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The *Cdx4* mutation affects axial development and reveals an essential role of Cdx genes in the ontogenesis of the placental labyrinth in mice

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Caudal related homeobox (Cdx) genes have so far been shown to be important for embryonic axial elongation and patterning in several vertebrate species. We have generated a targeted mutation of mouse *Cdx4*, the third member of this family of transcription factor encoding genes and the last one to be inactivated genetically. *Cdx4*-null embryos were born healthy and appeared morphologically normal. A subtle contribution of *Cdx4* to anteroposterior (AP) vertebral patterning was revealed in *Cdx1*/*Cdx4* and *Cdx2*/*Cdx4* compound mutants. Neither *Cdx4*-null nor *Cdx1*/*Cdx4* double mutants are impaired in their axial elongation, but a redundant contribution of *Cdx4* in this function was unveiled when combined with a *Cdx2* mutant allele. In addition, inactivation of *Cdx4* combined with heterozygous loss of *Cdx2* results in embryonic death around E10.5 and reveals a novel function of Cdx genes in placental ontogenesis. In a subset of *Cdx2*/*Cdx4* compound mutants, the fully grown allantois failed to fuse with the chorion. The remaining majority of these mutants undergo successful chorio-allantois fusion but fail to properly extend their allantoic vascular network into the chorionic ectoderm and do not develop a functional placental labyrinth. We present evidence that *Cdx4* plays a crucial role in the ontogenesis of the allantoic component of the placental labyrinth when one *Cdx2* allele is inactivated. The axial patterning role of Cdx transcription factors thus extends posteriorly to the epiblast-derived extra-embryonic mesoderm and, consequent upon the evolution of placental mammals, is centrally involved in placental morphogenesis. The relative contribution of Cdx family members in the stepwise ontogenesis of a functional placenta is discussed, with *Cdx2* playing an obligatory part, assisted by *Cdx4*. The possible participation of *Cdx1* was not documented but cannot be ruled out until allelic combinations further decreasing Cdx dose have been analyzed. Cdx genes thus operate in a redundant way during placentogenesis, as they do during embryonic axial elongation and patterning, and independently from the previously reported early *Cdx2*-specific role in the trophectoderm at implantation.

KEY WORDS: Cdx, Transcription factor, Mouse embryogenesis, Axial extension, AP patterning, Placental development, Allantois

INTRODUCTION

The vertebrate Cdx genes are the homologs of *Drosophila caudal* (*cad*) and encode homeodomain containing transcription factors. Together with the Hox genes, they are derived from a common ancestral Proto-Hox complex (Pollard and Holland, 2000). During early development the paralogous genes *Cdx1*, *Cdx2* and *Cdx4* are initially expressed similarly in the primitive streak, including the base of the allantois (Meyer and Gruss, 1993; Gamer and Wright, 1993; Beck et al., 1995; Chawengsaksophak et al., 2004; Lohnes, 2003). At somite stages, they exhibit nested expression patterns in the posterior axial and paraxial embryonic tissues emerging from the primitive streak and later from the tailbud. The expression of *Cdx1* extends most anteriorly and overlaps with the expression domains of *Cdx2* and *Cdx4* posteriorly so that all three Cdx genes are expressed in the tailbud (Meyer and Gruss, 1993; Gamer and Wright, 1993; Beck et al., 1995; Chawengsaksophak et al., 2004; Lohnes, 2003).

The phenotypes of *Cdx1* and *Cdx2* loss-of-function mutant mice generated by homologous recombination in part reflect the fact that the Cdx proteins are positive regulators of the Hox genes in embryonic tissues (for a review, see Deschamps and van Nes, 2005). $Cdx1^{-/-}$ animals show alterations in vertebral anteroposterior (AP) identity confined to the cervical region (Subramanian et al., 1995). $Cdx2^{-/-}$ embryos die at E3.5 because of implantation failure (Chawengsaksophak et al., 1997; Strumpf et al., 2005) but $Cdx2^{+/-}$ embryos implant successfully and, like $Cdx1^{-/-}$ homozygotes, display an anterior homeotic shift in the axial skeleton, although more subtle and situated more posteriorly. In addition, $Cdx2^{+/-}$ mice exhibit a slight shortening of the AP axis (Chawengsaksophak et al., 1997). Combined $Cdx1^{-/-}/Cdx2^{+/-}$ mutants have an axial phenotype showing abnormalities greater than either mutant separately thus reflecting functional redundancy due to gene co-expression in the presomitic mesoderm (van den Akker et al., 2002). The block to implantation of $Cdx2^{-/-}$ embryos, that is in keeping with the strong expression of the gene in the trophectoderm at E3.5 (Beck et al., 1995; Chawengsaksophak et al., 1997), can be overcome by tetraploid fusion (Nagy et al., 1993). Development then proceeds to E10.5 but embryos exhibit severe posterior truncation, there being little development posterior to the forelimb buds. The arrest of posterior tissue generation in $Cdx2^{-/-}$ embryos also concerns the extra-embryonic mesoderm (Chawengsaksophak et al., 2004).

Progenitors of extra-embryonic mesoderm originating from the posteriormost epiblast (Lawson et al., 1991; Kinder et al., 1999) express all three Cdx genes at the late primitive streak stage of development (Meyer and Gruss, 1993; Gamer and Wright, 1993; Beck et al., 1995; Davidson et al., 2003). *Cdx2* is the only Cdx gene expressed in the extra-embryonic ectoderm. Expression is demonstrable at E3.5 in the trophectoderm, subsequently in the

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extra-embryonic ectoderm of the ectoplacental cone (Beck et al., 1995), and eventually in the trophoblastic stem cells (Strumpf et al., 2005). *Cdx2* is also expressed in the chorion, though it is downregulated at the late chorionic plate stage. Expression of *Cdx2* in the E9.5 placenta is strong in the spongiotrophoblasts but is absent in the adjacent labyrinth (Beck et al., 1995). Thus, the *Cdx2*-positive cells of the chorion lose expression before they differentiate into labyrinthine syncytiotrophoblast.

We have inactivated the third mouse Cdx gene, the X-linked *Cdx4*, by homologous recombination in embryonic stem (ES) cells. $Cdx4^{-/0}$ mutants are born healthy and are fertile. A modest contribution of *Cdx4* in AP patterning, and an important role for this gene in posterior axial extension were revealed in compound $Cdx1^{-/-}/Cdx4^{-/0}$ and $Cdx2^{+/-}/Cdx4^{-/0}$ mutants. In addition, most $Cdx2^{+/-}/Cdx4^{-/0}$ compound mutant embryos die around E10.5, revealing a novel functional involvement of Cdx genes. We show here that a subset of these compound mutants is impaired in chorio-allantoic fusion, even though the allantois reaches a sufficient length to touch the chorion. The remaining majority of the $Cdx2^{+/-}/Cdx4^{-/0}$ compound mutants does undergo successful chorio-allantoic fusion, but subsequently exhibits deficient labyrinthine development, following the failure of allantoic vessels to branch and expand their network in the chorionic plate. This extends the role of the Cdx transcription factors, already known as modulators of embryonic anteroposterior patterning, to include the morphogenesis of the extra-embryonic mesoderm of the chorio-allantoic placenta, the most posterior of the structures originating from the epiblast.

MATERIALS AND METHODS

Gene targeting and mouse breeding

We isolated a 13.2 kb mouse *Cdx4* genomic DNA clone from BAC344L11 (kind gift of P. Avner, Paris) and used it to construct a targeting vector by standard techniques. A floxed *PGK-neo*^{r} cassette was inserted into the *Eco*RV site upstream of exon 2 and a flipped *PGK-hygro*^{r} including one loxp-site was inserted downstream of exon 3 into the *Hin*dIII site. We established *Cdx4* conditional knockout (*Cdx4*^{c}) ES cells (129/Ola) by double G418 (200 μg/ml) and Hygromycin (150 μg/ml) selection using standard ES-cell culture protocols and verified positive clones by Southern blot analysis with a unique upstream genomic probe. Chimeric mice were generated by blastocyst injection of two independently targeted *Cdx4*^{c} ES cells into E3.5 C57BL/6 mouse blastocysts using standard procedures. Chimeric males were mated to FVB females for generation of F1 animals carrying the *Cdx4*^{c} mutation. *Cdx4*$^{c/+}$ mice were mated to CMV-Cre to generate $Cdx4^{-/0}$ mice. $Cdx4^{-/0}$ mice generated from two independently targeted ES cell clones displayed similar phenotypes and both lines were used for further characterization. Further breeding of the mice was in C57BL/6×CBA F1 background. All other mice used were also in C57BL/6×CBA F1 genetic background.

Genotyping

Embryos or visceral yolk sacs were genotyped by PCR using the following primers: NEOF401 5′-GATTGCACGCAGGTTCTCC-3′ and NEOR802 5′-GATGTTTCGCTTGGTGGTC-3′ to detect the 401 bp *Cdx4* conditional allele. Wild-type *Cdx4* was genotyped with primer pair Cdx4FOR4723 5′-TTCCCTAAAAAGACCAAAATCAAA-3′ and Cdx4REV5161 5′-CCCCGGAGAACTGCCTAAC-3′ (438 bp), and the *Cdx4* null allele was detected with the Cdx4FOR4723 5′-TTCCC-TAAAAAGACCAAAATCAAA-3′ and Cdx4REV7900 5′-AGCG-CAAAACCTCACATCA-3′ (400 bp) primer combination. Male (hemizygous) *Cdx4*-null embryos were confirmed by the presence of the Y-chromosome using primer pair SryFOR 5′-TTATGGTGTGGTCCCGTG-GTGAG-3′ and SryREV 5′-TGTGATGGCATGTGGGTTCCTGT-3′ (302 bp). *Cdx1* and *Cdx2* mutant embryos and mice were genotyped by PCR as described previously (Subramanian et al., 1995; Chawengsaksophak et al., 1997).

Skeletal preparations

Skeletons of E15.5 embryos and newborn mice were stained according to the following procedure: newborn mice were skinned, and both newborn mice and E15.5 embryos were eviscerated, fixed overnight in 96% ethanol containing 1% glacial acetic acid and stained overnight in 0.5 mg/ml Alcian Blue (Sigma) dissolved in 80% ethanol/20% acetic acid. After rinsing twice for 1 hour in 96% ethanol, soft tissue was dissolved in 1.5% KOH for 1 and 6 hours, for E15.5 embryos and newborns, respectively. Bone was then stained overnight in 0.5% KOH containing 0.15 mg/ml Alizarin Red (Sigma). Newborns and embryos were destained in 0.5% KOH/20% glycerol for 1 day or longer and afterwards stored in 20% ethanol/20% glycerol.

Histology, in situ hybridization and immunohistochemistry

Paraffin embedding and processing of the sections, including Hematoxylin and Eosin, and PAS staining, was carried out using standard methods. Methods for in situ hybridization on sections have been described (Moorman et al., 2001). RNA probes used were: *Cdx1* (Meyer and Gruss, 1993); *Cdx2* (Beck et al., 1995); *Cdx4* (Gamer and Wright, 1993); *Mash2* (Guillemot and al., 1994); *Hand1* (previously *eHAND*) (Cserjesi et al., 1995); *Tpbp* (Lescisin et al., 1998). Immunohistochemistry was performed according to standard procedures. The following primary antibodies were used: Pecam (BD Biosciences, 1:50), anti-cleaved caspase 3 (BD Biosciences, 1:300) and anti-Ki67 (BD Biosciences, 1:500). Pecam signal was amplified using Tyramid signal amplification steps (Perkin Elmer). For cleaved caspase 3 and Ki67, Envision+ kit (DAKO) was used as a secondary reagent. Signal was developed with 3,3′ diaminobenzidine (DAB, Sigma) and sections were counterstained with Hematoxylin.

Morphometric analysis of placental vasculature

The diameter of Pecam-stained embryonic vessels that had penetrated the chorionic ectoderm and branched in the placental labyrinth was measured at their minimal width on tissue sections with Leica software. Representative sections from the central part of E9.5 placentas were analyzed in eight wild-type controls and eight $Cdx2^{+/-}/Cdx4^{-/0}$ compound mutants. Two-tailed *t*-test assuming equal variance was used to determine *P* for the total embryonic vessel number per placental labyrinth and for the average embryonic vessel width.

Allantois isolation and whole-mount immunofluorescent antibody staining

E7.5 and E8.25 allantoises were dissected from 0- to 3- and 5- to 7-somite embryos, respectively. The isolated allantois was fixed in immunohistochemistry-zinc-fixative (BD Biosciences). Fluorescent antibody staining was performed according to standard procedures. Briefly, allantoises were permeabilized with 0.2% Triton X-100 in TBS, blocked for 1 hour in TNB buffer (Amersham) at room temperature and incubated overnight at 4°C with primary antibody against Pecam (BD Biosciences, 1:100) or Flk1 (BD Biosciences, 1:100) in TNB buffer. After three washes with TBS, FITC-conjugated anti-rat IgG (Jackson Immunological, 1:100) secondary antibody in TNB was applied for 1 hour at room temperature. Allantoises were analyzed using a Leica confocal microscope, permitting 3D compilation of individual confocal planes.

RESULTS

Generation of *Cdx4* targeted ES cells and mice

We designed a strategy to conditionally inactivate *Cdx4* in order to circumvent the risks of creating an embryonic lethal phenotype (Fig. 1A,B). Mice bearing the *Cdx4* conditional allele and, more surprisingly, $Cdx4^{-/0}$ embryos developed to term and were born healthy, according to Mendelian frequencies. Some 20 adult *Cdx4*-null males have been used in successful breeding programs, half of them for more than a year. In addition, homozygous *Cdx4*-null females have been generated, and a homozygous mutant colony has been established, proving that these animals were physiologically healthy. Skeletal preparations of *Cdx4*-null and wild-type E15.5 embryos (n=5), and of *Cdx4*-null and wild-type

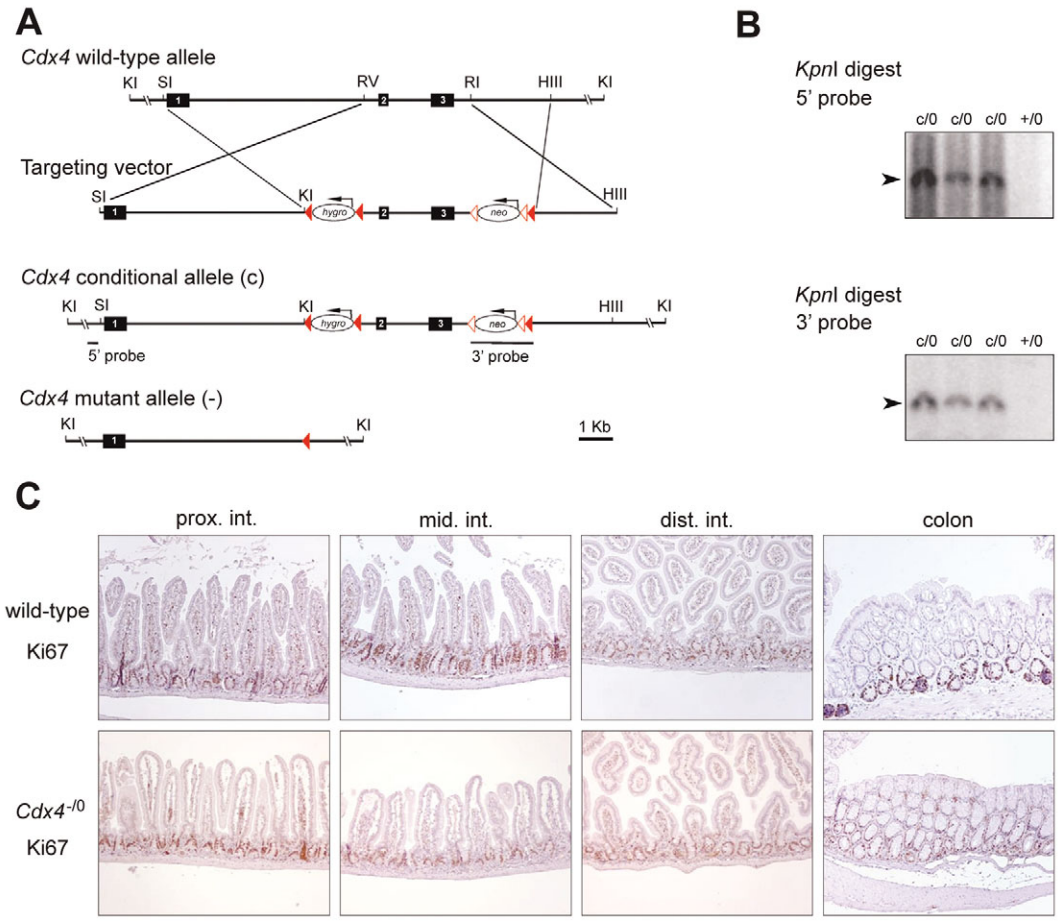

Fig. 1. Targeted disruption of the *Cdx4* gene. (**A**) The gene-targeting vector inserts an antisense floxed *PGK-hygro*^{r} cassette into an *Eco*RV site upstream of exon 2 and an antisense flipped *PGK–neo*^{r} cassette, including a single loxP site into an *Eco*RI site downstream of exon 3, resulting in a conditional allele of *Cdx4* (*Cdx4*^{c}). Cre-mediated deletion of the homeodomain in exon 2 and exon 3 generates the *Cdx4*-null allele (*Cdx4*$^-$). LoxP and FRT sites are represented by closed and open red triangles, respectively. HIII, *Hin*dIII; KI, *Kpn*I; RI, *Eco*RI; RV, *Eco*RV; SI, *Spe*I. (**B**) Southern blot analysis of male ES cell clones with a unique 5′ genomic probe reveals a 17 kb *Kpn*I fragment generated by the targeted conditional allele. A 3′ internal probe reveals correct 3′ targeting by identification of a 17 kb *Kpn*I fragment. (**C**) Histological evaluation of the digestive tract of adult *Cdx4*-null and control (wild-type) mice. Ki67 immunohistochemistry shows normal proliferation in the intestinal and colonic crypts (brown cells). Unlike previous findings in *Cdx2*$^{+/-}$ mice, no intestinal polyp-like lesions were observed.

newborns (n=3), established that the mutants have a wild-type vertebral phenotype at all axial levels (not shown). The only exception concerns a difference at the level of vertebra 15 (V15), where one (1/8) mutant had its eighth rib pair attached to the sternum whereas this rib pair was the first free-floating in the other mutants and the controls. This feature might be interpreted as a very mild anterior transformation at the level of V15 with an extremely low penetrance.

Histological analysis of the digestive tract of two 1-year-old adult *Cdx4*-null mice did not reveal any abnormalities. Given the role of Cdx factors in AP patterning, the complete gastrointestinal (GI) tract was investigated for anatomical and histological organization. Anatomical boundaries between esophagus, stomach, small intestine and colon were clearly defined, and no truncations or homeosis was observed. Stomach, intestine and colon exhibited normal histological structures based on Hematoxylin and Eosin staining. Proliferation was assessed by Ki67 staining and was restricted to the gland isthmus in the stomach (not shown) and to the crypt in the intestine and colon (Fig. 1C), showing no differences to control mice. PAS staining revealed a normal distribution of mucus cells in the stomach and goblet cells in the intestine (not shown). Thus, proliferation and differentiation was normal throughout the GI tract. Finally, unlike earlier observations in *Cdx2*$^{+/-}$ mice, no intestinal polyp-like lesions were observed.

Loss of *Cdx4* function synergizes with a reduction in Cdx dosage in AP patterning, axial elongation and placentogenesis

In view of early co-expression of *Cdx1*, *Cdx2* and *Cdx4*, and the functional redundancy between *Cdx1* and *Cdx2* in axial extension and AP patterning (van den Akker et al., 2002), we set out to investigate whether additional loss of *Cdx1* and *Cdx2* alleles would reveal functional cooperation between *Cdx4* and these other Cdx genes. E15.5 *Cdx1*$^{-/-}$/*Cdx4*$^{-/0}$ double-null mutant embryos exhibited very subtle changes in their vertebral phenotype compared with *Cdx1*-null littermates. Although 5/9 *Cdx1*-null embryos carried their first rib on the 9th vertebrae (V9) unilaterally instead of on V8 (thoracic to cervical transformation of V8, with the consequent loss of one rib), 5/7 *Cdx1/Cdx4* double null mutants did, of which 3/5 exhibited this transformation bilaterally, manifesting a slight

increase in severity and penetrance of the *Cdx1* phenotype at this V8 level. In addition, 5/7 double mutant embryos had their 8th rib pair attached to the sternum bilaterally, while only 2/9 *Cdx1*-null mutants did, and 3/9 unilaterally (not shown). This represents a slight increase in the severity and penetrance of the *Cdx1*-null phenotype at the V15 axial level. Compared with E15.5 *Cdx2* heterozygote mutant embryos (n=4), which did not manifest any vertebral transformations in our genetic background, 4/4 $Cdx2^{+/-}/Cdx4^{-/0}$ littermates had their 8th pair of ribs attached to the sternum, manifesting an anterior homeotic-like transformation at this V15 level. In addition, all four compound mutant embryos had a partial or complete rib attached to V21 bilaterally, at the position normally corresponding to the first lumbar vertebrae. Consequently, the compound $Cdx2^{+/-}/Cdx4^{-/0}$ mutants have 14 ribs. We conclude that the *Cdx4* mutation has a specific additive effect on the *Cdx1* loss of function, and on the loss of one active *Cdx2* allele, in patterning the vertebrae at discrete thoracic levels (respectively V8 and V15, and V15 and V21).

Cdx1-, *Cdx4*-null embryos and *Cdx1/Cdx4* double null mutants did not manifest any axial truncation (not shown). By contrast, *Cdx2* was found to strongly synergize functionally with *Cdx4* in posterior tissue generation during axial extension. $Cdx2^{+/-}/Cdx4^{-/0}$ compound mutant embryos exhibited truncation of embryonic structures posterior to the hindlimbs, resulting in a more anteriorly located tail bud, a phenotype much more severe than that of either mutant alone and reminiscent of, though less marked than that of *Cdx2*-null embryos (Fig. 2A-C).

In addition, and independently from the posterior truncations which are not expected to lead to early embryonic lethality, most compound $Cdx2^{+/-}/Cdx4^{-/0}$ embryos were growth retarded at E10.5 (see Fig. 2A) and died in utero shortly thereafter (Tables 1 and 2). The heartbeat and circulation in the embryo, as well as circulation in the yolk sac were normal at E9.5, but failed around E10.5. Taken together with the expression of *Cdx2* and *Cdx4* in the allantoic anlage during normal development, these findings suggested chorioallantoic placental failure. A first analysis of the phenotype of embryos from $Cdx2^{+/-}$ and $Cdx4^{+/-}$ intercrosses revealed that the allantois of a small number of $Cdx2^{+/-}$ (three out of 54), $Cdx2^{+/-}/Cdx4^{+/-}$ (two out of 28) and $Cdx2^{+/-}/Cdx4^{-/0}$ (seven out of 35) embryos had not fused with the chorion at E9.5 (Fig. 3B,C; data not shown). Measurement of allantoic length around the normal fusion stage of six to eight somites (E8.0) (Downs and Gardner, 1995; Downs, 2002) indicated that the reason for the lack of fusion of the mutant allantoises with the chorion was not a length reduction (see Fig. S1 in the supplementary material). Histological analysis of E9.5 unfused allantoises revealed that the chorio-adhesive mesothelium had formed, and that blood filled vessels were present in the distal allantoic mesenchyme (Fig. 3D). Moreover, in situ hybridization showed that *Vcam1* was expressed in the mutant E8.5 distal allantois that had failed to fuse (see Fig. S1 in the supplementary material). The mesodermal layer of the chorion was also present in the mutant, and expressed α4 integrin, the VCAM1 receptor in the chorio-allantoic fusion process. Expression of α4 integrin was still observed in the mutant at a stage when chorio-allantoic fusion and degradation of the chorionic mesoderm layer have occurred in the wild type (see Fig. S1 in the supplementary material). A subset of $Cdx2^{+/-}/Cdx4^{-/0}$ mutant allantoises is thus unsuccessful in fusing with the chorion in spite of the fact that they reach a sufficient length to touch it, and achieve an advanced degree of differentiation.

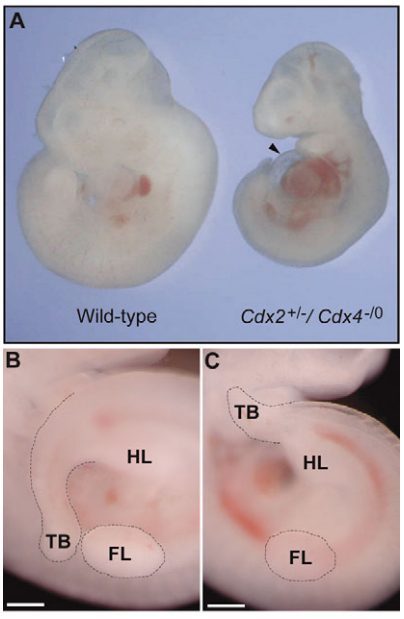

Fig. 2. Phenotype of compound $Cdx2^{+/-}/Cdx4^{-/0}$ mutant embryos. (**A**) Gross morphology of a wild-type and $Cdx2^{+/-}/Cdx4^{-/0}$ compound mutant littermate at E10.5, showing developmental retardation of the compound mutant embryo. Thoracic edema (arrowhead in A) indicates a defect in embryonic circulation. (**B**,**C**) Close up of the tail bud of a E10.25 wild type (B) and $Cdx2^{+/-}/Cdx4^{-/0}$ mutant (C), showing the posterior truncation. TB, tail bud; HL, hindlimbs; FL, forelimbs. Scale bar: 500 μm.

Table 1. Defective placental labyrinth after chorio-allantoic fusion at E10.5

	Defective placental labyrinth at 10.5 dpc
Wild type	0/14
$Cdx2^{+/-}$	6/62
$Cdx4^{+/-}$	0/14
$Cdx4^{-/0}$	0/12
$Cdx2^{+/-}/Cdx4^{+/-}$	2/11
$Cdx2^{+/-}/Cdx4^{-/0}$	18/18
$Cdx1^{-/-}/Cdx4^{-/0}$	0/8

Table 2. Number of new born pups recovered from crosses between *Cdx2* heterozygous ($Cdx2^{+/-}$) males and *Cdx4* heterozygous ($Cdx4^{+/-}$) females

	Postnatal observed (expected*)
Wild-type	52 (52)
$Cdx2^{+/-}$	39 (52)
$Cdx4^{+/-}$	28 (26)
$Cdx4^{-/0}$	22 (26)
$Cdx2^{+/-}/Cdx4^{+/-}$	11 (26)
$Cdx2^{+/-}/Cdx4^{-/0}$	4 (26)

*The expected numbers were calculated using the Mendelian rule, based on the fact that these crosses produced 52 wild-type pups (1/4), and should give about 52 (1/4) $Cdx2^{+/-}$ heterozygous pups (males and females together); 52 (1/4) pups carrying exclusively the *Cdx4* mutant allele, among which half would be $Cdx4^{-/0}$ males and half $Cdx4^{+/-}$ females; and 52 (1/4) pups carrying both mutant alleles, among which half would be $Cdx2^{+/-}/Cdx4^{+/-}$ females and half $Cdx2^{+/-}/Cdx4^{-/0}$ males.

From a second data set involving exclusively the 80% of E8.5 *Cdx2*$^{+/-}$/*Cdx4*$^{-/0}$ embryos that had undergone successful chorio-allantoic fusion, histological analysis revealed that virtually all (in this case 18/18) *Cdx2*$^{+/-}$/*Cdx4*$^{-/0}$ embryos displayed deficient placental labyrinth development at E10.5 (Fig. 4E-H; Table 1).

Cdx2/*Cdx4* compound mutant embryos initiate but do not maintain or expand their allantoic vascular network

In order to characterize the most frequent defect causing the death of *Cdx2*$^{+/-}$/*Cdx4*$^{-/0}$ embryos, we selectively recovered compound mutant embryos that had undergone successful chorio-allantoic fusion. At E9.5, blood vessels were present in the *Cdx2*$^{+/-}$/*Cdx4*$^{-/0}$ mutant allantois, and they had initiated penetration of the chorionic ectoderm (Fig. 4B, compare with 4A, and with enlargements in 4C and 4D). We observed some variation in the extent of allantoic vessel penetration into the chorion, and the mildest defects may correspond to the minority of *Cdx2*$^{+/-}$/*Cdx4*$^{-/0}$ mutants that survive (Table 2). In all E10.5 mutant placentas that we analyzed histologically, maternal blood pools and embryonic blood vessels were widely separated in E10.5 mutant placentas (compare Fig. 4F,H with 4E,G), indicating an increasingly more dramatic defect with age, and showing that the mutant phenotype is not a simple developmental delay. These findings are sufficient to explain the high midgestation mortality as being due to placental insufficiency.

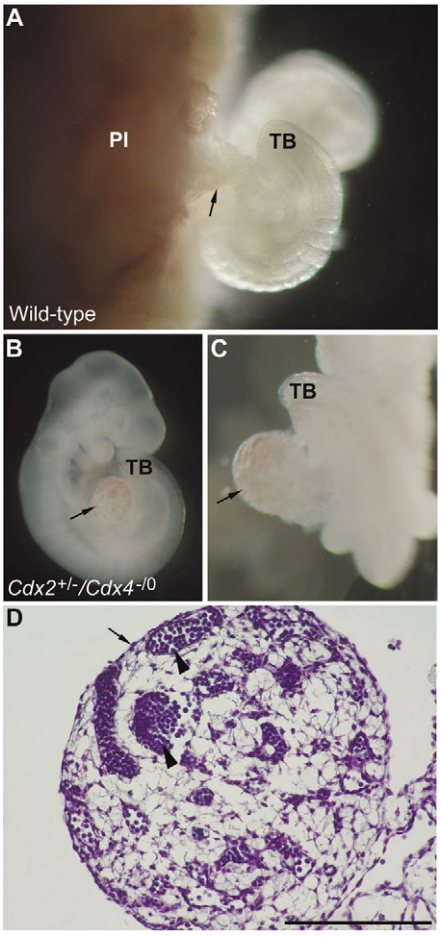

Fig. 3. A subset of *Cdx2*$^{+/-}$/*Cdx4*$^{-/0}$ mutant embryos fails to undergo chorio-allantoic fusion. (**A**) Lateral view of freshly dissected E9.5 wild-type embryo. The allantois has fused to the chorionic part of the placenta (arrow). (**B**) Lateral view of a E9.5 *Cdx2*$^{+/-}$/*Cdx4*$^{-/0}$ compound mutant littermate that is defective in chorio-allantoic fusion (arrow). (**C**) Frontal view of the embryo shown in B. The allantois appears to end as a massive ball of cells (arrow). (**D**) Histological analysis of the allantois shown in C. Several allantoic vessels, containing nucleated embryonic blood cells (arrowheads) and the outer mesothelial layer (arrow) are visible. Pl, placenta; TB, tail bud. Scale bar: 100 μm.

We studied the allantoic vasculature that, together with the chorion will generate the placental labyrinth. The allantoic vasculature is known to be initiated by vasculogenesis within the allantois before the latter fuses with the chorion (Downs et al., 1998; Downs, 1998). Following chorio-allantoic fusion, allantoic vessels penetrate the chorionic ectoderm and branch extensively to interdigitate between the maternal blood sinuses (reviewed by Cross, 2005; Rossant and Cross, 2001). We followed this process throughout development in mutant and wild-type placentas using several endothelial markers. In situ hybridization of *Flk1* transcripts (data not shown) and antibody staining for PECAM expression (Fig. 5C-F) both highlighted the limited expansion of allantoic vessels in the chorionic ectoderm in mutants compared with controls at E9.5 and E10.5. Quantification of both the number and the diameter of allantois-derived embryonic vessels that had penetrated the chorionic ectoderm at E9.5 clearly documented that these allantoic vessels branched much less extensively in Cdx mutants (Fig. 5A,B), and almost never came close to the maternal blood sinuses. The thin hemotrichorial membrane (Enders, 1965) normally developing in areas of close contact between fetal and maternal blood circulation is almost completely absent in the mutant (Fig. 4C,D). The defect in establishment of an interdigitated labyrinth was more extreme at E10.5, with maternal and embryonic blood widely separated (Fig. 5E,F and Fig. 4G,H).

All allantoic and placental molecular markers tested are expressed in Cdx compound mutants

Cdx2 is expressed in the placental spongiotrophoblasts but not in the labyrinth, in both wild type and *Cdx2*$^{+/-}$/*Cdx4*$^{-/0}$ mutants at E9.5 (Fig. 5G,H). *Cdx4* was not expressed in either placenta or labyrinth (data not shown).

We tested the expression of several endothelial markers [*Tie1*, *Tie2* (*Tek* – Mouse Genome Informatics), *Flk1* (*Kdr* – Mouse Genome Informatics), *Flt1*, *Pecam*, *Vegf*] by RT-PCR in the E8.25 allantois before chorio-allantoic fusion. All genes were expressed in compound mutants and controls (see Fig. S2A in the supplementary material). In addition we tested the expression of *Angpt1*, *Angpt2* (previously Agpt1 and Agpt2) and *Vegf*, genes known to be involved in angiogenesis during placental labyrinth development (reviewed by Rossant and Cross, 2001) by semi-quantitative RT-PCR at E9.25 and did not observe consistent changes in gene expression (see Fig. S2B in the supplementary material).

The expression of trophoblast markers was assayed as well in mutants and controls. *Mash2* (*Ascl2* – Mouse Genome Informatics) (Guillemot et al., 1994) was found to be expressed in the spongiotrophoblasts and in the differentiating labyrinthine trophoblasts at E9.5 (Fig. 5I,J). The expression of *Fzd5* expression in labyrinthine trophoblasts and *Flt1* in the spongiotrophoblast (reviewed by Rossant and Cross, 2001) were not changed (data not shown). Analysis of the expression of *Hand1* in the diploid trophoblasts and trophoblast giant cells (Cserjesi et al., 1995), and *Tpbp* in spongiotrophoblasts and their precursors in the ectoplacental cone (Lescisin et al., 1998), did not reveal any

change between Cdx compound mutants and controls (Fig. 5K-N). These results confirmed the presence of all placental cell types in E9.5 mutants and controls, and only reflected the lack of labyrinthine cell organization in *Cdx2*$^{+/-}$/*Cdx4*$^{-/0}$ mutants, compared with wild types.

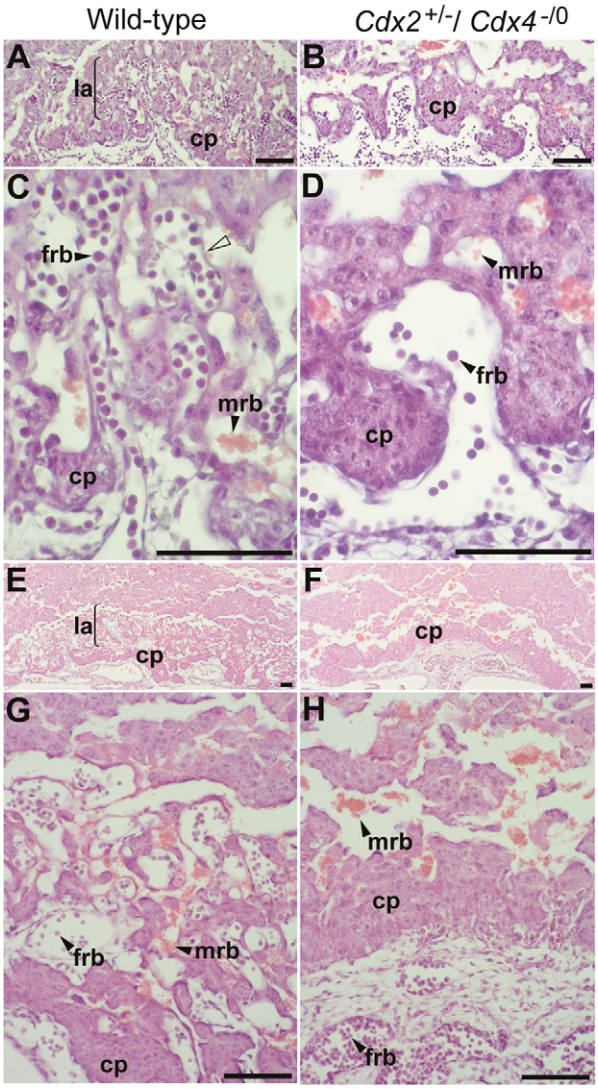

Fig. 4. Defective placental labyrinth development in *Cdx2*$^{+/-}$/*Cdx4*$^{-/0}$ compound mutant embryos. (A-H) Hematoxylin and Eosin-stained sections of placentas from wild-type (A,C,E,G; C is an enlargement of A; G is an enlargement of E) and *Cdx2*$^{+/-}$/*Cdx4*$^{-/0}$ compound mutant littermates (B,D,F,H; D is an enlargement of B; H is an enlargement of F). (**A-D**) At E9.5, only some *Cdx2*$^{+/-}$/*Cdx4*$^{-/0}$ allantoic vessels have started to penetrate the chorionic trophoblast layer without overt signs of branching morphogenesis, whereas wild-type placentas show branched vessels deeply penetrating the chorionic ectoderm. These vessels intermingle with maternal blood sinuses, from which they are separated in many places by only a thin haemotrichorial membrane (triangle in C) in the crucial part of the labyrinth (la in A) that is concerned with the vital interchanges between maternal and fetal circulation. (**E-H**) At E10.5, the defect becomes more severe, as revealed by the complete separation of maternal and embryonic blood flows (mrb and frb in H) and the virtual absence of the labyrinth (F, compare with E). Scale bars: 100 μm. cp, chorionic plate; mrb, maternal red blood cell; frb, fetal red blood cell; la, labyrinthine trophoblast.

We examined the expression of *Gcm1*, encoding a factor associated with specification of the sites of penetration of allantoic vessels into the chorionic ectoderm, and a marker of differentiating chorionic trophoblasts at later stages (Anson-Cartwright et al., 2000). Although no difference could be detected between the weak *Gcm1* expression in E9.5 control and mutant placentas by in situ hybridization (not shown), a slightly reduced expression was observed in semi-quantitative RT-PCR analysis (see Fig. S2B in the supplementary material).

The degree of proliferation of labyrinthine endothelial cells and the possible occurrence of apoptosis were tested at several developmental stages in control and mutant embryos. Ki67 labelling did not reveal impaired proliferation (see Fig. S3 in the supplementary material), and the use of caspase 3 did not indicate increased apoptosis (see Fig. S3 in the supplementary material) at E9.5, the stage at which the allantoic vasculature initiates penetration into the chorionic trophoblast in both mutants and controls. At E10.5, apoptotic cells were more numerous in mutant than control placenta, but this difference was not restricted to the endothelial cells as it concerned chorionic trophoblasts and fetal blood cells as well, and may only reflect developmental arrest around this stage (see Fig. S3 in the supplementary material).

Early Cdx expression overlaps with Flk1 positive endothelial precursors

Cdx genes are not expressed in the labyrinth. The only place and stage where progenitors for the allantoic vasculature are expected to express Cdx genes is the early allantoic bud at primitive streak stages. *Cdx4*, like *Cdx2*, is widely expressed in the early allantoic bud (E7.5), while expression of *Cdx1* is confined to the base of the allantois (Fig. 6A-C). In order to characterize the ontogenesis of the allantoic component of the labyrinth in the Cdx mutants, we analyzed the distribution of Flk1-positive endothelial precursor cells (Downs et al., 1998; Drake and Fleming, 2000; Yamashita et al., 2000) at an early stage. Flk1-positive cells are present in both mutants and controls (Fig. 6D,E), in an area and at a stage (E7.5) when *Cdx2* and *Cdx4* are still fully expressed in the allantois (Fig. 6B,C). This indicates that the precursors of allantoic endothelial cells could be affected by the Cdx mutations.

Mutant allantoic endothelial cells often exhibit a poorer primary vessel organization

At E8.0 and E8.5, Pecam-positive endothelial cells are abundant and are organized in a primary vessel network in both mutants and controls. Among the mutant specimens that we examined compared with age-matched controls (nine mutants and eight wild types), we found some variability in the extent of maturation of this network, which may correspond to the variability in the severity of the labyrinth defect at E9.5 described above. Fig. 6F,G shows an example of an E8.25 age-matched pair of allantoises shortly after chorio-allantoic fusion and reveal a lower degree of endothelial organization into a primary vessel network in the mutant compared with the wild type. In the (in this case) slightly smaller mutant allantois, the endothelial cells are locally less well organized than in the control (Fig. 6H,I). Only a minority (two out of nine) of *Cdx2*$^{+/-}$/*Cdx4*$^{-/0}$ mutant allantoises isolated at E8.25 after chorio-allantoic fusion show similar endothelial organization to the wild types (not shown). Thus, these data reveal that mutant allantoic endothelial cells often (seven out of nine) exhibit, to a variable extent, a poorer degree of primary vessel organization around the time of chorio-allantoic fusion. Although endothelial cells are abundant in both mutants and controls, they seem to be less

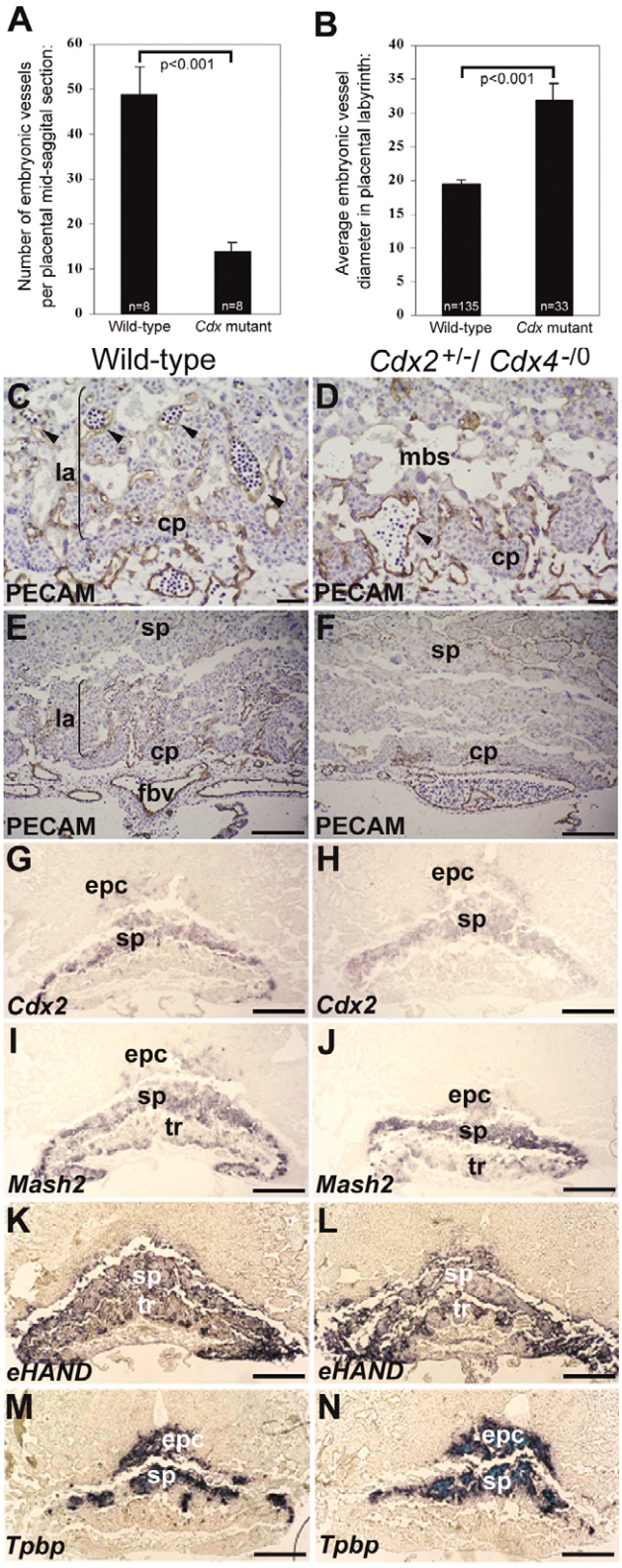

Fig. 5. Altered branching of allantoic vessels, and similar endothelial and trophoblast marker expression in Cdx mutant and control embryos. (**A**,**B**) Morphometric analysis of embryonic placental vessels reveals more vessels with smaller average diameter in wild-type placentas than in mutant placentas. (**C**,**D**) Immunohistochemical staining for Pecam marks endothelial cells (in brown) in the developing labyrinth. At E9.5, wild-type embryonic vessels have penetrated the chorionic ectoderm and branched extensively (arrowheads in C) while *Cdx2*$^{+/-}$/*Cdx4*$^{-/0}$ mutant endothelial cells can only be detected on one side of the chorionic plate (arrowhead in D). (**E**,**F**) A well-established labyrinth is present at E10.5 in wild types (la in E), whereas in *Cdx2*$^{+/-}$/*Cdx4*$^{-/0}$ mutant placentas, embryonic vessels are present only in the allantoic mesoderm (F). (G-N) Expression of trophoblast markers at E9.5 in *Cdx2*$^{+/-}$/*Cdx4*$^{-/0}$ compound mutant placentas and controls. (**G**,**H**) In situ hybridization of *Cdx2* shows expression in the ectoplacental cone and spongiotrophoblast. Expression is absent in the labyrinthine trophoblasts in both mutants and controls. (**I**,**J**) In situ hybridization of *Mash2* shows expression in the ectoplacental cone, spongiotrophoblasts and labyrinthine trophoblasts. The morphology of the labyrinthine trophoblast differs between mutant and control, owing to the absence of an extensively intermingled labyrinth in the compound mutant. (**K**,**L**) In situ hybridization of *Hand1* (*eHAND*) shows expression in the spongiotrophoblast and labyrinthine trophoblasts in wild-type and compound mutant placentas. (**M**,**N**) The ectoplacental cone and spongiotrophoblast marker *Tpbp* is expressed in wild-type and *Cdx2*$^{+/-}$/*Cdx4*$^{-/0}$ compound mutant placentas. epc, ectoplacental cone; sp, spongiotrophoblasts; tr, labyrinthine trophoblasts; cp, chorionic plate; la, placental labyrinth; mbs, maternal blood sinus; fbv, fetal blood vessel; Cdx mutant, *Cdx2*$^{+/-}$/*Cdx4*$^{-/0}$. Scale bars: 100 μm C-F; 500 μm in G-N.

organized in the *Cdx2*$^{+/-}$/*Cdx4*$^{-/0}$ compound mutants than in the wild type, suggesting an incipient defect expressed later as failure to extend and branch into the chorion between E9.5 and E10.5.

Thus, the data altogether reveal that an essential component of the labyrinthine defect observed in E9.5 and E10.5 Cdx mutants resides in the intrinsic property of the allantois to organize and extend its vasculature into the chorion.

DISCUSSION

A main requirement for Cdx proteins during placental labyrinth development

The data presented here on the phenotype of *Cdx2/Cdx4* double mutant embryos indicate that the Cdx genes are essential for the ontogenesis of a functional placental labyrinth. Their inactivation compromises chorio-allantoic fusion in a subset of the compound mutants. In the majority of these mutants, which successfully unite their allantois with the chorion, loss of Cdx function disables the allantoic vessel extension and branching within the chorionic ectoderm between E8.5 and E10.5. Neither *Cdx4* deficiency alone, nor, in most cases, *Cdx2* heterozygosity, compromises placental development crucially. However, *Cdx2* heterozygosity together with heterozygous or homozygous loss of *Cdx4* often result in placental failure. In the *Cdx2* heterozygote background, the penetrance of the labyrinth deficiency is dependent on the dose of *Cdx4* (Table 1), which is only expressed in the allantoic component of the labyrinth. The combined function of *Cdx2* and *Cdx4* in the placental labyrinth may appear to be specific. Nevertheless, considering the redundancy between Cdx functions in the embryo, we must await the analysis of more complex genotypes to determine whether *Cdx1* is, to some extent, assisting *Cdx2* and *Cdx4* in this function.

It will be interesting to find out whether Cdx genes exert their effect on the placenta by regulating target Hox genes. This is suggested by the reported biological activity of certain Hox genes to promote migratory behavior of adult endothelial cells in culture

(Boudreau et al., 1997) and to induce morphogenesis of new vascular sprouts in chick chorio-allantoic membranes in culture (Myers et al., 2000). Our data suggest that the regulatory function of Cdx genes in placentogenesis is an early one, acting at the level of the progenitors of endothelial cells in the early allantois, as Cdx genes, like Hox genes, are downregulated in the allantois at E8.5.

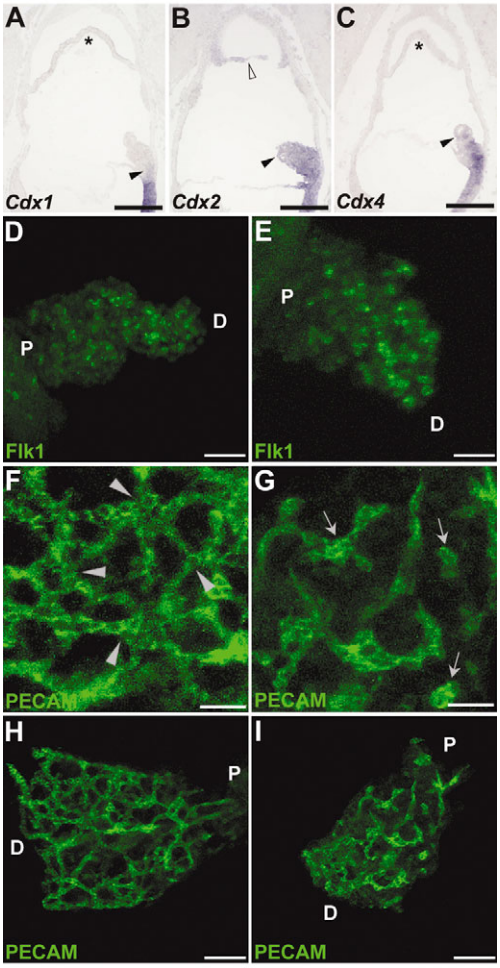

Fig. 6. Cdx expression and early vascular development in the allantois. (**A-C**) In situ hybridization on E7.5 embryo sections reveals strong expression of *Cdx1* in the primitive streak and weak expression at the base of the allantois (arrowhead in A). Expression is absent in the chorion (asterisk in A). (B) *Cdx2* is strongly expressed in the primitive streak and in the outgrowing allantois (arrowhead) and in the chorion (triangle). (C) *Cdx4* expression is restricted to the primitive streak and allantois (arrowhead), and is absent in the chorion (asterisk). (**D**,**E**) Flk1 staining marks endothelial precursors that are present throughout the E7.5 wild-type (D) and $Cdx2^{+/-}/Cdx4^{-/0}$ mutant allantois (E). The allantois of the wild type was slightly smaller than that of the compound mutant, explaining the lower Flk1 staining in D. (**F-I**) Pecam staining of allantoises isolated at E8.25 just after chorio-allantoic fusion. Primary endothelial networks in wild-type (F and H) and $Cdx2^{+/-}/Cdx4^{-/0}$ mutant (G and I) allantoises are visible. The wild-type E8.25 allantois in F shows organization of endothelial cells into vessels with numerous branching and connecting vessels (arrowheads). Endothelial cells are less well organized in $Cdx2^{+/-}/Cdx4^{-/0}$ compound mutant littermate shown in G, with clumps of Pecam-positive cells (arrows). The $Cdx2^{+/-}/Cdx4^{-/0}$ allantois in I is, in this case, smaller than the wild-type control (H). Scale bars: 200 μm in A-C; 40 μm in D,E; 20 μm in F,G; 80 μm in H,I. D, distal; P, proximal.

Function of *Cdx4* in mouse and fish

A possibly related function of *Cdx4* has been described in zebrafish (Davidson et al., 2003). *Cdx4*-null zebrafish embryos manifest a transient deficiency in generating erythroid progenitors (Davidson et al., 2003). The authors suggest an early function of *Cdx4* in making the posterior lateral plate mesoderm, containing early hematopoietic progenitors, competent to respond to genes that specify hematopoietic fate. The defect in zebrafish *Cdx4* mutants was a selective loss of blood progenitors but not of angioblast specification, thus affecting only one of the hemangioblast derivatives (Huber et al., 2004; Ema et al., 2003). Such a defect in blood development was not evident in the Cdx compound mutant mice examined so far, but a possible slight and/or transient delay in generating erythroid cells at any one of the stages when blood progenitors are born (successively yolk sac blood islands, placenta, AGM and fetal liver) (Gekas et al., 2005; Ottersbach and Dzierzak, 2005) might not have been noticed in our experiments. Alternatively, such a function for *Cdx4* might be redundant in mice, thus differing from the situation in zebrafish. An insufficiency may remain hidden until the Cdx dose has been lowered below a certain threshold. The fact that *Cdx4* rescues the hematopoietic colony-forming potential in mixed lineage leukemia (*Mll*) mutant ES cell-derived embryoid bodies and enhances the frequency of hematopoietic colonies in control embryoid bodies (Ernst et al., 2004) provides support for this hypothesis.

Vascular lineages were not affected by the *Cdx4* mutation in zebrafish but the absence of an allantois in fish leaves the possibility that, after the acquisition of the chorio-allantoic placenta in mammals, the gene became specifically involved in the ontogeny of a functional allantoic vasculature. An implication of these findings is that mutations in Cdx genes might lead to pregnancy failure in humans.

Specificity versus redundancy in function among Cdx members

Among the three Cdx family members, *Cdx2* is the only one that is expressed in the early extra-embryonic ectoderm, where it ensures establishment and maintenance of the trophectoderm lineage (Strumpf et al., 2005), mediating implantation of the conceptus (Chawengsaksophak et al., 1997). This specific expression and function only concerns *Cdx2* and may result from a trophectoderm-specific regulatory influence selectively relating to *Cdx2*, without counterpart on the other, unlinked, Cdx genes. This early *Cdx2* function requires one active allele, and is not relevant in the context of the compound mutants analyzed in the present work.

The common Cdx functions studied here concern epiblast-derived embryonic and extra-embryonic tissues. All three Cdx genes collaborate to pattern the axial skeletal structures [see van den Akker et al. (van den Akker et al., 2002) for *Cdx1/Cdx2*; see this work for *Cdx1/Cdx4* and *Cdx2/Cdx4,* where the contribution of *Cdx4* is modest but observable in the presence of the *Cdx1* and *Cdx2* mutations]. The distinct AP level of the vertebral changes in the double mutants may relate to the differences in rostral span of the expression domains of *Cdx1* and *Cdx2*. The three Cdx genes contribute to allow completion of posterior axial elongation (van den Akker et al., 2002) (this work). Again, their contributions are unequal, as the effect of inactivating *Cdx1* or *Cdx4* was visible only in combination with a mutation in *Cdx2,* whereas *Cdx2* heterozygocity alone affects axial elongation (van den Akker et al., 2002) (this work). In this case, we cannot tell whether these differences in contribution result from sequence differences in the proteins or from differences in the spatiotemporal regulation of their expression.

Two of the three Cdx genes (*Cdx2* and *Cdx4*) have been shown in this work to control the ontogenesis of the allantoic part of the placenta, and we cannot rule out that a contribution of *Cdx1* in this function might be revealed upon further lowering Cdx dose. All three Cdx genes are expressed in the posterior epiblast, including the most proximal area containing the presumptive extra-embryonic mesoderm. Although loss of function of *Cdx1* or *Cdx4* does not affect the extra-embryonic derivatives by themselves, *Cdx2*-null mutations have previously been shown to impair the generation of embryonic and extra-embryonic mesoderm. The Cdx2-null allantois remains extremely short, and does not touch the chorion (Chawengsakhophak et al., 2004). This reveals the earliest Cdx dependence of placental ontogeny, reflected by the fact that one active *Cdx2* allele is required for outgrowth of the early allantoic bud. The allantoises in $Cdx2^{+/-}$, $Cdx2^{+/-}/Cdx4^{+/-}$ and $Cdx2^{+/-}/Cdx4^{-/0}$ mutants reach a normal size. But these three genotypes exhibit specific subsequent defects that compromise the ontogenesis of a proficient chorio-allantoic placenta, with a penetrance that increases with the decrease in Cdx dose. A first defect is manifest by the fact that some of the fully grown allantoises reach the chorion but fail to unite with it. Several mutants in the Wnt and Fgf signaling pathways are impaired in chorio-allantoic fusion [Wnt7b (Parr et al., 2001); Tcf1/Lef1 (Galceran et al., 1999); Fgfr2 (Xu et al., 1998)]. This could indicate a functional link between these signaling pathways and Cdx genes in the chorio-allantois fusion process, in addition to the links previously documented in the processes of embryonic axial extension and patterning (reviewed by Lohnes, 2003; Deschamps and van Nes, 2005). The majority of mutant allantoises undergo chorio-allantoic fusion but exhibit a later defect, being impaired in the establishment of a functional endothelial network in the labyrinth. The penetrance is again dependent on the dose of Cdx: 10% for $Cdx2^{+/-}$, 20% for $Cdx2^{+/-}/Cdx4^{+/-}$ and ~100% for $Cdx2^{+/-}/Cdx4^{-/0}$ embryos. In each of these genotypic classes, the most frequently occurring labyrinth abnormalities are qualitatively indistinguishable and equally severe, suggesting that *Cdx2* and *Cdx4* function in an identical way, and that the Cdx-dependence operates at the level of reaching a threshold rather than touching different aspects of the events. A small subset of the numerous $Cdx2^{+/-}/Cdx4^{-/0}$ embryos examined were found to be more mildly affected than the majority of them, revealing a certain variability in the severity of this phenotype (these exceptions probably correspond to the occasional survivors among the embryos with this genotype). This variability, which we expect to be proportionally present in each genotypic class ($Cdx2^{+/-}$, $Cdx2^{+/-}/Cdx4^{+/-}$ and $Cdx2^{+/-}/Cdx4^{-/0}$) would possibly result either from stochastic events among parameters involved in the onset of the phenotype, or from the fact that the genetic background of embryos carrying the new *Cdx4* mutation is not homogenous.

In conclusion, our results reveal that the collaborative role of Cdx genes in posterior embryonic development extends to the stepwise establishment of a functional placental labyrinth, from allantois growth and chorio-allantoic fusion to the extension of the allantoic vascular network within the chorionic ectoderm. The data highlight a novel role for these posterior embryonic patterning genes in controlling a vital function that allows the fetus to survive through exchanges with its mother, a function presumably acquired during vertebrate evolution towards placental mammals.

The three Cdx genes exert redundant functions in mediating the generation and AP patterning of posterior embryonic structures. They might also operate redundantly in placentogenesis. In both cases, their functional contribution is quantitatively unequal, owing to either protein or regulatory differences. This suggests that the extra-embryonic mesoderm arising from the posterior part of the primitive streak is controlled by the same genetic system as the posterior axial and paraxial embryonic structures.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/133/3/419/DC1

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