in an 883 bp region of 5' flanking sequence (Fig. 7A), which has previously been shown to be functional as a promoter for human PTK7 (Jung et al., 2002). This sequence is 100% conserved between mouse and human. Consistent with this, ChIP analysis revealed occupancy of this promoter region by Cdx2 in E8.5 embryos (Fig. 7B). A functional relationship is further supported by transfection assays in P19 cells, which illustrated that these promoter sequences respond to both Cdx1 and Cdx2 (Fig. 7C). Taken together, these data are entirely consistent with Ptk7 as a direct Cdx target, and suggest a mechanistic basis by which Cdx regulates neural tube closure.

### Cdx and Scrib interaction

Scrib loss-of-function mutants exhibit CRS and other defects typical of PCP signaling mutants (Murdoch et al., 2001b) and have been shown to interact genetically and physically with other members of the PCP pathway (Montcouquiol et al., 2003; Montcouquiol et al., 2006b; Wansleeben et al., 2010). We therefore investigated the relationship of Cdx and PCP via analysis of an allelic series of Cdx-Scrib mutants.

Following tamoxifen-mediated deletion of Cdx2 at E5.5, Cdx1+/Cdx2+/Scrib+/+, Cdx1+/Cdx2+/Scrib+/ and Cdx1+/Cdx2+/Scrib++/E9.5 offspring were found to have modestly shortened AP axes, but did not display CRS (data not shown). LWR measurements at the seven-somite stage likewise revealed no significant difference in the Cdx-Scrib backgrounds relative to controls (see Fig. S2A in the supplementary material). It is possible that the single copy of Cdx1, and concomitant partial recovery of Cdx4 activity in these mutants (see Fig. S2B,C in the supplementary material), elicits sufficient Ptk7 expression to effect neural tube closure in the face of reduced Scrib function.

Genetic interaction between Ptk7 and Vangl2 has been suggested by an increase in the incidence of spina bifida, which manifests at later stages of development (Lu et al., 2004). However, we were unable to assess such NTD due to the lethality evoked by Cdx2 loss at mid-gestation (Chawengsaksophak et al., 2004; Savory et al., 2009a). We therefore treated pregnant females at E8.5 with a suboptimal dose of tamoxifen to circumvent lethality. This regimen reduced the severity of the Cdx2 mutant phenotype (Savory et al., 2009a), and offspring examined at E15.5 (n=36) did not exhibit spina bifida. However, most (90%) of the Cdx1+/Cdx2–/–/Scrib+/–
embryos exhibited a shortened or curly tail (e.g. arrow in Fig. 8D). This phenotype was seen in only one of seven Cdx1+/–Cdx2–/– mutants (arrow in Fig. 8B), and in none of the Scrib heterozygotes. Although the suboptimal tamoxifen treatment at E8.5 probably accounts for the relatively mild phenotype in the Cdx1+/–Cdx2–/–Scrib+/– mutants, nevertheless, these data are consistent with an interaction between Cdx function and the PCP pathway.

**DISCUSSION**

Central effectors in planar cell polarity are crucial to several fundamental events in vertebrate development, including neural tube closure (Montcouquiol et al., 2006a; Simons and Mlodzik, 2008). Although considerable information has emerged regarding PCP pathway members, our understanding of the nature of upstream transcriptional regulators of these core constituents is lacking. We used the Cre-loxP system to delete Cdx2 in the context of the Cdx1-null mutant background, and found that the resulting Cdx1/2 DKO offspring exhibited features consistent with an impact on PCP signaling, including CRS associated with a foreshortened and broader axis. Additional analyses suggest that the PCP member Ptk7 is a direct Cdx target gene, suggesting a novel role for Cdx function in regulating neural tube closure.

**Cdx members functionally overlap**

Functional overlap among Cdx members has been suggested by the finding that compound mutants typically exhibit more pronounced vertebral patterning phenotypes than seen in the corresponding single mutant backgrounds (van den Akker et al., 2002; Young et al., 2009). In addition, gene substitution approaches have shown that Cdx2 can fulfill Cdx1 function in regulating Hox gene expression and cervical vertebral patterning (Savory et al., 2009b). The phenotype of the Cdx1/2 DKO mutants described herein further highlights the functional overlap among Cdx family members, as neither cognate single mutant exhibits NTD (Chawengsaksophak et al., 2004; Savory et al., 2009a; Subramanian et al., 1995). This functional commonality is probably due to convergent regulation of common target genes, as evidenced by the marked reduction of expression of the Cdx targets T and Wnt3a in the double mutants, compared with Cdx2 single mutants.
This supposition is also consistent with ChIP studies which place all three Cdx members on these, and other, target promoters in vivo (Savory et al., 2009a).

Cdx genes exhibit extensive overlapping in expression in the caudal embryo. Given that Cdx4 expression is essentially lost in the Cdx1/2 DKO background, it is possible that the Cdx1/2 DKO phenotype is reflective of complete loss of Cdx function. However, Cdx4-null mutants appear morphologically normal and Cdx4 function is only apparent in the context of compound mutant backgrounds (van Nes et al., 2006). Thus, the contribution of Cdx4 to the Cdx1/2 DKO phenotype may be relatively modest.

Cdx function and neural tube closure

The importance of PCP signaling in neural tube closure, and in other developmental paradigms, has become increasingly clear with the characterization of loss-of-function mutants in various vertebrate models, including the mouse (Curtin et al., 2003; Darken et al., 2002; Jessen et al., 2002; Lu et al., 2004; Murdoch et al., 2003; Wang et al., 2006a; Wang et al., 2006b), Xenopus (see Tada and Smith, 2000; Wallingford et al., 2000) and zebrafish (see Jessen et al., 2002; Marlow et al., 1998). In both Xenopus (Darken et al., 2002; Tada, 2000; Wallingford et al., 2002) and zebrafish (see Hammerschmidt et al., 1996; Jessen et al., 2002; Marlow et al., 1998; Sepich et al., 2000; Solnica-Krezel et al., 1996), the PCP pathway impacts on convergent extension (CE), leading to CE-related defects such as a wider and shorter axis, a wider notochord and CRS. Although the morphogenic movements that create the elongated AP axis in the mouse are not as well characterized, the phenotypic similarities between these CE mutants and those of PCP mouse mutants suggest a similar mechanism at play. Indeed the wider midline in Vangl2 mutants has been attributed to a defect in CE (Ybot-Gonzalez et al., 2007), while Ptk7 mutants have been suggested to have a lesion in mesodermal CE (Yen et al., 2009).

A significant reduction in LWR is associated with neural tube closure defects in both Xenopus (Wallingford et al., 2002; Wallingford and Harland, 2002) and in several mouse PCP mutants (Wang et al., 2006a; Ybot-Gonzalez et al., 2007). A marked reduction in LWR was also observed in Cdx1/2 DKO embryos prior to normal initiation of neural tube closure. Although a decrease in the length of the DKO mutants may be due to impaired axial elongation, the increase in width cannot be explained by such a deficiency. In this regard, Cdx2 mutants exhibit precarious termination of axial extension, but no discernable medial widening or neural tube defects (Savory et al., 2009a). The reduced LWR in the Cdx1/2 DKO mutants is thus consistent with disruption of PCP function, and is entirely congruent with the other phenotypic commonalities shared by Cdx1/2 DKO and mouse PCP mutants, including a broadened midline and foreshortened somites (Garcia-Garcia et al., 2008; Greene et al., 1998; Lu et al., 2004).

Cdx and PCP interactions

Non-allelic non-complementation is typically taken as evidence for functional interaction between two gene products operating in a common process, and has been widely employed to position putative PCP components (Lu et al., 2004; Montcouquiol et al., 2003; Qian et al., 2007; Satoh et al., 2008; Wang et al., 2006a). We further assessed the relationship between Cdx and PCP by generation and analysis of Cdx1/2 and Scrib compound mutants (Murdoch et al., 2003); the Scrib mutant has been shown to interact with the Vangl2 mouse mutant in similar assays (Montcouquiol et al., 2003; Murdoch et al., 2001b).

We found a marked and highly significant increase in the incidence of caudal defects in Cdx-Scrib mutant offspring relative to individual Cdx or Scrib mutant backgrounds. Although the phenotype of the Cdx-Scrib mutants is relatively modest, owing to the necessity to effect late Cdx2 ablation, it nonetheless clearly illustrates an interaction between Cdx and a gene that interacts with core PCP members, and is comparable to the previously described interactions between Scrib and Vangl2 mutants. It is also apparent that minimal Cdx function suffices for neural tube closure, as CRS and other PCP-related defects were not observed in Cdx1/–/– Cdx2+/– offspring, nor in any other Cdx compound mutant reported to date.

Cdx-dependent regulation of PCP pathway members

PCP is governed by a set of conserved ‘core’ proteins as well as a number of upstream effectors, including non-canonical Wnts (Montcouquiol et al., 2006; Simons and Mlodzik, 2008). We surveyed the expression of a number of these genes in Cdx1/2 DKO embryos, and found that the transcript levels of Dvl1, Dvl2 and Ptk7 were all reduced in the mutant background, whereas the expression of Vangl2 and Scrib appeared to be unperturbed. The close phenocopy between the Cdx1/2 DKO and Ptk7 mutants prompted further investigation, leading to our finding that Cdx2 occupied the Ptk7 promoter in vivo, and that this promoter could be transcriptionally regulated by Cdx members in transfection assays. Although Ptk7 is not considered to be a core PCP gene, axis elongation fails in Ptk7 mutants, and the genetic interaction between Ptk7 and Vangl2 mutants indicate that they function in a common genetic pathway to regulate PCP (Lu et al., 2004). Taken together, these findings are entirely consistent with Cdx impacting on PCP, at least in part, via regulation of Ptk7.

Novel roles for Cdx in the mouse

Cdx transcription factors are well documented to affect vertebral patterning through regulation of Hox genes. Recent work has also identified Hox-independent functions for Cdx genes in both intestinal patterning and hindgut specification (Gao et al., 2009; Grainger et al., 2010) as well as in the ontogenesis of presomitic mesoderm and axial extension (Savory et al., 2009a). Our results reveal an additional novel role for Cdx members upstream of PCP in the mouse. Morpholino knockdown of Cdx genes in Xenopus results in posterior defects, but such manipulations have not been reported to impact on PCP (Faas and Isaacs, 2009), nor has a role for Cdx genes in PCP signaling been reported in zebrafish. These findings suggest either that a role for Cdx in PCP signaling is unique to the mouse or, more likely, that sufficient Cdx function remains in these other models to support PCP. Taken together with prior findings, our present observations place Cdx in a hierarchy wherein they coordinate the development and patterning of caudal mesodermal and endodermal derivatives, as well as PCP-dependent morphogenesis necessary for neural tube closure.

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Competing interests statement

The authors declare no competing financial interests.
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Supplementary material

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