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Cdx1 and Cdx2 are functionally equivalent in vertebral patterning

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ABSTRACT

The Cdx transcription factors regulate anterior–posterior (AP) vertebral patterning, at least in part, through direct regulation of *Hox* gene expression. Analysis of allelic series of *Cdx* mutant mice suggests functional overlap between these family members. However, the lack of a Cdx2 null mutant makes these analyses incomplete. Moreover, Hox proteins are sometimes redundant, making it difficult to discern whether Cdx members regulate identical *Hox* target genes in a redundant manner, or whether they regulate separate *Hox* genes which then converge on events related to vertebral patterning. To more directly assess this question, we developed a "knock in" model whereby *Cdx2* was substituted for *Cdx1*. Consistent with functional redundancy *Cdx2* "knock-in" mice exhibited perfect complementation of the *Cdx1*-null phenotype, as evidenced by the lack of skeletal defects or altered expression of *Hox* genes typically impacted by *Cdx1* loss-of-function.

It has been proposed that vertebral AP patterning is reliant on a gradient of the sum total of Cdx proteins, a posit that is consistent with functional redundancy between Cdx family members. To further assess this, we generated a gain-of-function model using BAC trangenesis to alter Cdx1 dosage. *Cdx1* BAC transgenic mice overexpressed *Cdx1* mRNA and protein, and fully complemented the *Cdx1* null allele. However, gain of Cdx1 dosage via this BAC transgene in an otherwise wild type background had no discernible effects on vertebral patterning or *Hox* gene expression, suggesting that a moderate alteration in the Cdx protein gradient is of no consequence.

Introduction

Hox gene products play key roles in patterning the vertebrate anteroposterior (AP) axis (McIntyre et al., 2007; van den Akker et al., 2001; Wellik, 2007). The 39 murine *Hox* genes are distributed in four clusters, *Hoxa* to *d* and encode transcription factors possessing a conserved 60 amino acid DNA-binding motif, the homeodomain (Gehring, 1993). In the mouse, *Hox* transcripts are first detected at embryonic day 7.5 (E7.5) in the primitive streak region with expression subsequently expanding anteriorly in the neural tube and mesoderm to reach a characteristic rostral limit (Deschamps et al., 1999; Oosterveen et al., 2003; Roelen et al., 2002). The onset, and eventual rostral limit, of expression of a given *Hox* gene is generally related to its chromosomal location within a cluster, with 3′ members expressed earlier and reaching a more anterior limit than 5′ members (Kondo and Duboule, 1999). This colinear pattern of expression results in staggered domains of *Hox* gene expression along the AP axis, with the combination of *Hox* genes expressed at a specific AP level believed to reflect a "Hox code" essential for normal vertebral patterning.

Somites are generated from the condensation of unsegmented paraxial mesoderm, and arise in an anterior to posterior order. Somites subsequently differentiate into dermamyotome and sclerotome, the latter being the anlage of the vertebrae and ribs. The morphological differences characteristic of many vertebrae necessitate patterning along the AP axis. Heterotopic grafting assays have revealed that such patterning is retained in vertebrae derived from unsegmented paraxial mesoderm (Kieny et al., 1972; Nowicki and Burke, 2000), indicating that patterning is imposed during or shortly after gastrulation. In this regard, such grafts retain *Hox* expression patterns typical of their original axial position, consistent with the concept that the cues upstream of Hox-dependent AP patterning operate early in vertebral ontogenesis (Christ et al., 1974; Christ and Ordahl, 1995; Kieny et al., 1972; Nowicki and Burke, 2000). These findings are also concordant with studies suggesting that the Hox proteins themselves establish their typical AP axial pattern of expression by influencing ingression during gastrulation (Iimura and Pourquie, 2006).

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Given their central role in numerous ontogenic processes, considerable work has focused on understanding the molecular mechanisms involved in establishing *Hox* expression. In the mouse, members of the vertebrate *Cdx* family, *Cdx1*, *Cdx2* and *Cdx4*, have emerged as important players in this programme (Beck et al., 1995; Chawengsaksophak et al., 1997; Gamer and Wright, 1993; Subramanian et al., 1995; Lohnes, 2003). *Cdx* genes encode homeodomain transcription factors related to the *Drosophila* gene *caudal*. Beginning at late streak stage (E7.5), *Cdx* genes are sequentially activated and subsequently form nested sets of expression domains with a caudal high distribution in the developing embryo. Expression of all *Cdx* members is extinguished in the caudal embryo around E12.5, although *Cdx1* and *Cdx2* are expressed at later stages in the posterior portion of the endoderm (Beck et al., 1995; Gamer and Wright, 1993; Meyer and Gruss, 1993).

Considerable work supports a role for Cdx members upstream of *Hox* genes in both the mesoderm and neuroectoderm. In particular, loss-of-function studies in the mouse have shown that *Cdx* genes are essential for normal vertebral AP patterning. Moreover, the vertebral defects seen in single and compound *Cdx* mutants closely phenocopy certain *Hox* null mutants, consistent with posterior shifts in the rostral limit of expression of relevant *Hox* genes (Chawengsaksophak et al., 1997; Subramanian et al., 1995; van den Akker et al., 2002). The finding of functional *Cdx* binding sites in the promoter of these, and other, *Hox* genes (Charité et al., 1998; Shashikant et al., 1995; Subramanian et al., 1995; Tabaries et al., 2005), some of which can impose spatial regulation *in vivo* (Charité et al., 1998; Shashikant et al., 1995), strongly supports a role for Cdx members in direct regulation of *Hox* expression.

Although considerable data supports a direct relationship between Cdx function and *Hox* gene expression, it is unclear precisely how the three Cdx members contribute to the spatial–temporal pattern of *Hox* expression. At least two, not necessarily exclusive, models have been proposed. One model suggests that a posterior-high gradient of Cdx protein differentially impacts on *Hox* activation, while the second suggests that Cdx members possess intrinsic differences in their ability to regulate a given *Hox* gene (Charité et al., 1998; Epstein et al., 1997; Gaunt, 2000; Gaunt et al., 2004). In support of the former model, several groups have reported caudal-high expression patterns of both *Cdx* mRNA (Gamer and Wright, 1993; Marom et al., 1997; Morales et al., 1996) and protein (Gamer and Wright, 1993; Gaunt et al., 2005; Meyer and Gruss, 1993). In addition, gain-of-function studies in mouse, chick and *Xenopus*, together with the haploinsufficiency seen in Cdx1 and Cdx2 heterozygous offspring, are consistent with Cdx members regulating *Hox* expression in a dose-dependent manner (Bel-Vialar et al., 2002; Charité et al., 1998; Chawengsaksophak et al., 1997; Ehrman and Yutzey, 2001; Epstein et al., 1997; Isaacs et al., 1998; Subramanian et al., 1995). Finally, the analysis of an allelic series of Cdx loss-of-function models is also consistent with functional redundancy between Cdx members, and a dosage-dependent mechanism of *Hox* gene regulation by these transcription factors (van den Akker et al., 2002; van Nes et al., 2006).

To investigate further the means by which the Cdx members regulate AP vertebral patterning, we developed and assessed two novel models. The first addressed the question of functional redundancy between Cdx1 and Cdx2, and utilized a "knock in" approach whereby Cdx2 was substituted for Cdx1. The second model addressed the consequence of increasing the hypothetical Cdx gradient using BAC transgenesis to increase Cdx1 dosage while maintaining the regulatory circuitry implicated in *Cdx1* expression. The knock-in mouse model exhibited completely normal vertebral morphology, consistent with functional equivalency between *Cdx1* and *Cdx2* in patterning paraxial mesoderm. The BAC-transgenic gain-of-function model likewise exhibited no overt vertebral defects, suggesting that increasing Cdx dosage also has no impact on *Hox* expression or vertebral patterning.

Materials and methods

Generation of Cdx2 "knock-in" mice

Using genomic sequences previously described (Houle et al., 2003), standard cloning methods were used to insert the *Cdx2* cDNA open reading frame into the first exon of the *Cdx1* locus such that translation initiated at the *Cdx1* ATG. A floxed *neo* selectable marker was subsequently cloned in the first intron to generate the targeting vector which was linearized by ScaI restriction and electroporated into RI ES cells. G418 resistant colonies were expanded and screened for homologous recombination by genomic Southern blot analysis using 5′ and 3′ probes external to the targeting vector. Two targeted clones were used to generate germ line chimaeras by standard means, following which the floxed *neo* cassette was excised by crossing the heterozygous founders with a CMV Cre deletor. Heterozygous knock-in offspring were subsequently intercrossed to yield homozygous offspring expressing *Cdx2* from *Cdx1* regulatory sequences, designated $Cdx1^{2ki/2ki}$ hereafter.

Cdx1 BAC-transgenic mice

Two BACs harboring *Cdx1* sequences were recovered from a mouse Sv129 strain library (Roswell Park Cancer Institute, Buffalo, USA) by conventional hybridization methods. Inserts were confirmed by end-sequencing. Pulsed field electrophoresis and Southern blot hybridization were utilized to assess the integrity of the inserts. One was chosen for subsequent studies.

Cdx1 BAC transgenic lines were generated by microinjection according to standard procedures, yielding three founders identified by Southern blot analysis of tail biopsy DNA using a Not I fragment from the BAC backbone vector pBACe3.6 (Frengen et al., 1999) as a probe. Homozygous BAC offspring generated from two of these lines by heterozygous intercross were identified by quantitative Southern blot analysis and confirmed by test breeding. Both lines gave indistinguishable results.

Whole mount in situ hybridization and skeletal analysis

For *in situ* hybridization analysis, mice were mated overnight and noon of the day of vaginal plug detection was considered as E0.5. *In situ* hybridization and skeletal preparations were performed as previously described (Allan et al., 2001; Prinos et al., 2001). Embryos to be compared were processed in parallel to control for variation in signal intensity and stage-matched according to established criteria. Probes for *in situ* hybridization were generated from previously described plasmids: *Hoxb3* (Manley and Capecchi, 1995) and *Hoxd4* (Featherstone et al., 1988).

Western immunoblotting and whole mount immunohistochemistry

Rabbit polyclonal anti-Cdx1, anti-Cdx2 and anti-Cdx4 antibodies were generated by immunization with GST fusion protein using sequences N-terminal to the Cdx homeodomains (Cdx1 amino acids 1 to 159; Cdx2 amino acids 1–179; Cdx4 amino acids 1–172). The specificity of antibody preparations was assessed by Western blot analysis of COS7 cell extracts prepared from cells transfected with expression vectors for Cdx1, Cdx2 or Cdx4. The Cdx1 expression vector has been described previously (Beland et al., 2004). Cdx2 and Cdx4 expression vectors were generated by subcloning the relevant coding sequences into a modified pCEP4 (Invitrogen) or pSG5 (Stratagene) plasmids, respectively.

For Cdx protein quantification in embryos, tissue lysates were prepared from pooled, staged matched, E8.5 embryos. Extracts were run on 12% SDS/PAGE gels and then transferred to Immobilon-P

(Millipore) membranes. Membranes were blocked for 1 h with 5% skim milk in PBS:0.1% Tween 20 and incubated with antibody solution overnight at 4 °C. Secondary antibodies were HRP-conjugated anti-rabbit or anti-mouse IgG (Santa Cruz Biotechology) with detection by ECL (Perkin Elmer) according to the manufacturer's instructions. The fold overexpression of Cdx protein was calculated by scanning densitometry using the NIH ImageJ programme (NIH), using β-actin (mAbcam8226) as a loading control. Similar results were obtained from two separate experiments.

Whole mount immunohistochemistry was performed on E8.5 embryos as previously described (Qiu et al., 1997) using stage matched embryos. Note that all embryos for a given antibody were processed in parallel to control for relative signal intensity.

Semi-quantitative RT-PCR

RNA extracted from E8.5 embryos using Trizol reagent (Invitrogen) was used to generate cDNA by standard methods. cDNA was subsequently amplified by PCR using oligonucleotides for Cdx1 (F:5′-CCCTACGAATGGATGCGGCGC-3′; R:5′-AAGCCAGCTGCCC-AGCATTCAAGT-3′), Cdx2 (F:5′-GACGCTCAACCTCGGCCCC-3′;R:5′-GCTTCAGACCACGGGAGGGGT-3′), Cdx4 (F:5′-CCCGGGAAGA-CTGGAGTACATAC-3′; R:5′-GTGAAGAAAGCGTTAGGCAGTTCTCC-3′) or β-actin (F:5′-AGCCATGTACGTAGCCATCC-3′; R:5′-CTCTCAGCTGTGGT-GGTGAA-3′) as a loading control. PCR products were resolved by electrophoresis on a 1.5% agarose gel, quantified by densitometry and normalized relative to β-actin. PCR was performed in triplicate from independent samples.

Results

Cdx1 and Cdx2 are functionally equivalent

Cdx1 null mutants and Cdx2 heterozygous offspring exhibit vertebral homeoses. The specific region affected reflects the differential onset of expression of these transcription factors, with Cdx1 loss impacting on more anterior vertebral elements relative to Cdx2 (Chawengsaksophak et al., 2004; Subramanian et al., 1995). Subsequent analysis of Cdx1–Cdx2 compound mutants, however, suggests a considerable degree of functional overlap between these family members (van den Akker et al., 2002; van Nes et al., 2006). In this regard, it is notable that Hox proteins can exhibit considerable functional redundancy in vertebral patterning (Chen et al., 1998; Davis and Capecchi, 1996; Fromental-Ramain et al., 1996; Horan et al., 1995, Wellik and Capecchi, 2003). It is therefore difficult to conclude that the Cdx compound mutant phenotype is indicative of functional redundancy per se or is the result of convergent input from different, Cdx-type specific, targets.

To more precisely address the question of functional redundancy between Cdx members, we utilized a "gene swap" approach to insert the *Cdx2* open reading frame into the first exon of the *Cdx1* locus. This was done such that Cdx2 translation utilized the Cdx1 start codon, and is therefore governed by *Cdx1* regulatory elements including any 5′ UTR-dependent mechanisms; Cdx1 translation cannot occur due to loss of the initiator codon. Mice generated from this line, denoted $Cdx1^{2ki/2ki}$ in the homozygous form (Fig. 1), were viable and fertile with the mutant allele transmitted at a Mendelian frequency.

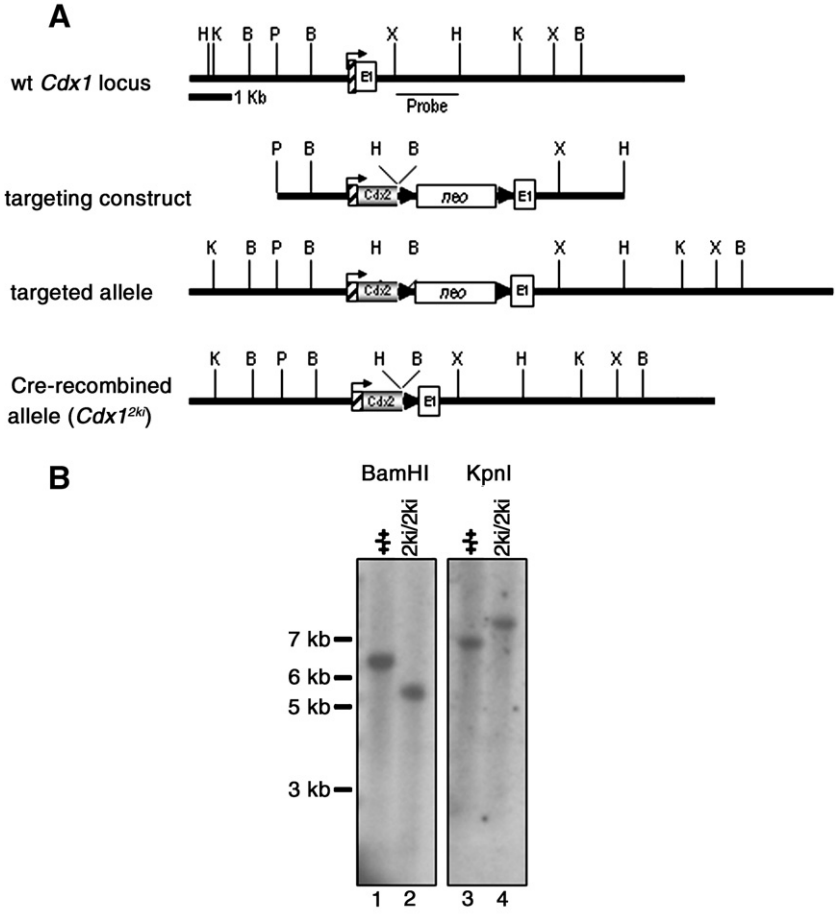

Fig. 1. Insertion of *Cdx2* cDNA into the *Cdx1* locus. (A) Schematic representation of the *Cdx1* locus. The targeting construct, the targeted allele and the expected Cre recombination product are shown below. E1, exon 1 of *Cdx1*; B, BamHI; K, KpnI; H, HindIII; P, PstI; X, XhoI. (B) Multi-enzyme Southern blot demonstrating the fidelity of targeting.

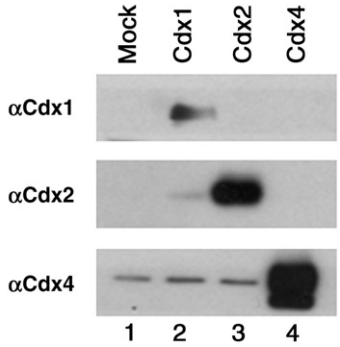

Fig. 2. Specificity of Cdx antibodies. COS7 cells were transfected with expression vectors encoding Cdx1, Cdx2 or Cdx4 and lysates analyzed by Western blotting using antibodies raised against the N-terminus of each Cdx family member.

To characterize the models used in this study, antibodies were generated against all three Cdx proteins using sequences N-terminal to the homeodomain; this region exhibits limited sequence homology between family members (Marom et al., 1997). Analysis of these antibodies by Western blot revealed only a small degree of cross-reactivity of the Cdx2 antibody with Cdx1, and modest non-specific interaction with the Cdx4 antibody (Fig. 2).

Immunohistochemical analysis of Cdx1 in E8.5 wild type embryos was consistent with previous studies, with protein observed in the caudal embryo and in the neurectoderm with an anterior limit in the hindbrain region (Fig. 3A). Also notable was a diffuse staining in the cranio-facial region (bracket in Fig. 3A), consistent with prior description of *Cdx1* in presumptive neural crest cell populations (Meyer and Gruss, 1993). This staining is specific, as no signal was observed in the $Cdx1^{2ki/2ki}$ background which is anticipated to be null for *Cdx1* (Fig. 3B). Signal was also absent in *Cdx1* null mutants at comparable stages (data not shown) further validating the specificity of this antibody.

Wild type E8.5 embryos also exhibited a caudal-high distribution of Cdx2. However, in contrast to Cdx1, Cdx2 was not observed in more rostral domains (Fig. 3C). These observations are consistent with prior work, which have shown that *Cdx2* exhibits a more restricted pattern of expression along the AP axis relative to *Cdx1* (Beck et al., 1995).

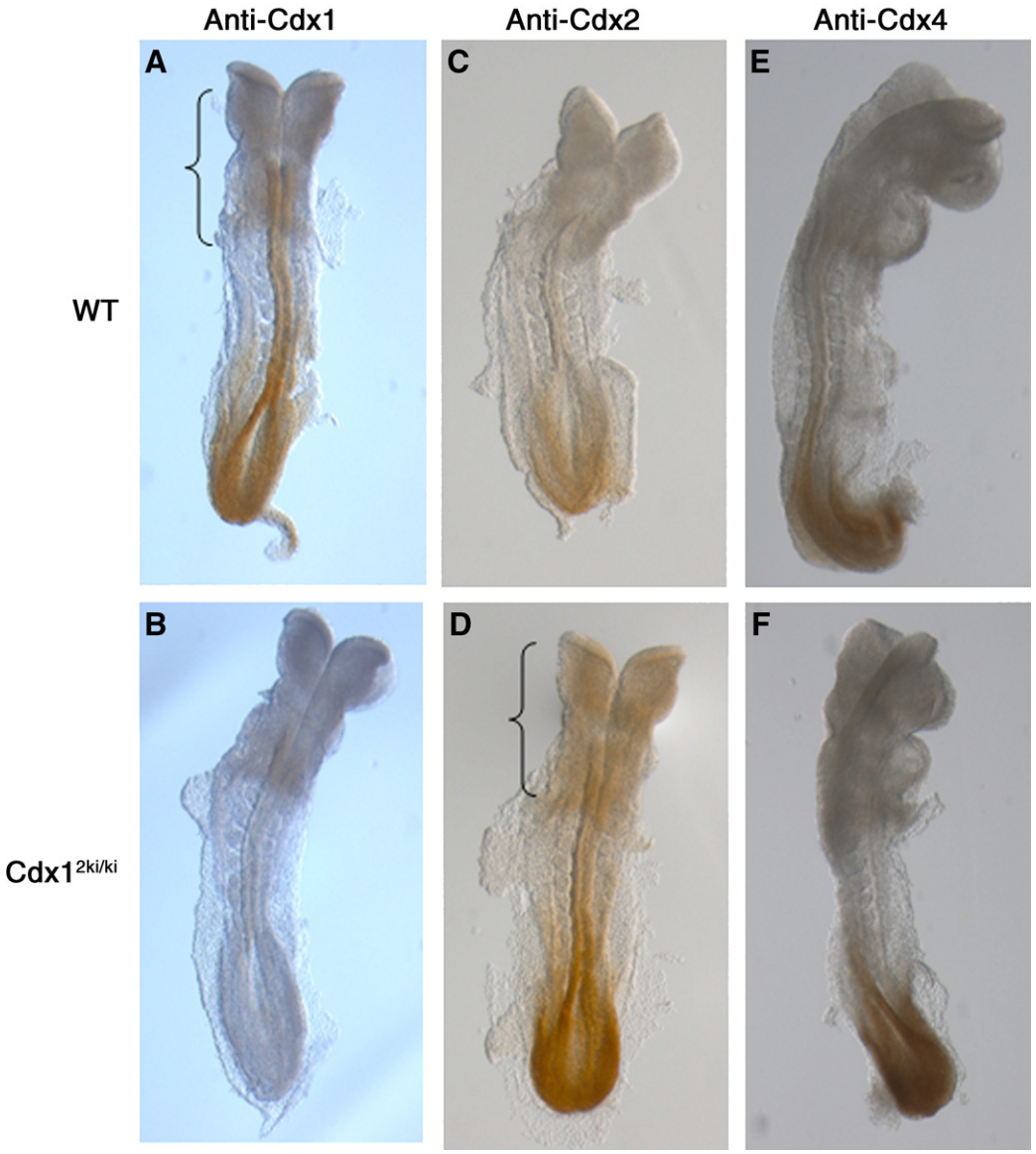

Fig. 3. Cdx2 recapitulates Cdx1 distribution in $Cdx1^{2ki/2ki}$ embryos. Cdx1, Cdx2 and Cdx4 protein expression in wild type (A, C, E) and $Cdx1^{2ki/2ki}$ (B, D, F) embryos revealed by whole mount immunohistochemistry. Bracket in D indicates the expansion of the Cdx2 expression domain in the $Cdx1^{2ki/2ki}$ embryos. Note also the loss of Cdx1 expression in the $Cdx1^{2ki/2ki}$ background in panel B.

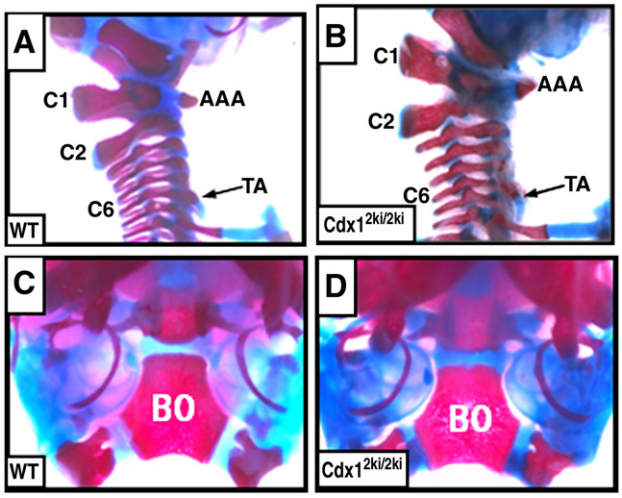

Fig. 4. Skeletal morphology of *Cdx1*$^{2ki/2ki}$ mice. Lateral (A and B) and ventral (C and D) view of the cervical region of whole-mount skeletal preparations from wild-type (A, C) and *Cdx1*$^{2ki/2ki}$ (B, D) E18.5 offspring. Note the normal cervical regions of *Cdx1*$^{2ki/2ki}$ (B, D) skeletons. Abbreviations: AAA, anterior arch of the atlas; TA, tuberculum anterior; C, cervical vertebrae; BO, basioccipital.

Moreover, and consistent with the fidelity of the targeting exercise, Cdx2 expression in *Cdx1*$^{2ki/2ki}$ embryos was altered to reflect the Cdx1 distribution profiles, including an anterior domain of expression in the hindbrain and craniofacial region (brackets in Fig. 3D).

Cdx1–Cdx2 compound mutants exhibit an increased incidence in vertebral defects relative to either single mutant allele (van den Akker et al., 2002) suggestive of functional overlap between these family members. The *Cdx1*$^{2ki/2ki}$ model appears to represent an accurate replacement of Cdx2 by Cdx1, and therefore the degree of any functional overlap between these transcription factors should be reflected in the vertebral patterning of *Cdx1*$^{2ki/2ki}$ offspring. In this regard, examination of over 20 *Cdx1*$^{2ki/2ki}$ mice revealed no overt skeletal abnormalities (Fig. 4). The lack of overt patterning defects was not likely due to compensation by *Cdx4*, as the expression of this family member was not affected in the *Cdx1*$^{2ki/2ki}$ background at the level of protein or message (Figs. 3E, F and data not shown). Finally, the expression of *Hoxb4* and Hox*d3*, both of which are posteriorized in *Cdx1* null mutants (Houle et al., 2003), was unperturbed in *Cdx1*$^{2ki/2ki}$ embryos (Supplemental Fig. 1), consistent with the lack of vertebral patterning defects. These observations are in agreement with functional equivalency between Cdx1 and Cdx2, at least as regards vertebral patterning.

Cdx1 gain-of-function

The above data suggest that Cdx members regulate *Hox* expression in a manner independent of functional specificity. One model that has been proposed relies on a Cdx protein gradient which differentially regulates expression in a manner dependent on the sensitivity of a given *Hox* target gene to Cdx transactivation (Charité et al., 1998; Gaunt, 2001; Gaunt et al., 2004, 2005). To investigate this model, we used a gain-of-function approach to alter the levels of this presumed Cdx protein gradient and assessed the impact of this manipulation on vertebral patterning.

In order to avoid potential complications which could arise when using heterologous expression strategies, we altered Cdx protein levels within normal spatial–temporal domains, and under the control of endogenous regulatory pathways, by BAC transgenesis. To this end, a BAC clone containing *Cdx1* sequences was isolated by conventional hybridization techniques. End sequencing and genomic Southern blot analysis revealed that this BAC contained the *Cdx1* locus in the middle of an approximately 150 kb genomic insert (Fig. 5). Standard microinjection methods were used to derive two transgenic founders from this BAC, each of which exhibited the anticipated restriction pattern, suggesting no gross rearrangements of the transgene occurred upon integration (Fig. 5 and data not shown).

The BAC transgene complements Cdx1 loss-of-function

Whole mount immunohistochemistry, *in situ* hybridization, semi-quantitative RT-PCR and Western blot analysis were all consistent with an increase in Cdx1 protein and message in the BAC transgenic relative to wild type controls (Fig. 6 and data not shown). To assess functionality of the transgene, we monitored its ability to rescue the *Cdx1* loss-of-function phenotype. To this end, hemizygous transgenics, denoted *BAC*$^{+/-}$, were crossed with *Cdx1* null mutants and the skeletons of the resultant *BAC*$^{+/-}$*Cdx1*$^{+/-}$ and *Cdx1*$^{+/-}$ offspring, as well as wild type controls, were assessed. Consistent with prior

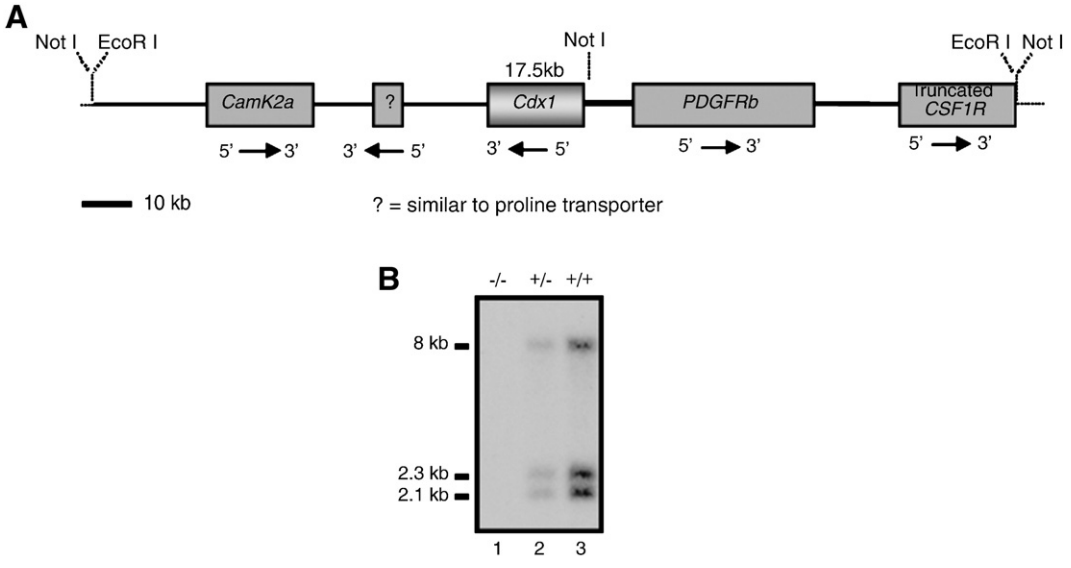

Fig. 5. Schematic of the *Cdx1*-containing 375K21 BAC clone and genotyping of transgenic mice. (A) Map of the 375K21 BAC insert obtained by sequencing and BLAST search of the mouse genome using NCBI's Mouse Genome Resources. (B) Genotyping of 375K21 BAC transgenic mice by quantitative Southern blot. 10 μg of genomic DNA was digested with Bgl II and probed with the BAC vector, pBACe3.6. Genotypes: −/−, wild-type; +/−, heterozygous transgenic; +/+, homozygous transgenic.

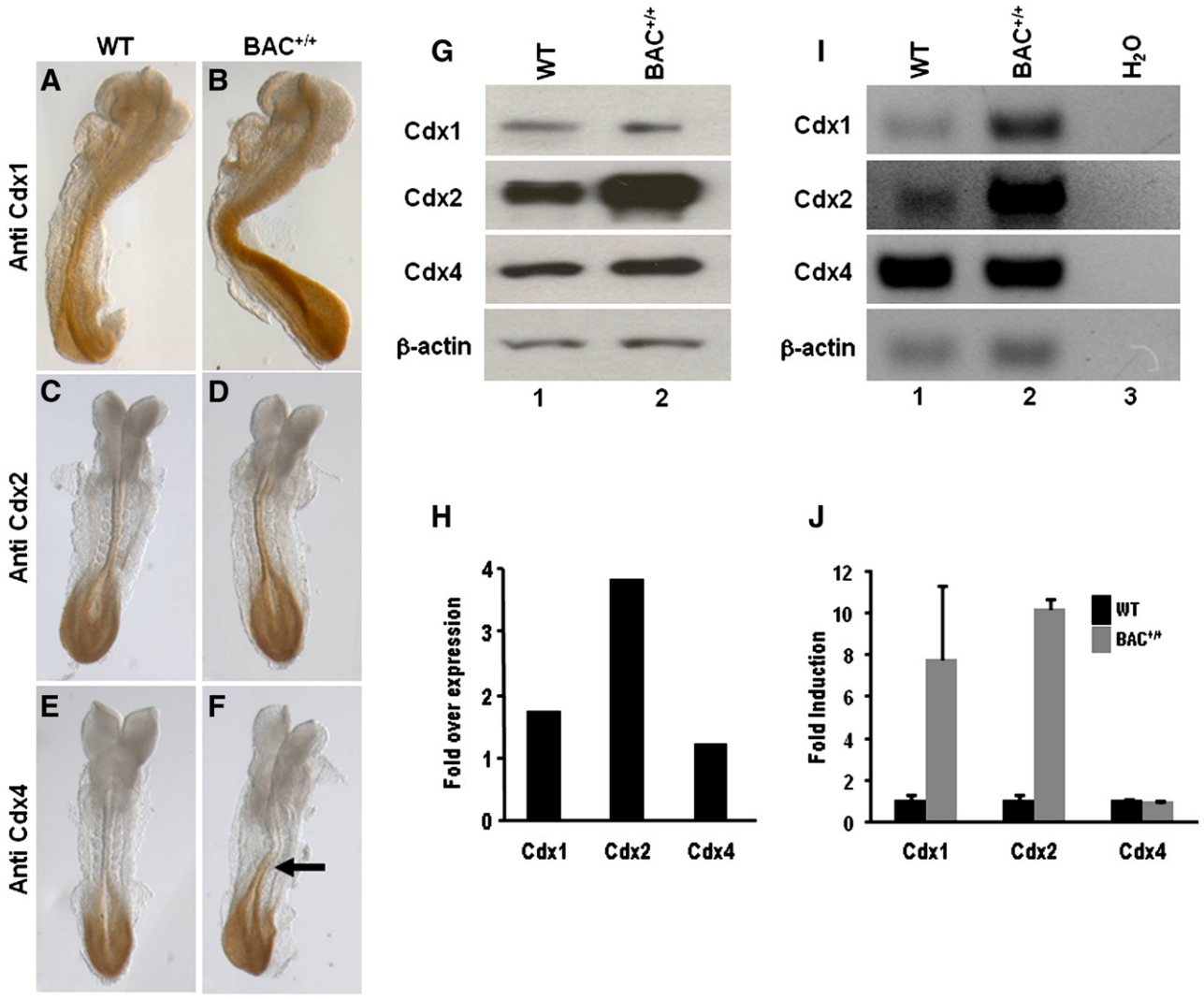

Fig. 6. Cdx1, Cdx2 and Cdx4 expression in $BAC^{+/+}$ transgenic embryos. (A–F) Cdx1 (A, B), Cdx2 (C,D) and Cdx4 (E, F) protein in wild-type (A, C, E) and $BAC^{+/+}$ (B, D, F) transgenic embryos revealed by whole mount immunohistochemistry. (G, H) Western blot analysis of Cdx protein levels in extracts from staged-matched wild-type and $BAC^{+/+}$ transgenic embryos. Relative expression was calculated by scanning densitometry using β-actin signal as an internal control. (I, J) Relative transcript abundance of *Cdx1*, *Cdx2* and *Cdx4* in WT (J, black bars) and $BAC^{+/+}$ (J, gray bars) embryos as assessed by semi-quantitative RT-PCR. (I) Agarose gel of representative RT-PCR analysis.

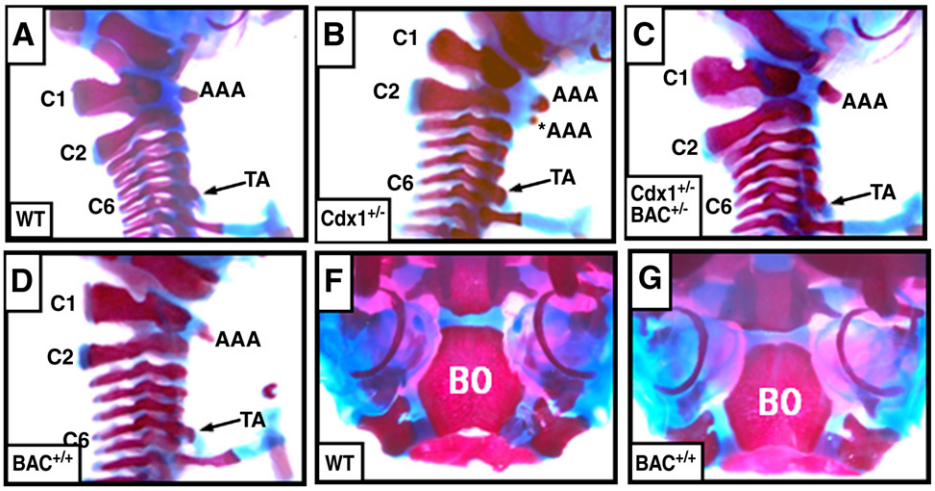

Fig. 7. Skeletal morphology of $BAC^{+/+}$ transgenic mice. Lateral (A–D) and ventral (E, F) views of the cervical region of whole-mount skeletal preparations from wild-type (A, F); $Cdx1^{+/-}$ (B); $BAC^{+/-}$ $Cdx1^{+/-}$ (C); and $BAC^{+/+}$ (D, G) neonates. Note the C2 to C1 anterior homeotic transformation in the $Cdx1^{+/-}$ sample evidenced by the presence of an ectopic anterior arch of the atlas (*AAA) and a broader C2 neural arch (B). Note also the normal cervical regions of $BAC^{+/-}Cdx1^{+/-}$ (C) and $BAC^{+/+}$ (D, G) offspring. Abbreviations: AAA, anterior arch of the atlas; *AAA, ectopic anterior arch of the atlas; TA, tuberculum anterior; C, cervical vertebrae; BO, basioccipital.

observations (Allan et al., 2001; Houle et al., 2003; Subramanian et al., 1995), approximately 50% of the *Cdx1* heterozygous offspring exhibited one or more vertebral defect, the most frequent being a partial anterior homeotic transformation of the second cervical vertebra (C2) to a first vertebral (C1) identity (Fig. 7B; Table 1). In contrast to *Cdx1* heterozygotes, $BAC^{+/-}Cdx1^{+/-}$ littermates exhibited no such vertebral anomalies (Fig. 7C; Table 1), suggesting that the BAC harbored complete wild type *Cdx1* function.

Cdx1 gain-of-function *via BAC transgenesis does not impact on vertebral patterning*

To assess the consequence of *Cdx1* gain-of-function, $BAC^{+/-}$ and $BAC^{+/+}$ neonates were generated and assessed for vertebral defects. In marked contrast to prior models (Bel-Vialar et al., 2002; Charité et al., 1998; Ehrman and Yutzey, 2001; Epstein et al., 1997; Gaunt et al., 2008; Pownall et al., 1996), both $BAC^{+/-}$ and $BAC^{+/+}$ offspring were devoid of any overt vertebral patterning defects (Fig. 7 and data not shown). Consistent with this, expression of *Hoxb4* and *Hoxd3*, both of which are posteriorized by one somite in $Cdx1^{-/-}$ mutants (Houle et al., 2003; Subramanian et al., 1995), were unaffected, with anterior expression reaching the level of somite 6 and somite 5, respectively, in both wild type and $BAC^{+/+}$ backgrounds (Supplemental Fig. 1).

It is conceivable that compensatory mechanisms could lead to the lack of a phenotype in the *Cdx1 BAC* offspring. In this regard, both auto-regulation and cross-regulation among all three Cdx members have been described (Charité et al., 1998; Chawengsaksophak et al., 2004; Lorentz et al., 1997; Prinos et al., 2001). In addition, *Cdx2* has been shown to inhibit β-catenin-stimulated expression of *Cdx1* (Domon-Dell and Freund, 2002). To investigate potential compensatory mechanisms we examined the expression of *Cdx2* and *Cdx4* in wild type and $BAC^{+/+}$ embryos. *Cdx2* transcript and protein levels were found to be elevated (Figs. 6G, I), while protein distribution was unaffected (Figs. 6C, D). Cdx4 message and protein levels, in contrast, were unaffected (Figs. 6G, I), although ectopic expression was observed in a restricted region of the neural tube (arrow in Fig. 6F). While the increase in Cdx2 is consistent with prior work suggesting it is a direct Cdx target (Xu et al., 1999), these findings do not support a Cdx-dependent compensatory mechanism as a basis for the lack of a vertebral phenotype in the BAC transgenics.

Discussion

Models including functional specificity and an instructive gradient have been proposed to explain how Cdx family members contribute to *Hox* expression patterns relevant to vertebral AP patterning (Charité et al., 1998; Epstein et al., 1997; Gaunt, 2000; Gaunt et al., 2004; Lohnes, 2003). We evaluated functional specificity using a gene swap approach, and found that *Cdx2* could substitute for *Cdx1* with complete fidelity suggesting that mechanisms other than functional specificity underlie Cdx-dependent vertebral patterning. This led us to develop a BAC transgenic gain-of-function model to assess the impact of alteration of a presumptive Cdx morphogenetic gradient on axial patterning. The BAC transgene compensated for *Cdx1* loss, and transgenic offspring exhibited increased levels of both Cdx1 and Cdx2. However, this increase in Cdx dosage had no observable impact on vertebral patterning; compensatory alteration in *Cdx2* or *Cdx4* expression was also not observed. These data suggest that Cdx members contribute to vertebral patterning via mechanisms other than an instructive gradient or Cdx-specific target gene regulation.

Table 1
Vertebral phenotypes of $Cdx1^{+/-}$ and $BAC^{+/-}$–$Cdx1^{+/-}$ offspring.

Phenotype	Genotype		
	WT ($n=26$)	$Cdx1^{+/-}$ ($n=25$)	$BAC^{+/-}$–$Cdx1^{+/-}$ ($n=23$)
Basioccipital			
Fusion to AAA	–	3 (12)	–
Caudal Extension	–	4 (16)	–
Vertebrae 2			
Partial C1 identity			
AAA	–	7 (28)	–
Thick NA	–	8 (32)	1 (4)
Malformed NA	–	2 (8)	–
Vertebrae 6			
No TA	–	1 (4)	–
Vertebrae 7			
TA	–	1 (4)	–

Summary of the vertebral phenotypes observed in $Cdx1^{+/-}$ and $BAC^{+/-}Cdx1^{+/-}$ mutants. The number of embryos displaying each phenotype is noted, with the percentage of offspring exhibiting that defect in parentheses. Abbreviations: AAA, anterior arch of the Atlas; C1, cervical vertebra 1; NA, neural arch; TA, tuberculum anterior.

Cdx1 and Cdx2 are functionally redundant

Outside of the homeodomain, hexapeptide motif and a small region near the N-terminus involved in subcellular trafficking, Cdx members exhibit poor sequence homology, including the regions that comprise the transactivation domain (Marom et al., 1997; Lohnes, 2003). This observation is consistent with the hypothesis of specific transactivation capacity between Cdx proteins. Alternatively, Cdx members have been proposed to differentially regulate target genes via an instructive gradient (Marom et al., 1997; Gaunt et al. 2004; Gaunt et al. 2008). This is consistent with the phenotypes of Cdx compound mutants, relative to the cognate single mutants, which suggest that all three Cdx members exhibit functional overlap in vertebral patterning (van den Akker et al., 2002). However, given the functional redundancy between Hox proteins in patterning vertebrae (Horan et al., 1995; McIntyre et al., 2007; van den Akker et al., 2001; Wellik, 2007; Wellik and Capecchi, 2003), it is difficult to conclude whether this latter observation is indicative of functional redundancy or is the result of regulation of different target genes in a Cdx-type specific manner, with these target gene products then converging on vertebral patterning. In this regard, evidence suggests that Cdx1 and Cdx2 exhibit specific, and sometimes opposed, gene regulation properties in enterocytes (Alkhoury et al., 2005), consistent with functional specificity.

To more definitively assess functional overlap between Cdx family members, we used homologous recombination to effect replacement of *Cdx1* by *Cdx2*. Immunohistochemistry indicated the fidelity of this exercise, with complete loss of Cdx1 and concomitant gain of Cdx2 in the Cdx1 expression domain in knock-in offspring. Homozygous offspring were viable, fertile, of normal longevity and exhibited no paraxial mesoderm patterning defects characteristic of Cdx1 null mutants, consistent with functional equivalence between *Cdx1* and *Cdx2*, at least as regards vertebral patterning. Moreover, we have observed no overt defects impacting on the intestinal tract. While this is consistent with the lack of a reported intestinal phenotype in *Cdx1* null offspring, it also suggests that increased *Cdx2* dosage is of no significant consequence in this lineage.

Cdx gain-of-function does not affect vertebral patterning

Prior gain-of-function studies in diverse vertebrate models are consistent with Cdx dosage impacting on *Hox* expression and subsequent AP patterning (Bel-Vialar et al., 2002; Charité et al., 1998; Ehrman and Yutzey, 2001; Epstein et al., 1997; Isaacs et al., 1998). The finding that multimerization of a Cdx response element can anteriorize reporter gene expression *in vivo* (Gaunt et al., 2004), together with analysis of *Cdx* expression patterns (Beck et al., 1995; Gamer and Wright, 1993; Meyer and Gruss, 1993; Marom et al., 1997)

and the haploinsufficiency of *Cdx1* and *Cdx2* in vertebral patterning (Charité et al., 1998; Chawengsaksophak et al., 1997; Subramanian et al., 1995), led to the hypothesis that an instructional Cdx gradient contributes to the establishment of *Hox* expression along the AP axis (Charité et al., 1998; Gaunt et al., 2004, 2005).

We tested the effect of increasing Cdx dosage, and presumably altering the hypothetical Cdx gradient, by generating a Cdx1 BAC transgenic line. This approach was chosen in order to maintain the Cdx transgene under the control of endogenous regulatory mechanisms. The BAC line efficiently complemented Cdx1 loss-of-function, demonstrating that it harbored a functional allele. However, *Cdx1–BAC* offspring exhibited normal vertebral patterning, suggesting that a modest increase in the presumed Cdx protein gradient does not impact on *Hox* expression along the AP axis. This is in contrast to prior conclusions showing that Cdx gain-of-function impacts on *Hox* expression and/or vertebral patterning in the mouse (Charité et al., 1998; Gaunt et al., 2004; Gaunt et al., 2005; Gaunt et al., 2008). However, with one exception (see below), these studies utilized heterologous expression strategies, and it is therefore difficult to ascertain if the impact of these exercises were due to an alteration of the Cdx gradient or through other means, such as precocious activation of Cdx targets.

In contrast to heterologous transgenic drivers, more recent work utilized homologous promoters to examine the consequence of overexpression of *Cdx1*, *Cdx2* and *Cdx4*. This exercise resulted in an increase in Cdx protein confined largely to the spatio-temporal domains of the endogenous proteins, and reported an impact on both the expression of certain *Hox* genes as well as vertebral patterning defects (Gaunt et al., 2008). These findings are entirely consistent with alteration of a Cdx gradient as an important mediator of AP patterning. A number of differences, compared to the current study, may have contributed to this finding. Perhaps most notable is the relative difference in expression levels, with the previous study yielding up to a fifty fold increase in Cdx protein. This suggests that the approximately two fold increase in Cdx1 protein reported in the present study does not suffice to alter the purported gradient sufficiently to impact on Hox expression. Alternatively, markedly high levels of Cdx could conceivably lead to titration of a limiting co-factor common to other transcription factors involved in vertebral patterning, leading to non-specific effects on vertebral ontogenesis.

Consistent with prior work describing potential auto- and cross-regulation among Cdx members (van den Akker et al., 2002; Beland et al., 2004; Xu et al., 1999) we found elevated levels of Cdx2 protein and mRNA in Cdx1 BAC transgenic offspring. As Cdx members appear to be functionally redundant, this suggests that the phenotype seen in Cdx1 and Cdx2 heterozygotes could reflect concomitant reduced expression from the other allele and/or of other family members. Further study is necessary to determine if this prediction holds.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2009.03.016.

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