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Cdx1 and *Cdx2* have overlapping functions in anteroposterior patterning and posterior axis elongation

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SUMMARY

Mouse Cdx and Hox genes presumably evolved from genes on a common ancestor cluster involved in anteroposterior patterning. *Drosophila caudal* (*cad*) is involved in specifying the posterior end of the early embryo, and is essential for patterning tissues derived from the most caudal segment, the analia. Two of the three mouse Cdx paralogues, *Cdx 1* and *Cdx2*, are expressed early in a Hox-like manner in the three germ layers. In the nascent paraxial mesoderm, both genes are expressed in cells contributing first to the most rostral, and then to progressively more caudal parts of the vertebral column. Later, expression regresses from the anterior sclerotomes, and is only maintained for *Cdx1* in the dorsal part of the somites, and for both genes in the tail bud. *Cdx1* null mutants show anterior homeosis of upper cervical and thoracic vertebrae. *Cdx2*-null embryos die before gastrulation, and *Cdx2* heterozygotes display anterior transformations of lower cervical and thoracic vertebrae. We have analysed the genetic interactions between *Cdx1* and *Cdx2* in compound mutants. Combining mutant alleles for both genes gives rise to anterior homeotic transformations along a more extensive length of the vertebral column than do single mutations. The most severely affected *Cdx1* null/*Cdx2* heterozygous mice display a posterior shift of their cranio-cervical, cervico-thoracic, thoraco-lumbar, lumbo-sacral and sacro-caudal transitions. The effects of the mutations in *Cdx1* and *Cdx2* were co-operative in severity, and a more extensive posterior shift of the expression of three Hox genes was observed in double mutants. The alteration in Hox expression boundaries occurred early. We conclude that both Cdx genes cooperate at early stages in instructing the vertebral progenitors all along the axis, at least in part by setting the rostral expression boundaries of Hox genes. In addition, Cdx mutants transiently exhibit alterations in the extent of Hox expression domains in the spinal cord, reminding of the strong effects of overexpressing Cdx genes on Hox gene expression in the neurectoderm. Phenotypical alterations in the peripheral nervous system were observed at mid-gestation stages. Strikingly, the altered phenotype at caudal levels included a posterior truncation of the tail, mildly affecting *Cdx2* heterozygotes, but more severely affecting *Cdx1/Cdx2* double heterozygotes and *Cdx1* null/*Cdx2* heterozygotes. Mutations in *Cdx1* and *Cdx2* therefore also interfere with axis elongation in a cooperative way. The function of Cdx genes in morphogenetic processes during gastrulation and tail bud extension, and their relationship with the Hox genes are discussed in the light of available data in *Amphioxus*, *C. elegans*, *Drosophila* and mice.

Key words: Mouse Cdx genes, Hox genes, Anteroposterior patterning, Axial elongation

INTRODUCTION

caudal (*cad*) plays a role in anteroposterior (AP) patterning of the early fly embryo, and in specifying the identity of posteriormost structures. It encodes a protein of which the homeodomain shares approximately 50% amino acid sequence identity with the homeodomain of Antennapedia class proteins (Antp) and the two also partially share a conserved 'hexapeptide' motif upstream of the homeodomain. *cad* is initially expressed maternally. RNA is first transcribed ubiquitously but it is differentially degraded by the maternal *Bicoid*, to generate a posterior gradient. Later in development, the maternal RNA is replaced by zygotic transcripts, which also accumulate at the posterior end of the embryo (Mlodzik et al., 1985; Mlodzik and Gehring, 1987; MacDonald and Struhl, 1986). The maternal and the zygotic *cad* gradient are necessary for proper segmentation of the posterior blastoderm (MacDonald and Struhl, 1986). They

control the expression of gap and pair-rule genes, which in turn regulate the homeotic (*HOM-C*) genes (Dearolf et al., 1989; Rivera-Pomar et al., 1995; Schulz and Tautz, 1995). After gastrulation, the zygotic expression domain of *cad* immediately flanks the posterior expression boundary of the most posteriorly expressed *HOM-C* gene, *Abd-B*. *cad* is necessary for the specification of the most posterior segment, the analia, and in this respect behaves as a *HOM-C* gene (Moreno and Morata, 1999). *cad* like genes have been found in a variety of species. The *Amphioxus* homologue *AmphiCdx* has been found to be linked with the homeobox genes *AmphiXlox* and *AmphiGsx*, in a recently defined ParaHox cluster, which is presumed to be an ancient paralogue of the Hox clusters. The Hox and ParaHox clusters have probably arisen by duplication of an original 'ProtoHox' cluster (Brooke et al., 1998). In the mouse, a remnant of a ParaHox cluster consists of *Gsh1*, *Pdx1* and the *cad* homologue *Cdx2* (Chawengsaksophak and Beck, 1996; Beck et al., 2000). Like the Hox genes, ParaHox genes display a conserved co-linear expression along the AP axis in neural tube, in mesoderm and in the gut endoderm (Beck et al., 1995; Guz et al., 1995; Li et al., 1996; Brooke et al., 1998). No link between the two other mouse *cad* homologues, *Cdx1* and *Cdx4*, with potential paralogues of *Pdx* and *Gsh* on possible duplicates of the ParaHox cluster has been reported.

Cdx1 starts to be expressed at the late primitive streak stage (day 7.2) in ectodermal and mesodermal cells of the primitive streak (Meyer and Gruss, 1993). Expression of *Cdx2* begins at day 3.5 in the trophectoderm of the blastocyst and later continues in tissues derived from the extra-embryonic ectoderm. *Cdx2* transcripts are detected in the embryo proper slightly later than *Cdx1* (Beck et al., 1995) (E. Tolner, B. Roelen and J. D., unpublished). Initiation of *Cdx4* expression follows, in the allantois and the posterior part of the primitive streak in the late streak stage embryo (Gamer and Wright, 1993) (E. Tolner, B. Roelen and J. D., unpublished). The three Cdx genes, although paralogous, exhibit overlapping expression patterns in the neural tube and mesoderm at day 8.5 of development. Their expression domains in these tissues form nested sets, with *Cdx1* showing most anterior and *Cdx4* most posterior rostral expression boundaries, compatible with a possible differential role in AP patterning (Gamer and Wright, 1993; Meyer and Gruss, 1993; Beck et al., 1995). This has been confirmed by the generation of loss-of-function mutants. *Cdx1* null mutant mice are viable and show anterior homeotic transformation of axial skeletal elements from the level of the first cervical (C1) to the level of the eighth thoracic vertebra (T8) (Subramanian et al., 1995). *Cdx2* null mutant mice die at the time of implantation, but heterozygous mice are viable and display anterior homeosis of vertebrae from the level of C6 to the level of T8, and slightly shorter tails. *Cdx2* and *Cdx4* are expressed in posterior gut endoderm at day 8.5 (Gamer and Wright, 1993; Beck et al., 1995). *Cdx2* expression has been shown to be maintained in the gut epithelium at later stages, and to persist during adulthood (James and Kazenwadel, 1991). *Cdx1* starts to be expressed later (from day 14.5) in developing intestinal epithelium where it becomes restricted and is maintained in the proliferative crypt compartment (Duprey et al., 1988; Meyer and Gruss, 1993; Subramanian et al., 1998). *Cdx2*$^{+/-}$ mice develop polypoid lesions in the intestine secondary to homeotic transformation of the distal intestine, showing that Cdx genes have a function in gut development (Chawengsaksophak et al., 1997; Beck et al., 1999).

Experiments in mouse and *Xenopus* suggest that Cdx genes may be directly involved in the regulation of Hox genes (Pownall et al., 1996; Epstein et al., 1997; Isaacs et al., 1998; Charité et al., 1998). *Cdx1* null mice showed posterior shifts in the mesodermal expression boundary of a number of Hox genes (Subramanian et al., 1995). Cdx-binding sites are present in Hox regulatory sequences (Shashikant et al., 1995; Subramanian et al., 1995; Charité et al., 1998) and *Cdx1* can transactivate *Hoxa7* reporter constructs (Subramanian et al., 1995). We have previously shown that Cdx proteins in vitro bind Cdx-binding sites in a regulatory element of Hoxb8. These sites were shown to be necessary for the ability of this element to drive Hox-like expression in transgenic embryos. Expression of both transgene and endogenous *Hoxb8* was anteriorised by rostral overexpression of Cdx genes in neurectoderm and mesoderm at day 8.5 (Charité et al., 1998).

In an attempt to elucidate the relationship between Cdx genes and the Hox genes, we have investigated the effect of loss-of-function mutations in Cdx genes on AP patterning and Hox gene expression. We show that *Cdx1*$^{-/-}$/*Cdx2*$^{+/-}$ double mutants exhibit skeletal defects along the complete vertebral column, with an increased severity compared with single mutants. An associated posterior shift of Hox gene expression is also more extensive in these double mutants than in single mutants, demonstrating a parallel functional redundancy between Cdx genes in regulating Hox gene transcription. Furthermore, we show that mesodermal and neurectodermal expression boundaries of trunk Hox genes are already affected at day 8.5/9.0 in Cdx mutants, suggesting that Cdx genes are involved in modulating the early establishment phase of Hox expression at these levels. However, *Cdx1* co-operates with *Cdx2* in ensuring proper posterior axis elongation from the tail bud at later stages. We discuss the possibility that Cdx genes could, depending on the time and A-P position, influence early patterning by regulating Hox genes, and act as later mediators of posterior axial extension, directly or as regulators of 5′ Hox genes.

MATERIALS AND METHODS

Generation of mutant mice and embryos

The generation of *Cdx1* and *Cdx2* loss-of-function mutants and of the *Hoxb8lacZneo*$^{-}$ 'knock in' line have been described previously (Subramanian et al., 1995; Chawengsaksophak et al., 1997; van den Akker et al., 1999). *Cdx1* mutant mice were from a mixed background, and have been re-derived from crosses with C57Bl6/CBA F_1 hybrids. They were then bred with these F_1 hybrids for about six generations, and were subsequently made homozygous. The *Cdx2* mutant mice underwent the same history, except that they continued to be bred as heterozygotes with C57Bl6/CBA F_1 mice. The *Hoxb8* knock in strain was originally generated from 129 Ola ES cells, and the mutation has been kept on an FVB background after the CRE deletion step.

Mice heterozygote for the *Hoxb8* null allele wherein *lacZ* is inserted in frame in the *Hoxb8* locus are named *Hoxb8lacZ*$^{+/-}$. *Cdx1*-null mice were crossed with *Cdx2*$^{+/-}$ animals to obtain

transheterozygous offspring. These were mated with *Cdx1*-null mice and the progeny was used for skeletal staining. We also used these crosses to recover single and double mutant day 8.5 and 9.5 embryos for whole mount in situ hybridisation, and day 14.5 embryos for histological analysis on sections. For analysis of the *Hoxb8* expression pattern in the *Cdx2* mutant background, *Hoxb8lacZ*$^{+/-}$ mice were crossed with *Cdx2*$^{+/-}$ mice and the embryos were recovered at day 11.5 and X-gal stained. For analysis of the *Hoxb8lacZ* pattern in the *Cdx1* single and *Cdx1/Cdx2* double mutant backgrounds, *Cdx1*-null mice were first crossed with *Hoxb8lacZ*$^{+/-}$ mice. *Cdx1*$^{+/-}$/*Hoxb8lacZ*$^{+/-}$ mice were subsequently crossed with *Cdx1*-null mice or with *Cdx1*$^{+/-}$/*Cdx2*$^{+/-}$ mice. The resulting *Cdx1*$^{-/-}$/*Hoxb8lacZ*$^{+/-}$ and *Cdx1*$^{+/-}$/*Cdx2*$^{+/-}$/*Hoxb8lacZ*$^{+/-}$ mice were crossed with, respectively, *Cdx1*$^{+/-}$/*Cdx2*$^{+/-}$ and *Cdx1*-null mice, and embryos were recovered at day 8.5-12.5. The day of the plug was considered as day 0.5 of development, except when the mother had a *Cdx1*-null genotype. In this case, because we observed a consistent delay in development of approximately 1 day, the day after the day of the plug was considered as day 0.5.

Genotyping mice and embryos

Cdx1 genotypes were determined using PCR as described previously (Subramanian et al., 1995). *Hoxb8* genotypes were determined by PCR as described elsewhere (van den Akker et al., 2001). For *Cdx2* genotyping, reverse primer 5′-TAAAAGTCAACTGTGTTCGG-ATCC (primer 1) could be used in one PCR reaction with forward primers 5′- AGGGACTATTCAAACTACAGGAG (primer 2) and 5′-ATATTGCTGAAGAGCTTGGCGGC (primer 3). The combination of primers 1,2 and primers 1,3 makes it possible to identify the wild-type (443 bp product) and targeted (636 bp product) *Cdx2* loci, respectively. Amplification conditions were denaturation at 96°C for 30 seconds, annealing at 61°C for 60 seconds and extension at 72°C for 120 seconds for 35 cycles.

Comparative amino acid sequence analysis

Amino acid sequences of Cdx, Hox and Evx gene products were compared using Lasergene software (DNASTAR, Madison, WI).

X-gal staining and histology

For analysis of the *Hoxb8lacZ* expression patterns, day 8.5-12.5 embryos were stained with X-gal as whole mounts (Vogels et al., 1993). Paraffin wax-embedded sections of whole mount X-gal stained embryos were counterstained with Neutral Red. For analysis of the phenotypes, paraffin wax-embedded sections of day 14.5 embryos were stained with Haematoxylin and Eosin.

Skeletal staining

Newborns were stained for bone and cartilage as described previously (van den Akker et al., 2001).

In situ hybridisation

Whole mount in situ hybridisation was performed as previously described (Wilkinson, 1992). The brachyury (*T*) probe has been described by Wilkinson et al. (Wilkinson et al., 1990). The *Hoxd4* probe was from Featherstone et al. (Featherstone et al., 1988) and the *Hoxb9* probe was from Graham et al. (Graham et al., 1989).

RESULTS

AP patterning defects in *Cdx1*/*Cdx2* compound mutants reveal functional cooperativity between the genes all along the vertebral axis

Bone and cartilage of single and compound *Cdx1/Cdx2* mutant newborns (ten of each genotype) was analysed. The skeletal phenotype of *Cdx1*$^{-/-}$ mice has been described previously (Subramanian et al., 1995). The *Cdx1*$^{-/-}$ newborn mice that we analysed in this study showed malformations of the axial skeleton indicative of anterior transformation of vertebrae in a slightly larger AP domain than described previously. Transformations were observed from the level of the first cervical vertebra (C1; the atlas) to the level of the first lumbar vertebra (L1) (see Table 1; Fig. 1D, Fig. 2D, Fig. 3D and Fig. 4D). Heterozygous *Cdx1* mutant newborns (Fig. 1C, Fig. 2C, Fig. 3C and Fig. 4C) frequently carried an eighth (pair of) rib(s) attached to the sternum (T8 towards T7 transformation, Fig. 3C) and an incomplete (pair of) rib(s) on the first lumbar vertebra (L1 towards T13 transformation, not shown, see Table 1). In addition, less frequent transformations of cervical and thoracic vertebrae were observed for this genotype (not shown, see Table 1). Skeletal abnormalities of *Cdx2* heterozygous mutant mice have been described previously (Chawengsaksophak et al., 1997). The data obtained in the present study are in complete agreement with those of an earlier study, showing anterior transformation of vertebrae from the level of C6 to the level of T8 with incomplete penetrance (see Table 1, and Fig. 1B, Fig. 2B, Fig. 3B and Fig. 4B). Most frequently we observed a one- or two-sided shift in the position of the anterior tuberculum from C6 to C7, and a shift in the position of the long spinous process from T2 to T3 (Fig. 2B). Fusion of the first and second rib, and presence of an eighth pair of ribs at the sternum (Fig. 3B) were also observed in these animals but less frequently.

We found more severe skeletal defects in *Cdx1*$^{+/-}$/*Cdx2*$^{+/-}$ double heterozygotes than in *Cdx* single heterozygous mutants (Table 1). Phenotypes that were observed frequently with incomplete penetrance in each of the single heterozygotes or only in *Cdx1*$^{+/-}$ mice became almost fully penetrant in the double heterozygotes (Table 1 and Fig. 1B,C,E, Fig. 2B,C,E, Fig. 3B,C,E and Fig. 4B,C,E). In three of these mice, we observed anterior transformation of the first sacral vertebra (S1 to L6 transformation) and an associated shift in the position of the hindlimbs (Fig. 4E), a phenotype never observed in the single mutants. The frequency at which the transformations were observed in double heterozygotes was at all AP levels higher than the sum of the frequencies observed in each single heterozygote. At thoracic, lumbar and sacral levels, phenotypes were even observed with equal or higher frequencies in double heterozygotes than in *Cdx1* homozygote mutants, suggesting equal or stronger contribution of *Cdx2* at these levels. By contrast, cervical defects were found at a clearly lower frequency in the *Cdx1*$^{+/-}$/*Cdx2*$^{+/-}$ mutants compared with the *Cdx1*$^{-/-}$ mice (Table 1), indicating a predominant role of *Cdx1* at rostral vertebral levels. The defects in the upper cervical area are, however, more severe in *Cdx1*$^{-/-}$/*Cdx2*$^{+/-}$ double mutants than in *Cdx1*-null mice (Table 1 and Fig. 1F). In all cases, dorsal and lateral parts of the atlas were missing, the axis had an atlas like morphology and C3 had an axis-like morphology (Fig. 1F, Fig. 2F). In five cases, C4 also was axis-like (C4 to C2 transformation, Fig. 2F). This shows that *Cdx2* does participate in patterning the upper cervical area, even if the *Cdx2* mutation on its own does not lead to a phenotype at that level. In more posterior areas, a clear increase in penetrance and severity of defects was observed

Table 1. Percentage of axial skeletal abnormalities in *Cdx1/Cdx2* single and double mutant mice

	Type of transformation	Wild type	*Cdx1*$^{+/-}$	*Cdx1*$^{-/-}$	*Cdx2*$^{+/-}$	*Cdx1*$^{+/-}$*Cdx2*$^{+/-}$	*Cdx1*$^{-/-}$*Cdx2*$^{+/-}$
Abnormalities of vertebra 1		-	10	100	-	30	100
Slightly malformed atlas or atlas fused with basioccipital	C1→bo	-	10	-	-	30	-
Dorsal and lateral structures (partially) present, ventral structures fused to basioccipital, no AAA	C1→bo	-	-	30	-	-	-
Dorsal and lateral structures absent, ventral structures fused to basioccipital, no AAA	C1→bo	-	-	70	-	-	100
Abnormalities of vertebra 2		-	10	100	-	90	100
Malformed (split) axis	C2→C1	-	10	-	-	30	-
Atlas like morphology (sometimes fused with vertebra 1)	C2→C1	-	-	100	-	60	100
Abnormalities of vertebra 3		-	10	100	-	80	100
Normal but fused to vertebra 2	C3→C2	-	10	-	-	-	-
Axis like morphology (sometimes split dorsally and fused to vertebra 2)	C3→C2	-	-	100	-	80	100
Abnormalities of vertebra 4		-	10	-	-	-	60
Normal but fused with vertebra 3	C4_C3	-	10	-	-	-	10
Axis like morphology	C4→C2	-	-	-	-	-	50
Anterior tuberculi on							
V6 bilaterally	No	100	80	-	40	-	-
V6+V7 unilaterally	C7→C6	-	10	-	50	10	-
V7 bilaterally	C7→C6	-	10	90	10	90	-
V7+V8 unilaterally	C7,C8→C6	-	-	10	-	-	10
V8 bilaterally	C8→C6	-	-	-	-	-	90
First vertebra with rib attached to top of sternum							
V8 bilaterally	No	100	100	10	100	-	-
V8+V9 unilaterally	T1→C7	-	-	30	-	-	-
V9 bilaterally	T1→C7	-	-	60	-	100	-
V9+V10 unilaterally	T1,T2→C7	-	-	-	-	-	-
V10 bilaterally	T2→C7	-	-	-	-	-	100
Long spinous process on							
V9	No	100	90	30	30	-	-
V10	T3→T2	-	10	70	70	100	-
V11	T4→T2	-	-	-	-	-	100
Most caudal thoracic vertebra with rib attached to sternum							
V14 bilaterally	No	100	30	-	60	-	-
V15 unilaterally	T8→T7	-	20	10	30	10	-
V15 bilaterally	T8→T7	-	50	90	10	90	50
V15+V16 unilaterally	T9,T8→T7	-	-	-	-	-	40
V16 bilaterally	T9→T7	-	-	-	-	-	10
Transitional vertebra							
V17	No	100	90	80	100	60	-
V18	T11→T10	-	10	20	-	40	100
Most caudal vertebra-bearing (partial) ribs							
V20 two complete ribs	No	100	60	50	100	20	-
V21 one partial rib	L1→T13	-	30	10	-	30	-
V21 two partial ribs	L1→T13	-	10	10	-	30	-
V21 one partial and one complete rib	L1→T13	-	-	20	-	10	30
V21 two complete ribs	L1→T13	-	-	10	-	10	70
First vertebra contributing to sacrum							
V27 bilaterally	No	100	100	100	100	70	30
V27+V28 unilaterally	S1→L6	-	-	-	-	20	-
V28 bilaterally	S1→L6	-	-	-	-	10	70
Fusions							
First and second ribs unilaterally	T2→T1	-	-	40	10	-	20
First and second ribs bilaterally	T2→T1	-	10	30	20	-	-
Fusion of more caudal ribs		-	-	-	-	10	40
Fusion of thoracic vertebrae		-	-	-	-	-	40

For all genotypes n=10. AAA, anterior arcis atlantis (the ventral arch of the atlas); V, vertebra.

as well in the double mutant compared with the single and double heterozygous mutants (Fig. 2F, Fig. 3F and Fig. 4F, and see Table 1). In summary, compound *Cdx1* and *Cdx2* mutants exhibit anterior transformations along the complete AP axis. *Cdx1*$^{-/-}$/*Cdx2*$^{+/-}$ double mutant mice exhibit the most severe alterations and their anatomical boundaries were shifted more posteriorly, as a result of these anterior transformations all along the axis.

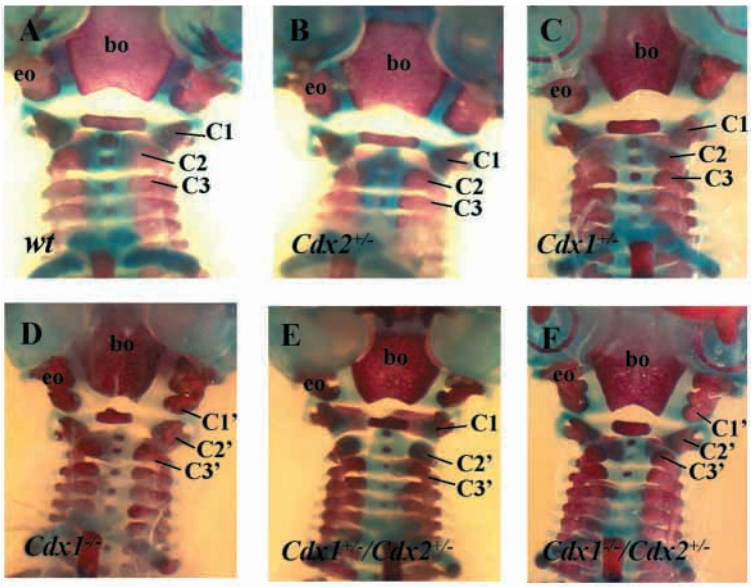

Fig. 1. (A-F)Ventral views of the upper cervical area in a wild type (A) and in different genotypic combinations between *Cdx1* and *Cdx2* mutant alleles (B-F). Rostral is towards the top. The position of the basioccipital (bo), exoccipital (eo) and the first three cervical vertebrae (C1-C3) is indicated. In *Cdx1*$^{-/-}$ (D) and *Cdx1*$^{-/-}$/*Cdx2*$^{+/-}$ (F) mutants, fusion of C1 to the exoccipital and basioccipital bones is clearly visible (anterior transformation of C1′) and the third vertebra has an axis-like morphology (C3′), indicating C2 to C1 and C3 to C2 transformations, respectively.

Cdx double mutants exhibit a more extensive caudal shift of Hox expression boundary in paraxial mesoderm than do single mutants

The skeletal phenotypes of *Cdx1* null mutants had been shown previously to be accompanied by posterior shifts in the anterior expression boundary of several Hox genes in paraxial mesoderm (Subramanian et al., 1995). *Hoxb8* is one of the Hox genes with a rostral expression boundary at AP levels affected by the *Cdx1* and *Cdx2* mutations. Its expression boundaries had not been examined so far in either of the two mutants. The upper thoracic vertebrae T1 and T2, which are affected by inactivation of *Hoxb8* (van den Akker et al., 1999), are also affected in *Cdx1* and *Cdx2* single and double mutants. In particular, fusion between the 1st and 2nd ribs was a characteristic of both *Hoxb8* null animals and *Cdx1/Cdx2* double mutants (Table 1). Previous work has indicated that Cdx proteins can positively regulate *Hoxb8* in vivo (Charité et al., 1998). We analysed the expression of the *Hoxb8lacZ* ‘knock in’ allele (van den Akker et al., 1999) in *Cdx1*/*Cdx2* single and double mutants. We examined sagittal sections of three day 11.5/12.0 embryos of each genotype. Expression boundaries were identical at day 11.5 and 12 for each genotype. *Hoxb8* expression is normally maintained at low levels in prevertebra (pv) 7 and at higher levels in more posterior prevertebrae (van den Akker et al., 1999). In wild-type mice and *Cdx1*$^{+/-}$ mice, which generally did not present any defect in the upper thorax, the expression of *Hoxb8lacZ* was clearly visible in pv7 (Fig. 5A,C). In *Cdx1*$^{-/-}$ and *Cdx2*$^{+/-}$ mutants, which show relatively mild upper thorax defects we observed either very weak or total loss of *Hoxb8lacZ* expression in pv7 (Fig. 5B,D). In double heterozygotes, the most anterior *Hoxb8lacZ*-expressing vertebra was always pv8 (Fig. 5E). In *Cdx1*$^{-/-}$/ *Cdx2*$^{+/-}$

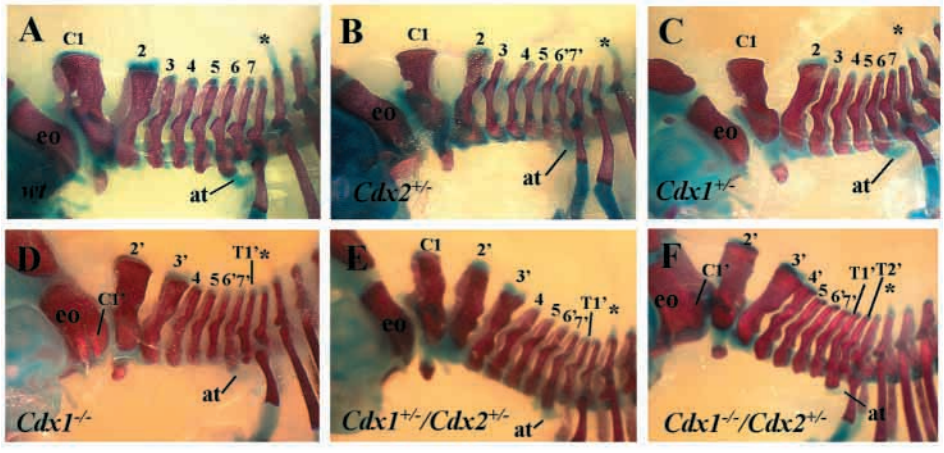

Fig. 2. Side views of the cervical and upper thoracic region of the same wild-type (A) and *Cdx* mutant newborns (B-F) as in Fig. 1. Rostral is towards the left, dorsal towards the top. The first rib emanates from the eighth vertebra and the long spinous process (asterisk) is located on the ninth vertebra in both the wild-type and *Cdx1*$^{+/-}$ mutant. Like the wild type, the *Cdx1*$^{+/-}$ newborn displayed in C has seven cervical vertebrae (indicated by numbers C1-7) and a normal atlas and axis. The *Cdx2*$^{+/-}$ animal (B) shown here displays a posterior shift of the anterior tuberculum (at) from the sixth to the seventh vertebra (C7 to C6 transformation) and the long spinous process is visible on the tenth instead of the ninth vertebra (T3 to T2 transformation). The *Cdx1*$^{-/-}$ animal in D lacks dorsal and lateral parts of the first vertebra and the remaining ventral part is fused to the exoccipital bone. Clearly visible are the atlas (with ventral arch) and axis-like morphologies of the second and third cervical vertebrae. As in the *Cdx2*$^{+/-}$ mutant in B, the position of the anterior tuberculum and the long spinous process is shifted posteriorly by one vertebra. The ninth vertebra (T2) is the first vertebra bearing ribs (T1 to C7 transformation – T1′). In the *Cdx1*$^{+/-}$/*Cdx2*$^{+/-}$ mutant shown in (E) the atlas looks normal. The second vertebra has a partial atlas-like and the third vertebra an axis-like morphology (respectively indicating C2 to C1 and C3 to C2 transformations). At lower levels, similar transformations are visible as in the *Cdx1*$^{-/-}$ mutant (D). More severe transformations are visible in the *Cdx1*$^{-/-}$/*Cdx2*$^{+/-}$ mutant (F) compared with the *Cdx1*$^{-/-}$ mutant. The first vertebra in F also lacks dorsal and lateral structures but is more completely fused to the exoccipital bone than the first vertebra in D, and not only the third but also the fourth vertebra has an axis-like morphology (C4 to C2 transformation). The anterior tuberculum is present on the eighth vertebra (T1′), the ninth vertebra has lost its rib (T2′), and the long spinous process is present on the eleventh vertebra, all indicating anterior transformation of vertebrae by two segments in the *Cdx1*$^{-/-}$/*Cdx2*$^{+/-}$ mutant.

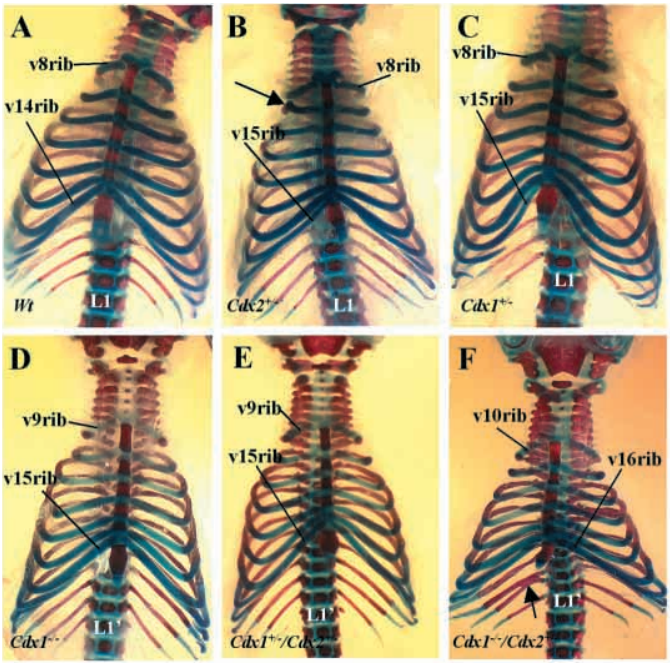

Fig. 3. Ventral views of the ribcage of the same animals as in Fig. 1 and Fig. 2. Rostral is towards the top. Wild types (A) have seven ribs emanating from the 8th to 14th vertebrae (T1 to T7), attached to the sternum. The *Cdx2*$^{+/-}$ (B) and *Cdx1*$^{+/-}$ (C) animals shown here have an extra rib attached to the sternum at one side (T8 to T7 transformation). The arrow in B indicates a slight second rib defect (rib partly anteriorised) in the *Cdx2*$^{+/-}$ mutant. The *Cdx1*$^{-/-}$ (D) and *Cdx1*$^{+/-}$/*Cdx2*$^{+/-}$ (E) mutants displayed here show bilateral attachment of ribs, emanating from the 9th to 15th vertebrae, to the sternum and bilateral presence of (partial) ribs on the 21st vertebra (L1 to T13 transformation: L1'). In the *Cdx1*$^{-/-}$/*Cdx2*$^{+/-}$ mutant shown in F, ribs are emanating from the 10th to 16th vertebrae (the latter indicating T9 to T7 transformation) and a complete pair of ribs is visible on L1. The arrow points at two fused ribs. v, vertebra.

mutants, which exhibit more severe upper thorax defects, we observed either very weak expression or no expression at all in pv8, the most rostral strongly expressing vertebra being pv9 (Fig. 5F). The increasing posterior shift of the anterior boundary of *Hoxb8*, taken as a representative of the Hox genes with rostral boundaries in the upper thorax, correlates well with the progressively more severe abnormal upper thorax phenotypes that we observed in *Cdx1Cdx2* compound mutants.

We also examined the expression domains of Hox genes with expression boundaries more anterior (*Hoxd4*) and more posterior (*Hoxb9*) than *Hoxb8*. Each single mutant showed a slight posterior shift in the rostral expression boundary of *Hoxb9* at day 9.5 (23-29 somites), the effects being additive in the compound mutant (Fig. 6E-H). The first somite strongly expressing *Hoxd4* was somite 6 for the wild type and for *Cdx2* heterozygotes, and somite 7 for *Cdx1* null and the compound mutants (Fig. 6A-D). The slight posterior shift of the *Hoxd4* boundary in the compound Cdx mutant is therefore likely to result from the loss of the *Cdx1* alleles, whereas both the *Cdx1* and *Cdx2* mutations contribute to the anterior restriction of the expression domains of *Hoxb8* and *Hoxb9*.

Cdx gene products affect rostral Hox expression boundaries in mesoderm and neural tissues at an early stage

We examined expression of the Hoxb8βgal knock in fusion protein in *Cdx* mutants at stages when *Hoxb8* expression is still in the early establishment phase (reviewed by Deschamps et al., 1999). The *Hoxb8lacZ* expression level in the mesoderm at day 9 appeared to be reduced in the *Cdx1*$^{-/-}$/*Cdx2*$^{+/-}$ mutants compared with *Cdx1*$^{+/-}$ taken as a control, and the anterior boundary was detected at the level of S12 and S10/11, respectively (Fig. 7A-B). In addition, the boundary of *Hoxb8lacZ* expression in the neurectoderm was shifted posteriorly by the extent of one somite in a *Cdx1*$^{-/-}$/*Cdx2*$^{+/-}$ double mutant embryo (and of half a somite in *Cdx1*$^{-/-}$ and *Cdx1*$^{+/-}$/*Cdx2*$^{+/-}$ double heterozygotes, not shown) compared with a stage-matched *Cdx1*$^{+/-}$ control (Fig. 7A,B). No clear shift of the *Hoxb8* expression boundary appeared to persist in the neurectoderm at later stages (day 10.5 and day 12.5, not shown). A slight shift of the *Hoxb9* rostral expression boundary in the spinal cord was observed as well. This boundary was located at an AP level between somites 6 and 7 for the wild-type and *Cdx2* heterozygotes, between somites 7 and 8 in *Cdx1*-null embryos, and in the middle of somite 8 in double mutants. The expression boundary of *Hoxd4* in the neural tube was not affected by *Cdx* mutations in 8.5- and 9.5-day embryos (not shown).

We also investigated whether the *Cdx* mutations could have an effect during the initial expression of two Hox genes, *Hoxb1* (at day 7.2) and *Hoxb8* (at day 7.75). We could not detect any significant difference in the timing of initiation of expression of those two Hox genes in the *Cdx1*/*Cdx2* single and compound mutant backgrounds (data not shown).

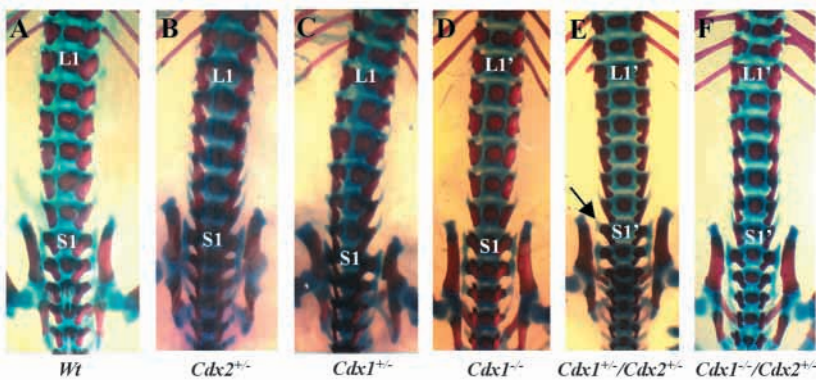

Fig. 4. Dorsal view of the lumbosacral region of the same animals as in Figs 1-3. Rostral is towards the top. The wild type (A), *Cdx2*$^{+/-}$ (B) and *Cdx1*$^{+/-}$ (C) mutants shown have the last pair of ribs attached to T13 (see also Fig. 3) and 6 lumbar vertebrae. In the *Cdx1*$^{-/-}$ mutant (D), one complete and one incomplete rib is visible on L1 (L1′). More caudally no abnormalities are present. In the *Cdx1*$^{+/-}$/*Cdx2*$^{+/-}$ (E) and *Cdx1*$^{-/-}$/*Cdx2*$^{+/-}$ (F) mutants, in addition to the partial and complete ribs present on L1, partial (arrow in E) and complete (F) shifts in the position of the sacrum (S1 to L6 transformation: S1′) are visible.

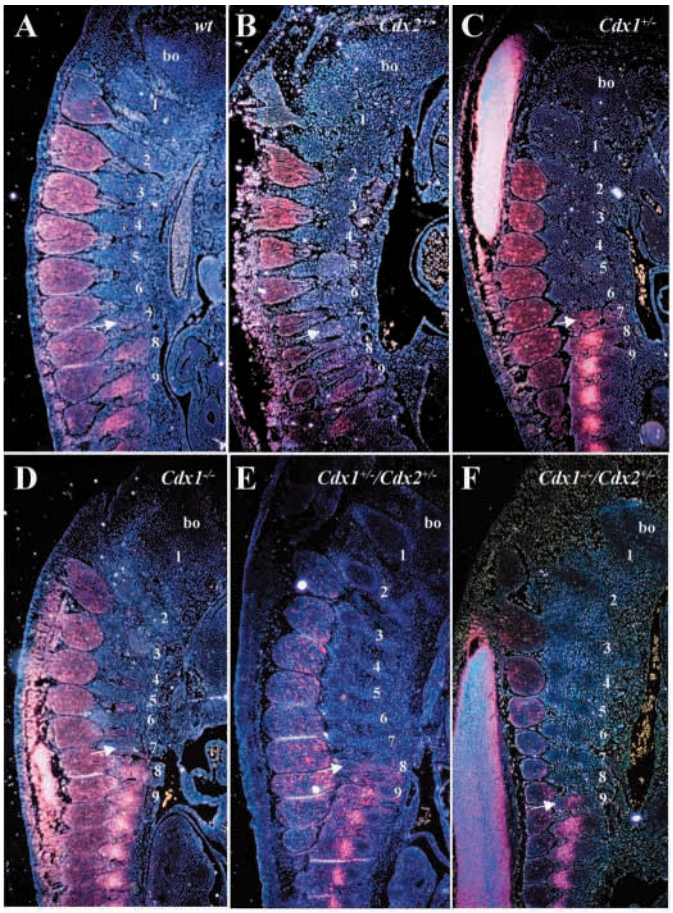

Fig. 5. Loss of *Cdx* functional alleles causes a shift in the anterior expression boundary of *Hoxb8* in the paraxial mesoderm. Sagittal sections of day 11.5-12 whole-mount *Hoxb8lacZ*$^{+/-}$ embryos stained with X-gal are viewed under dark field conditions. A and B are slightly younger than C-F, but our analysis has shown that the rostral *Hoxb8* and *Hoxb8lacZ* expression boundaries were identical at both stages. In the wild type (A), *Hoxb8lacZ* expression is visible in prevertebra (pv) 7. In the *Cdx2*$^{+/-}$ mutant (B), expression is almost undetectable in pv7 and expression in pv8 appears to be reduced. In the *Cdx1*$^{+/-}$ (C) embryo, the first *Hoxb8lacZ* expressing prevertebra is pv7 (arrow), as in wild type (A). In *Cdx1*$^{-/-}$ (D) mutant embryos, weak expression is visible in pv7. This expression has disappeared in *Cdx1*$^{+/-}$/*Cdx2*$^{+/-}$ mutant embryos (E), the first expressing vertebra being pv8 (arrow) In *Cdx1*$^{-/-}$/*Cdx2*$^{+/-}$ mutants, either weak or no (F) expression was visible in pv8. Note the lower expression of *Hoxb8lacZ* in the prevertebrae in E,F (compared with C,D at the same stage). This lower expression correlates with the compound *Cdx* mutant genotype. Vertebral abnormalities [close approximation of pv1 and the basioccipital (bo)] are already apparent at this stage in the *Cdx1*$^{-/-}$ and *Cdx1*$^{-/-}$/*Cdx2*$^{+/-}$ mutants.

The Cdx1 mutation reinforces the defect of Cdx2 heterozygotes in posterior axis elongation

Cdx2$^{+/-}$ mice have been reported to have slightly shortened tails (Chawengsaksophak et al., 1997). We counted a total number of 30-32 caudal (of 60-62 total) vertebrae in wild-type animals. Only 26-28 caudal (of 56-58 total) vertebrae were observed in *Cdx2*$^{+/-}$ mutants. In the double heterozygotes, the number of discernible caudal vertebrae was 15-20 (of a total of 45-50), indicating a more severe truncation of the axis compared with the *Cdx2*$^{+/-}$ heterozygotes. In *Cdx1*$^{-/-}$/*Cdx2*$^{+/-}$ compound mutants, the number of discernible caudal vertebrae was further reduced to 6-11 (of a total of 36-41). In the most extreme case, only a few caudal vertebrae were observed immediately below the hindlimbs and the rest of the tail consisted of a short continuous cartilaginous structure (see Fig. 8). The severe tail truncation observed in the *Cdx1*$^{-/-}$/*Cdx2*$^{+/-}$ double mutants is reminiscent of the tail defect in brachyury (*T*) heterozygous mice (Dobrovolskaia-Zavadskaia, 1927; Herrmann et al., 1990). Expression of *T* (Herrmann et al., 1990) was compared in controls and *Cdx* double mutants at day 7.5 and 8.5. The *T* expression level was not altered in Cdx double mutants. The anterior boundary of *T* expression, which is downregulated when somites form, was examined in several embryos of each genotype that had between 7 and 15 somites. It was always located at a distance one somite length more posteriorly than the last formed somite in both *Cdx1* heterozygotes, considered as controls, and in *Cdx* compound mutants. The *T* expression domain is less extended in compound mutants compared with controls because of the loss of posteriormost tail bud tissues in the posteriorly truncated *Cdx1*$^{-/-}$/*Cdx2*$^{+/-}$ mutants (Fig. 9). Because the anterior limit of *T* expression co-localises with the abnormal tail bending in the mutants, it seems that *T* expression defines the territory affected by the *Cdx* mutations in the tailbud. This suggests that both *T* and Cdx genes may be concerned with the same anatomical/morphogenetical territory but that *T* expression is not directly affected by the *Cdx1* and *Cdx2* mutations.

Fusion of vertebrae and spinal ganglia in Cdx mutants

The peripheral nervous system is a site where phenotypical abnormalities were found in *Hox* loss- and gain-of-function mutants (Charité et al., 1994; van den Akker et al., 1999). We repeatedly observed abnormalities in the peripheral nervous system of Cdx mutants. Fusions between spinal ganglia were observed at cervical and thoracic levels at day 12.5 (Fig. 10A-C) and 14.5 (Fig. 10D-F). Possibly relevant to ganglia abnormalities is the fact that vertebral processes were occasionally found to be fused in Cdx mutants (Fig. 3F, Fig. 4F, Fig. 11D), suggesting the occurrence of earlier segmentation, or neural crest migration defects possibly causally connected with the fusion of spinal ganglia.

Other phenotypical traits of Cdx mutants suggest an involvement of both genes in gut and limb patterning

We also noticed that at least some of the *Cdx1*$^{-/-}$/*Cdx2*$^{+/-}$ newborns had swollen fluid-filled abdomens (not shown). Histological analysis of the gut epithelium of double mutant and control day 13.5 embryos did not allow us to discover any meaningful alterations, and a more rigorous study of this phenotype in double mutant mice will be performed.

Examination of the appendicular skeleton revealed that one transheterozygous animal (of a total of 34), and one out of 14 *Cdx1*$^{-/-}$/*Cdx2*$^{+/-}$ double mutant newborns displayed abnormal limb patterning. Digit 1 (the big toe) of one of the hindlimbs was split (Fig. 11A,B, respectively). This polydactyly is compatible with Cdx genes playing a role in patterning the lateral plate mesoderm involved in limb outgrowth, possibly in

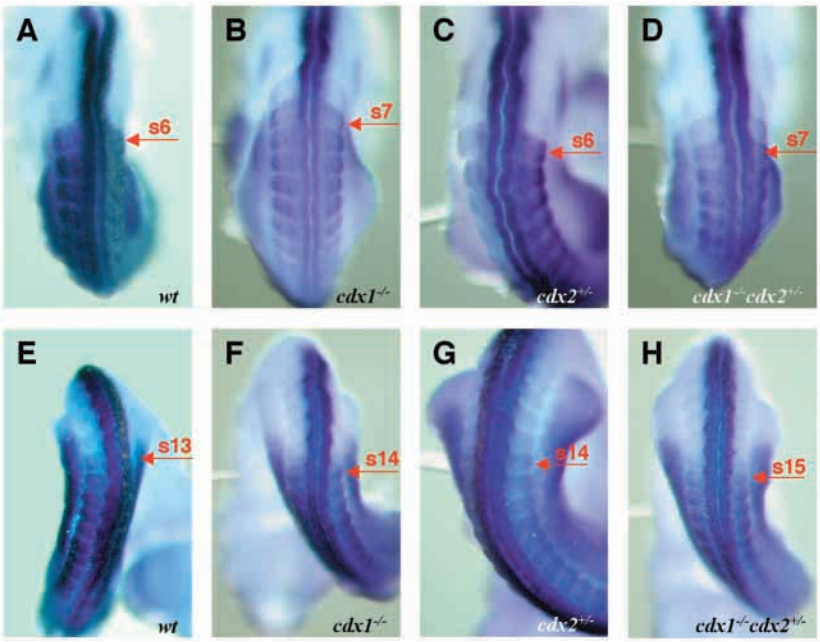

Fig. 6. *Cdx* mutations lead to a slight posterior shift of the rostral expression boundaries of *Hoxd4* and *Hoxb9*. Whole-mount 9.5-day embryos (23-29 somites) were hybridised with a *Hoxb4* (A-D) or a *Hoxb9* probe (E-H). (A,E) Wild types; (B,F) *Cdx1*-null mutants; (C,G) *Cdx2* heterozygotes; and (D,H) compound *Cdx1*null/*Cdx2* heterozygotes. The expression boundaries in the somitic mesoderm are indicated, the arrows pointing at the most rostral strongly expressing somite. For *Hoxb9* (E-H), a slight posterior shift in the neural tube was noticed as well, the expression boundary being located at the level between somites 6 and 7 (wild type and Cdx2 heterozygotes), between somites7 and 8 (*Cdx1* null), and in the middle of somite 8 (double mutants).

conjunction with their role in transducing AP positional information to structures along the axis via the Hox genes.

DISCUSSION

Cdx1 and *Cdx2* cooperate in regulating axial skeletal patterning all along the A-P axis

In agreement with the early expression of *Cdx1* and *Cdx2* in paraxial mesoderm, both genes appear to participate in patterning the vertebral column from cervical to caudal levels. While the function of *Cdx2* at rostralmost levels only became manifest in the absence of *Cdx1*, the participation of *Cdx1* at more posterior levels also became clear once one *Cdx2* allele was inactivated. Combining mutations in both genes affects patterning more severely and along a more complete length of the vertebrate column than single mutations. $Cdx1^{-/-}/Cdx2^{+/-}$ embryos exhibit the most severe phenotype, consisting in anterior homeotic-like transformations from cervical to caudal levels, implying a posterior shift of the sacrum and associated hindlimbs. In addition, the altered phenotype at caudal levels was a posterior truncation of the tail, which mildly affected *Cdx2* heterozygotes but more extensively affected $Cdx1^{-/-}/Cdx2^{+/-}$ mice. Mutations in *Cdx1* and *Cdx2* are accompanied by posterior shifts of Hox expression domains (Subramanian et al., 1995) (this work), and combining these mutations has a more severe effect on the Hox expression boundaries. It is therefore likely that Cdx genes affect AP patterning at least in part by regulating the Hox genes.

Cdx expression and function in the nervous system

Cdx genes are expressed in the early CNS and dorsal root ganglia along a restricted AP domain, similar to their relatives the Hox genes. In addition, Cdx genes modulate the extent of the Hox expression domains in the CNS at early stages, as shown by the loss-of-function studies presented here, and by the gain-of-function experiments reported earlier (Charité et

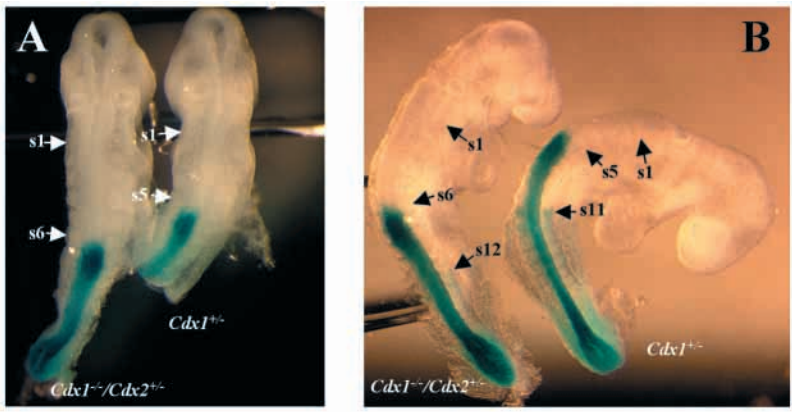

Fig. 7. The anterior expression boundary of *Hoxb8* in both the neurectoderm and paraxial mesoderm is located more posteriorly in $Cdx1^{-/-}/Cdx2^{+/-}$ compared with $Cdx1^{+/-}$ mutants during an early phase of *Hoxb8* expression. (A) Day 9 (17 somites) $Cdx1^{-/-}/Cdx2^{+/-}/Hoxb8lacZ^{+/-}$ and $Cdx1^{+/-}/Hoxb8lacZ^{+/-}$ mutant embryos were stained overnight with X-gal. The neurectodermal boundary of *Hoxb8lacZ* expression in the $Cdx1^{+/-}$ embryo (right) is located at a level halfway up the fifth somite, while in the $Cdx1^{-/-}/Cdx2^{+/-}$ embryo (left), the boundary is located halfway the sixth somite. (B) The level of *Hoxb8lacZ* expression in the mesoderm is lower in the $Cdx1^{-/-}/Cdx2^{+/-}$ (left) than in the $Cdx1^{+/-}$ (right) embryo, and the rostral boundary of expression in the paraxial mesoderm is located more posteriorly (somite 12 compared with 11, respectively). The embryo on the right in A is curved in such a way that only its rostral part is visible.

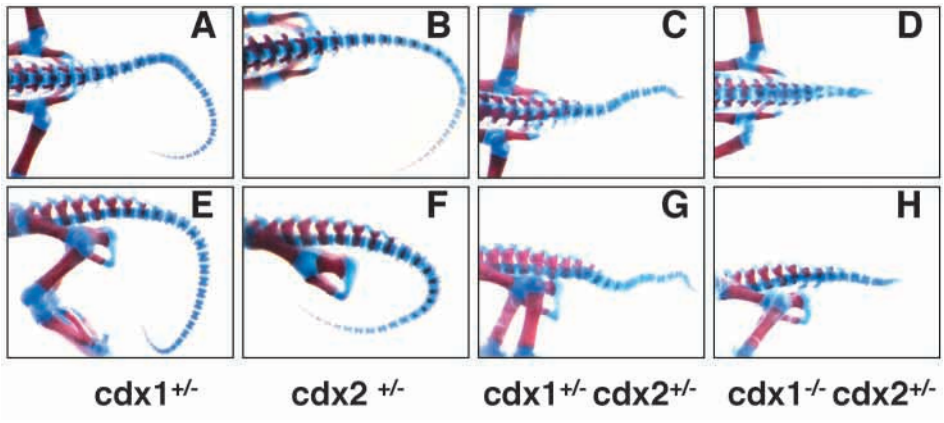

Fig. 8. Tail truncations in *Cdx1*/*Cdx2* compound mutants. (A-D) Ventral and (E-H) lateral view of the same newborn individuals of genotypes indicated below, stained for bone and cartilage. (A,E) Mice heterozygous for the *Cdx1* mutation have the same tail length as wild types, and have 30-32 caudal, of a total of 60-62 vertebrae. (B,F) Mice heterozygous for the *Cdx2* mutation have 26-28 caudal, out of a total of 56-58 vertebrae. (C,G) Mice that are double heterozygotes $Cdx1^{+/-}/Cdx2^{+/-}$ were found to have 15-20 caudal from a total of 45-50 vertebrae. (D,H) $Cdx1^{-/-}/Cdx2^{+/-}$ mutant mice were found to have only 6-11 caudal, out of a total of 36-41 vertebrae. The anatomical boundaries between each type of vertebrae are shifted 1 or 2 pv more posteriorly in these mice (due to anterior transformations at all axial levels). In addition, they have a severely truncated tail. In B,F, parts of the hindlimbs were removed for photographic purposes.

al., 1998). The positive regulation of Cdx gene products on Hox expression in all three germ layers was revealed by the complete loss of expression of a *Hox/lacZ* transgene upon mutation of the Cdx binding sites present in a crucial enhancer (Charité et al., 1998). Cdx loss-of-function mutants exhibit a slight posterior shift of the expression boundaries of at least some Hox genes in the neurectoderm. However, this effect is only observed transiently, a subsequent level of regulation probably taking over once Cdx gene expression is downregulated in this tissue. Whether the transient posterior shift of Hox gene expression in the CNS of compound Cdx mutants leads to patterning or neurological consequences is not known.

Cdx genes are expressed in the early CNS, including the neural crest precursors of the spinal ganglia. The fusion of spinal ganglia could result from a function at early stages in these neural crest progenitors, either directly or indirectly via the Hox genes. Gain- and loss-of-function Hox mutants indeed have revealed an involvement of Hox proteins in patterning spinal ganglia (Charité et al., 1994; van den Akker et al., 1999). However, *Cdx* newborn mutants also exhibit fusions between vertebral processes, suggesting either an earlier segmentation defect or partial sclerotome fusion. Such segmentation defects had already been reported for *Cdx* gain-of-function mutant embryos (Charité et al., 1998). Fusion between spinal ganglia therefore also could result from abnormal somitic properties interfering with migration of neural crest cells or causing mechanical compression of the spinal ganglia.

Cdx gene products modulate AP vertebral patterning at an early stage

The expression domains of Cdx genes at early somite stages encompass the paraxial mesoderm precursors of the complete vertebral column. The early expression phase of the Cdx genes during gastrulation probably accounts for their patterning action at rostral levels of the vertebral column. The paraxial mesoderm progenitors of the cervical vertebral column (somites 5-11) are found in the epiblast lateral to the anteriormost part of the primitive streak at the late streak to head fold stages (Tam and Beddington, 1987; Tam 1988) (K. Lawson, personal communication). Cdx and Hox genes are co-expressed in the nascent paraxial mesoderm lateral to the anterior part of the streak at these embryonic stages. It is therefore likely that the Cdx/Hox regulatory interactions begin at these early, presomitic stages. Interactions between Cdx proteins and Hox genes may go on in areas of co-expression in the unsegmented paraxial mesoderm corresponding to progressively more posterior future somites/vertebrae. Concomitantly with embryonic progression, Cdx proteins would affect more posterior paraxial mesoderm, that express gradually more 5′ Hox genes. These interactions would lead

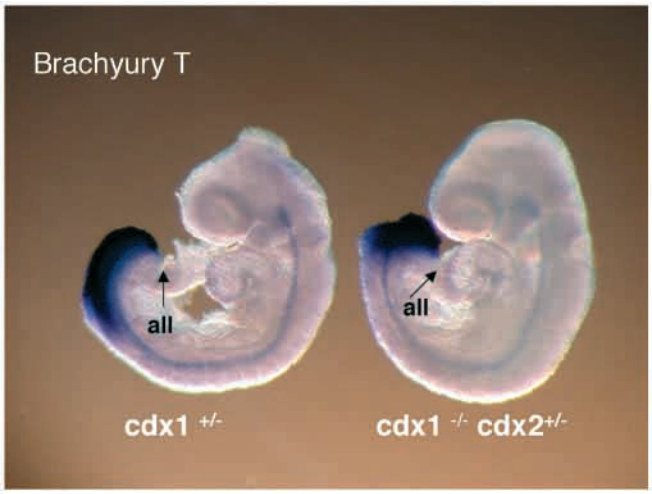

Fig. 9. Whole-mount in situ hybridisation of a $Cdx1^{+/-}$ (left) and a $Cdx1^{-/-}/Cdx2^{+/-}$ (right) 8.5-day embryos with a brachyury (*T*) probe. The double mutant had 15 somites, and the heterozygote *Cdx1* 13 somites. Clearly visible is the abnormally bent and posteriorly truncated tail bud in the double mutant. The posterior neuropore is also more widely open in the double mutant than in the $Cdx1^{+/-}$ embryo. The *T* expression domain in both embryos, and in other mutants and controls examined extends anteriorly up to a position located one somite more posterior than the last formed somite. The *T* expression level is also unaltered in double mutants compared with $Cdx1^{+/-}$, but the expression domain is posteriorly truncated. *T* expression encompasses the abnormally curved and shorter tail bud in the mutant, labelling the territory affected by the *Cdx* compound mutation at this stage.

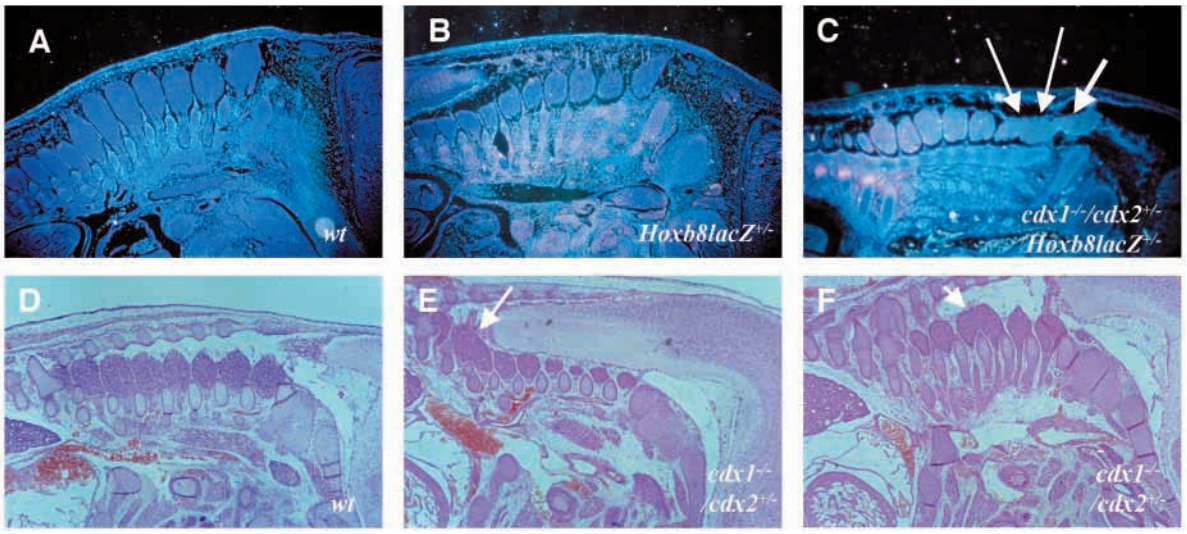

Fig. 10. Fusion between spinal ganglia in Cdx compound mutants. Sections of 14.5 day Cdx double mutants reveal fusions between spinal ganglia at cervical and upper thoracic levels (arrows in E,F). A section of a wild-type embryo is shown as a control (D). A section of a 12.5 day double mutant embryo carrying a *Hoxb8lacZ* knock in allele also reveal fusions between spinal ganglia at cervical levels (C, arrows). It is compared with a wild-type (A) and with a *Hoxb8* heterozygote knock-in (B) embryo section.

to a modulation of Hox gene expression, thereby contributing to the positional identity of the somitic progenitors.

From day 8.5 onwards, these domains rapidly regress away from the sclerotomes at cervical and thoracic levels. Nevertheless, definitive vertebral patterning in the latter areas is affected by the Cdx mutations. The time of action of Cdx products instructing positional identity at rostral and trunk levels therefore must be exclusively early. In agreement with this hypothesis, Cdx mutations were shown to alter the Hox expression boundaries at early stages. It has been shown previously that an early perturbation of Hox expression boundaries could lead to altered vertebral patterning, even if the perturbation is only transient (van der Hoeven et al., 1996; Kondo and Duboule, 1999). Altogether, the data are therefore compatible with Cdx affecting early AP somitic patterning via the Hox genes. As expected from the pleiotropic effect of Cdx mutations on many *Hox* genes (Subramanian et al., 1995) (this work), the phenotypes in *Cdx* mutants extend along a more extensive AP domain than that of single Hox mutants. At a single AP level, the upper thorax, for example, the effects of mutations in *Cdx1* and *Cdx2* on morphogenesis were found to be co-operative, as they were on the posterior shift of the expression of a Hox gene involved in patterning at that level. According to these observations, it is possible that Cdx proteins modulate AP patterning at trunk levels exclusively via the Hox genes.

Cdx function, Hox expression domains and AP vertebral patterning appear well buffered against mutational alterations

As documented in this work, Hox gene expression and AP vertebral patterning show a dose dependence on Cdx function. Instead of the five active Cdx alleles (*Cdx4* is X-linked) in wild-type mice, the compound *Cdx1*$^{-/-}$/*Cdx2*$^{+/-}$ mutants carry two functional Cdx alleles. A more severe impact on Hox expression and patterning can be expected in a Cdx-less situation. Nevertheless, the correlation between the loss of functional *Cdx* alleles and the severity of axial patterning phenotypes absolutely supports a role for Cdx genes in transduction of A-P positional information. The effects of Cdx mutations, together with the impact of *Cdx* experimental gain of function (Charité et al., 1998) on Hox gene expression support a role for Cdx gene products as Hox regulators. The comparatively stronger effect of Cdx deregulation on Hox gene expression in the neurectoderm in the Cdx gain-of-function transgenic experiments most probably arises from the relatively more extensive change in Cdx dose by the strong *RARbeta* promoter driving the transgene, than in the Cdx loss-of-function mutants. The subtlety of the effect of the Cdx

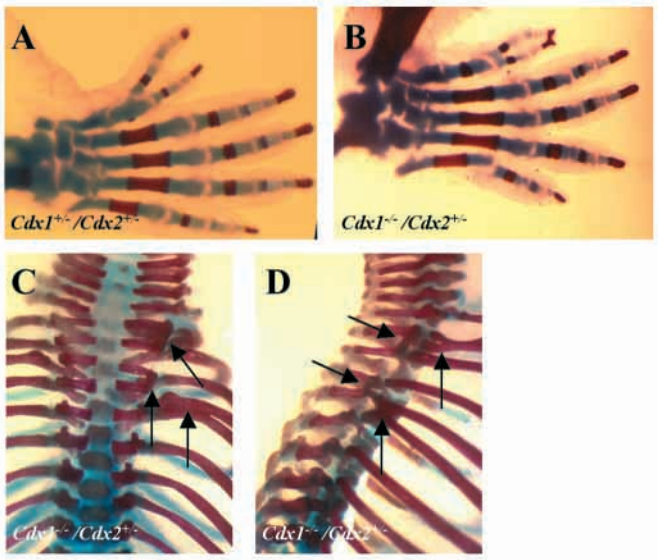

Fig. 11. Other skeletal abnormalities observed in *Cdx1*$^{+/-}$/*Cdx2*$^{+/-}$ and *Cdx1*$^{-/-}$/*Cdx2*$^{+/-}$ mutants. Split digit 1 in one of the hindlimbs of a *Cdx1*$^{+/-}$/*Cdx2*$^{+/-}$ (A) and a *Cdx1*$^{-/-}$/*Cdx2*$^{+/-}$ mutant (B). (C,D) Fusion of ribs and vertebrae (arrows) in a *Cdx1*$^{-/-}$/*Cdx2*$^{+/-}$ mutant.

mutations on the extent of the Hox expression domains may originate from the probable compensation by the remaining functional Cdx alleles. In addition, Cdx gene products are not the only regulators affecting the establishment of the Hox expression domains, and it seems that the Hox gene patterning system is well buffered against deleterious effects of mutations at one of the numerous regulatory levels.

Are Cdx homeotic genes?

Work on *Drosophila* and *C. elegans* has shown that *cad/pal-1* is involved in posterior development and patterning only partly by regulating Hox genes. *cad* is the homeotic gene that mediates correct patterning of the most posterior fly segment, the analia (Moreno and Morata, 1999), which still develops normally in a *HOM*-less fly. *pal-1* in *C. elegans* regulates the Hox gene *mab-5*, but total absence of *pal-1* causes more severe, posterior-less worm phenotypes (originally called nob – no back end) than *mab-5* mutations. *cad* therefore seems to have a homeotic function by itself. *Amphioxus Cdx* has been shown to belong to the ParaHox cluster, which would be historically related to the Hox cluster (Brooke et al., 1998). It is therefore likely that *cad*-related loci still exert a homeotic function by themselves. If this is so, mouse Cdx genes may be expected to directly pattern the most caudal embryonic structures, as *cad* does in the fly.

The patterning effect of Cdx genes at rostral levels is more likely to result from the regulatory action of Cdx on 3′ Hox genes than from a 'posterior' homeotic role of the Cdx gene products. Work in *Drosophila* and *C. elegans* has strongly suggested that Cdx gene products positively regulate several genes of the Hox cluster in the ancestral situation: *cad* regulates *ftz* in the fly (Moreno and Morata, 1999), and *pal-1* regulates *mab-5* and *vab-7* in worms (Edgar et al., 2001). Cdx target sequences probably already existed in the ancestral Hox cluster, as witnessed by the direct transcriptional activation of *mab-5* by *pal-1* in the V6 cells of *C. elegans* (Hunter et al., 1999). In the mouse, Hox genes with rostral expression boundaries at the level of cervical to sacral levels contain potential Cdx-binding sites in their regulatory regions (Subramanian et al., 1995). The existence of this molecular crosstalk would have given Cdx gene products the possibility to regulate the 5′ Hox genes and posterior development, as well as 3′ Hox genes and more anterior patterning. Direct Cdx/Hox regulatory interactions have been observed in vertebrates (Subramanian et al., 1995; Pownall et al., 1996; Epstein et al., 1997; Isaacs et al., 1998; Charité et al., 1998). Loss of expression of a *Hoxb8/lacZ* transgene in mesoderm and neurectoderm upon inactivation of the Cdx-binding sites (Charité et al., 1998) may indicate a fundamental requirement of Cdx gene products in aiding trunk Hox genes to achieve their correct expression patterns. Whether the Cdx genes directly contribute positional information to paraxial mesoderm cells, or whether they transduce this information via the Hox genes is not easy to establish at this point, in the absence of total Hox disruption, or without inactivating all Cdx binding sites in the Hox clusters.

Evolutionary relationship between Cdx and Hox genes

The early, maximally extending expression domain of *Cdx1* corresponds to that of the most 3′ Hox genes, with a rostral expression boundary at the level of the preotic sulcus, the limit between rhombomeres 2 and 3 (Meyer and Gruss, 1993). *Cdx1* and *Cdx2* are initially and transiently expressed as early as *Hoxb1* in the posterior part of the primitive streak at the late streak stage (Meyer and Gruss, 1993; Beck et al., 1995). These Cdx genes therefore display features of 3′-most Hox genes, in spite of the fact that they are later involved in generating and patterning posteriormost structures. According to Moreno and Morata (Moreno and Morata, 1999), *cad* in the ParaHox cluster might be paralogous to the 5′ neighbour of *AbdB* in the Hox cluster, *eve*. *evx2* has in fact been shown to function as a posterior *Hoxd* gene in distal structures of the mouse limbs (Herault et al., 1996). Nevertheless, comparative analysis of the amino acid sequence of the homeodomains reveals that Cdx1 and Cdx2 are closer to Hox paralogy groups 8 and 9, and even to Hox paralogy group 1 and 2 than to the most posterior paralogy group 13 and to Evx proteins. In addition, the Cdx gene products possess a Pbx recognition motif, which is absent in 5′-most Abdb Hox proteins, such as paralogy group 13, and in Evx gene products. This motif in Cdx1 shares four of the five consensus residues with that of Hoxb4. It therefore seems that mammalian Cdx genes are relatively closely related to 3′ Hox genes, although to a lesser extent than their 3′ neighbours on the ParaHox cluster, *Gsh1* and *Pdx1*. This could possibly explain the existence of similarities in their regulation.

Homeotic versus truncation phenotypes: a biphasic function of Cdx proteins?

Whether or not the Cdx gene products play a homeotic role on their own in the posterior part of the vertebral column, they definitely have a homeotic function along most of the axis, by modulating the position of the expression domain of Hox genes at relatively early stages.

From day 8.5/9.0 onwards, Cdx genes are not expressed any more in sclerotomes and neural tube at rostral and trunk levels, whereas these genes remain expressed in these structures at posterior levels until late embryonic stages. This late phase of Cdx expression may correspond to a different function of Cdx proteins in posterior development and patterning from the tail bud, where axial extension continues in a second phase of gastrulation (Gont et al., 1993). Persistent *Cdx* expression in the posteriormost part of the embryo would affect the maintenance and/or instruction of a progenitor population of tail bud-derived caudal structures (Gofflot et al., 1997; Gont et al., 1993). Strikingly, the phenotypical traits of Cdx mutants in the posterior structures are no longer homeotic-like anterior transformations but posterior truncations, much more severe in $Cdx1^{-/-}/Cdx2^{+/-}$ than in $Cdx2^{+/-}$ mutant mice. The posterior truncations in these mice are reminiscent of those found in *T* heterozygotes (Dobrovolskaia-Zavadskaia, 1927; Herrmann et al., 1990), *Wnt5A* null (Yamaguchi et al., 1999), *Lef1/Tcf1* double mutants (Galceran et al., 1999), and *Wnt3A* (Takada et al., 1994) and *Fgfr1* (Partanen et al., 1998) hypomorph mutants. This suggests that these transcription factors and signalling molecules all participate in posterior axial elongation, either by facilitating convergence/extension movements (reviewed by Sokol, 2000), or by maintaining the progenitor cell population in a proliferative state [as shown for the neural progenitors by Mathis et al. (Mathis et al., 2001)]. A function of Cdx gene products in proliferation maintenance may apply as well to the 5′ *AbdB*-related *Hoxd* and *Hoxa* genes,

the removal of which leads to truncations of distal limb, caudal gut and external genital structures (Zákány et al., 1997; Kondo et al., 1997; Warot et al., 1997). The role of Cdx gene products in posterior axial extension, like its role in modulating AP patterning of the complete vertebral axis, cannot therefore be claimed to be either *Hox*-dependent or independent until the phenotype of extensive Hox deletions is available. Whatever it may be, the data presented in this paper suggest that *Cdx* genes influence early AP patterning all along the complete vertebral column, and act as later mediators of posterior axial elongation. It could well be that these two functions are intimately linked during the progress of morphogenesis and patterning, which are known to be interdependent, as recently shown in the analysis of the function of FgfR1 (Ciruna and Rossant, 2001).

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