

## CORRIGENDUM

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**Concerted involvement of Cdx/Hox genes and Wnt signaling in morphogenesis of the caudal neural tube and cloacal derivatives from the posterior growth zone**

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There was an error in the ePress version of *Development* **138**, 3451-3462 published on 13 July 2011.

The first names of one of the authors were listed incorrectly. The final print and online versions are correct, and the correct author list appears above.

The authors apologise to readers for this mistake.

# Concerted involvement of Cdx/Hox genes and Wnt signaling in morphogenesis of the caudal neural tube and cloacal derivatives from the posterior growth zone

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## SUMMARY

Decrease in Cdx dosage in an allelic series of mouse Cdx mutants leads to progressively more severe posterior vertebral defects. These defects are corrected by posterior gain of function of the Wnt effector Lef1. Precocious expression of Hox paralogous 13 genes also induces vertebral axis truncation by antagonizing Cdx function. We report here that the phenotypic similarity also applies to patterning of the caudal neural tube and uro-rectal tracts in Cdx and *Wnt3a* mutants, and in embryos precociously expressing Hox13 genes. *Cdx2* inactivation after placentation leads to posterior defects, including incomplete uro-rectal septation. Compound mutants carrying one active *Cdx2* allele in the *Cdx4*-null background (*Cdx2/4*), transgenic embryos precociously expressing Hox13 genes and a novel *Wnt3a* hypomorph mutant all manifest a comparable phenotype with similar uro-rectal defects. Phenotype and transcriptome analysis in early Cdx mutants, genetic rescue experiments and gene expression studies lead us to propose that Cdx transcription factors act via Wnt signaling during the laying down of uro-rectal mesoderm, and that they are operative in an early phase of these events, at the site of tissue progenitors in the posterior growth zone of the embryo. Cdx and Wnt mutations and premature Hox13 expression also cause similar neural dysmorphology, including ectopic neural structures that sometimes lead to neural tube splitting at caudal axial levels. These findings involve the Cdx genes, canonical Wnt signaling and the temporal control of posterior Hox gene expression in posterior morphogenesis in the different embryonic germ layers. They shed a new light on the etiology of the caudal dysplasia or caudal regression range of human congenital defects.

**KEY WORDS:** Mouse Cdx genes, Canonical Wnt pathway, Caudal regression syndrome, Ano-rectal stenosis, Neurectoderm/mesoderm generation

## INTRODUCTION

The *Drosophila* gene *Caudal* (*Cad*) (Mlodzik et al., 1985) has three mammalian homologues: *CDX1*, *CDX2* and *CDX4* in human and *Cdx1*, *Cdx2* and *Cdx4* in mouse. *Cdx4/CDX4* is X-linked in both mouse and human. In the mouse, all three genes are expressed in embryos at the primitive streak stage of development. *Cdx1* transcripts appear at embryonic stage (E) 7.2 in the posterior part of the streak, extending posteriorly to the base of the allantois; *Cdx2* and *Cdx4* start transcription at about the same stage in an overlapping region extending into the base of the allantois (Deschamps and van Nes, 2005). All three genes remain highly expressed in and along the primitive streak and later in the tailbud until E10.5 (*Cdx4*), E11.5 (*Cdx1*) and E12.5 (*Cdx2*). All three genes are also expressed in the developing hindgut endoderm, but only *Cdx1* and *Cdx2* remain expressed there into late gestation and postnatally (Beck, 2002).

All three Cdx genes are involved in antero-posterior patterning of the embryonic axis. *Cdx1*<sup>-/-</sup> mice exhibit anterior homeotic shifts in vertebral identity that involve the upper cervical vertebrae (Subramanian et al., 1995), whereas *Cdx2*<sup>+/-</sup> animals manifest similar homeotic defects more posteriorly, in the lower cervical and upper thoracic regions (Chawengsaksophak et al., 1997). *Cdx2*-null embryos fail to implant because the gene is essential to trophoctoderm development (Strumpf et al., 2005). *Cdx4*-null mice exhibit only a mild anterior transformation at a specific thoracic position with a very low penetrance and no other abnormality. In *Drosophila*, *Cad* has been found to be the homeotic gene that specifies the identity of the last abdominal segment, the analia (Moreno and Morata, 1999).

Cdx genes have been shown to play an essential role in posterior axial elongation in the mouse (Savory et al., 2009; van den Akker et al., 2002; Young et al., 2009) and in several insect and arthropod species that also extend their body axis by posterior addition of tissues (Copf et al., 2004). This mode is not used by long germband insects such as the fruit fly. Although each of the three Cdx genes contributes to posterior axial extension, the contribution of *Cdx2* is the most obvious, as heterozygote *Cdx2* mutants have a slightly shorter axis. Using a *Cdx2* conditional allele (Gao et al., 2009; Savory et al., 2009; Young, 2009) and an epiblast restricted Cre transgene (Hayashi et al., 2002), it was possible to inactivate *Cdx2* specifically in the embryo proper. E10.5 *Cdx2*-null embryos are posteriorly truncated in a similar manner to the *Cdx2*-null embryos that were rescued by tetraploid fusion following intercrossing of

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*Cdx2* heterozygotes (Chawengsaksophak et al., 2004). These *Cdx2*-null embryos do not develop a chorio-allantoic labyrinth and die in utero at E10.5. They lack axial tissues posterior to the forelimbs. Conditional *Cdx2* mutants at E8.5 bypass the placental failure, which allows the embryos to develop up to birth (Savory et al., 2009). Inactivation of *Cdx2* exclusively in the endoderm from its initial specification using *Foxa2 Cre* leads to blunt ending of the gut at the caecum (Gao et al., 2009) and demonstrates a role of *Cdx2* in posterior endoderm expansion. Compound mutants carrying one *Cdx2* null allele and homozygous null for *Cdx4* (referred to later in the manuscript as *Cdx2/4* mutants), fail to generate their posterior tissues caudal to the hindlimbs (Young et al., 2009). Most of these embryos die around E10.5 from deficient placental labyrinthine development (van Nes et al., 2006), but the penetrance of the phenotype is variable, leaving about 10% of the *Cdx2/4* embryos to progress to full term. These latter animals die shortly after birth.

The caudal regression syndrome, also called caudal dysplasia (Welch and Aterman, 1984) encompasses a range of congenital defects of varying severity. These may involve malformations of the lumbar vertebrae, partial or complete sacral agenesis, caudal neural tube defects and abnormalities of cloacal derivatives. The latter include recto-anal atresia, recto-urinary or recto-vaginal fistulae and abnormalities of the bladder outflow tract. Animal models have been described for caudal regression or related syndromes such as ano-rectal malformations (ARMs) and were found to involve sonic hedgehog (Mo et al., 2001), retinoic acid (RA) (Padmanabhan, 1998) and the non canonical *Wnt5a* (Nakata et al., 2009; Tai et al., 2009). A role for *Gdf11* and its associated pro-protein convertase *Pcsk5* has also been suggested, as inactivating the latter causes the VACTERL-like phenotype (Szumska et al., 2008), which comprises vertebral and ano-rectal anomalies. Recently, ano-rectal malformations induced by ethylenethiourea in rat embryos were reported to be accompanied by downregulation of *Cdx1* (Zhang et al., 2009).

We analyzed the morphogenetic defects of an allelic series of *Cdx* mutants, at different stages of embryogenesis and found them to exhibit (besides posterior vertebral truncations) phenotypical traits that mimic caudal regression defects with respect to uro-rectal morphogenesis and neurectoderm patterning. We previously reported that the caudal vertebral truncation of *Cdx2/4* mutants was corrected by a posterior gain of *Lef1* expression activating the canonical *Wnt* pathway (Young et al., 2009). We now have carried out a detailed study of the relationship between *Cdx* and *Wnt* in posterior neural and ano-rectal tissue morphogenesis. Rescue of the *Cdx2/4* vertebral truncation phenotype by a gain of activated *Lef1* re-established development of a separated urogenital sinus and rectum in mutant animals. We also analyzed posterior tissues in a novel hypomorph *Wnt3a* mutant and found that it exhibits a vertebral truncation phenotype of intermediate severity between the *Wnt3a*-null mutants (Takada et al., 1994) and the mild *Wnt3a* hypomorph mutants *Vestigial tail (Vt)* (Greco et al., 1996). In this mutant, we found similar defects in the uro-rectal region and in the caudal neural tube to those we saw in *Cdx2/4* and *Cdx2* mutants (induced at stages to bypass placental defects). We also observed similar defects in posterior neural tube patterning and in uro-rectal septation in transgenic embryos that precociously express *Hox13* genes. These findings point to the participation of *Cdx* genes and canonical *Wnt* signaling not only in the generation of the vertebrae, but also in posterior neural tube morphogenesis and cloacal development. The data also stresses the importance of the correct timing of *Hox* gene expression for these events. We propose that the site of action of this

network centered on *Cdx* and *Wnt* resides in the posterior growth zone in the tail bud, and reveals a unifying function of *Cdx* genes in posterior morphogenesis of tissues in the three germ layers. This suggests that the etiological nature of human caudal dysplasia and ano-rectal malformations (ARMs) may often be the result of a shortage of growth stimulation of the progenitors of posterior tissues in the tail bud at earlier stages of development.

## MATERIALS AND METHODS

### Mice

All mice were in the C57Bl6j/CBA mixed background. *Cdx2* heterozygotes and *Cdx4*-null mutant mice as well as the protocols to genotype them have been described previously (Chawengsaksophak and Beck, 1996; van Nes et al., 2006). As *Cdx4* is X-linked, *Cdx2<sup>+/-</sup>/Cdx4<sup>0/0</sup>* and *Cdx2<sup>+/-</sup>/Cdx4<sup>+/-</sup>* embryos and pups were generated by crossing *Cdx2* heterozygote and *Cdx4*-null mice. *Cdx2<sup>+/-</sup>/Cdx4<sup>+/-</sup>* female embryos and pups were generated by crossing *Cdx2<sup>+/-</sup>/Cdx4<sup>+/-</sup>* females with *Cdx4<sup>0/0</sup>* males.

Transgenic mouse lines and embryos expressing *Hoxb13* and *Hoxc13* under the control of the *Cdx2* promoter (Benahmed et al., 2008) have been described previously (Young et al., 2009). Embryos and animals were analyzed at embryonic stages E8.5, E9.5, E10.5, E12.5, E15.5 and E18.5, at birth (P0), and two days after birth (P2). *Cdx2<sup>+/-</sup>/Cdx4<sup>0/0</sup>* male and *Cdx2<sup>+/-</sup>/Cdx4<sup>+/-</sup>* female animals are referred to as *Cdx2/4* compound mutants.

The generation of *Cdx2* conditional mutants has been reported (Young, 2009) and will be described in detail elsewhere. Epiblast specific *Cdx2*-null mutants were obtained by crossing *Cdx2* Cond homozygotes and *Cdx2<sup>+/-</sup>/Sox2Cre* transgenic mice (Hayashi et al., 2002). Post-placental inactivation of *Cdx2* was achieved by using the *Rosa26CreER<sup>12</sup>* (a generous gift from Austin Smith, CSCR, Cambridge, UK) and tamoxifen intraperitoneal injection at E7.5. The time delay in effective action of tamoxifen in inducing the *Cre* recombinase in the embryos in our experiments allowed the embryos to develop beyond placentogenesis. This *Cre* allele was genotyped using the following *Cre* primers: forward, CCGGGCTGCCACGACCAA; reverse, GGCGCGGCAACACCATTTTT (fragment size: 445 bp). *Wnt3a*-null mice were obtained from S. Takada (Takada et al., 1994). A new *Wnt3a* loss of function hypomorph mutant was recently described (Wansleben et al., 2011). Mice were treated according to the 'Law on animals in experiments', under the licenses required in The Netherlands.

### Tissue treatment

For histological analysis, tissues were fixed with 4% paraformaldehyde (PFA) overnight at 4°C and embedded in paraffin wax. Sections (10 µm) were stained with Hematoxylin and Eosin. For immunostaining, fixation was for 2 hours with 2% PFA.

### In situ hybridization

Whole-mount in situ hybridization of mutant and control embryos was performed according to Young et al. (Young et al., 2009). Probes were generated against *Cdx2*, *Cdx4*, *Axin2*, *Wnt3a*, *Wnt5a*, *Raldh2* (*Aldh1a2* – Mouse Genome Informatics), *Cyp26a1*, *Shh*, *Ihh*, *Hoxa13*, *Hoxb13*, *Hoxc13* and *Hoxd13* (Abu-Abed et al., 2002; Aulehla et al., 2003; Beck et al., 1995; Bogarad et al., 1989; Dolle et al., 1991a; Echelard et al., 1993; Niederreither et al., 1997; Peterson et al., 1994; Roelink and Nusse, 1991; Warot et al., 1997). Hybridization on sections from paraffin-embedded embryos was carried out according to van Nes et al. (van Nes et al., 2006).

### Antibody staining

Antibody staining on 50 µm vibratome sections of agarose-embedded embryos was performed with anti-*Sox2* (Millipore cat #AB5603) and anti-α6 integrin (Bajanca et al., 2004). Counterstaining was with DAPI (Invitrogen cat #D3571).

### Genome-wide transcriptome analysis of *Cdx2* mutants versus controls

Microarray screens of downregulated and upregulated genes in *Cdx2*-null mutant versus wild-type embryos were performed at the 4/5 somite and 7/8 somite stages. RNA was isolated from the posterior part of the embryos (20

embryos of each genotype and stage), dissected at the same axial levels by using the last somite boundary and the base of the allantois as landmarks. Treatment of tissues and microarray hybridization and analysis were performed as described previously (Young et al., 2009). Microarray data have been deposited in GEO with Accession Number GSE30113.

## RESULTS

### Ano-rectal malformation in Cdx mutants and in embryos precociously expressing Hox13 genes

Fetuses that lack *Cdx4* and one allele of *Cdx2* (*Cdx2/4* mutants) usually (90%) exhibit lethal placental defects (van Nes et al., 2006). Ten percent overcome this defect and develop further but all die within a few days of birth (Young et al., 2009). Inspection of neonates with this genotype revealed an imperforate anus (Table 1). Morphogenesis of ano-rectal and urethral tissues was analyzed further. Surviving *Cdx2<sup>+/-</sup>/Cdx4<sup>0/-</sup>* full-term males were growth retarded with short or absent tails. The anal opening was absent in all animals. The abdomen was often distended and on transillumination this was seen to be due to an enlarged fluid-filled bladder (see Fig. S1 in the supplementary material). There was no evidence of defecation and postnatal survival was not possible. On serial sectioning, the bladder was dilated and thin walled. The urethral outflow tract was patent but appeared somewhat distorted, and probably not functional because of pressure from the dilated hindgut. The hindgut ended blindly at the level of the bladder neck and a fistula between the urinary and intestinal tracts was present in all the adult specimens examined ( $n=4$ ). The region at which the gut terminated was variable (see Fig. 1D-F for E10.5 embryos). We also studied serial sections of *Cdx2<sup>+/-</sup>/Cdx4<sup>0/-</sup>* male embryos at E15.5. These exhibited similar features to those described for full-term specimens with the exception that the urinary outflow tract failed to open externally (Fig. 1A,B). We conclude that in the newborn male (*Cdx2<sup>+/-</sup>/Cdx4<sup>0/-</sup>*) compound mutants described above, some, but possibly diminished, continuity of the urinary outflow tract is re-established with the development of the terminal (glandular) region of the urethra. This normally develops to maintain continuity of the urinary tract with the exterior following closure of the male urethral folds in the midline on the lower surface of the penile shaft. This re-establishment is, however, defective and is insufficient to relieve the accumulation of urine in the bladder. We also examined serial sections of a *Cdx2<sup>+/-</sup>/Cdx4<sup>-/-</sup>* female mouse fetuses at E18.5. Once again, we found anal atresia, though a recto-urinary fistula did not

develop due to interposition of the utero-vaginal canal and its mesentery. The bladder in these animals was enormously dilated and the urinary tract did not open to the exterior. There was no evidence of hydronephrosis or of hydroureter. We examined the genital system in all the serially sectioned animals and found no abnormalities in either the gonads or in the gonadal ducts of either sex (not shown).

We analyzed embryos in which *Cdx2* was inactivated after placentogenesis, using the conditional *Cdx2* allele and *Rosa26CreER<sup>T2</sup>*. They were less caudally truncated than the *Cdx2*-null embryos generated with *Sox2Cre*. At E15.5, they all manifested ano-rectal abnormalities of the type observed in *Cdx2/4* compound embryos (Fig. 1C). Their cloacal development was incomplete, again causing a persistent communication between the urogenital and hindgut outflow tracts and death after birth.

A variation in severity of the loss-of-function phenotypes is therefore manifest both in different allelic combinations of Cdx mutations, or by varying the time point of gene inactivation during embryogenesis. This demonstrates a dose and time dependence on Cdx during anteroposterior development, and in particular during cloacal development. In these experiments, as in the work on vertebral axis extension, *Cdx2* plays a more prominent morphogenetic role than *Cdx1* or *Cdx4*.

Some of the *Cdx2PHoxb13* transgenic founder mice expressing the *Hoxb13* gene precociously, under the *Cdx2* promoter (Young et al., 2009), were found to manifest anal atresia. We generated transgenic fetuses expressing *Hoxc13* under the *Cdx2* promoter, and observed that they all exhibit ano-rectal agenesis and abnormal communication between the bladder and the hindgut (Fig. 1G,H). Premature expression of Hox13 genes thus leads to a phenotype similar to that resulting from a decrease in Cdx activity.

### Uro-rectal septation, Cdx and Wnt signaling

Posterior axial defects of *Cdx2/4* mutants are partially corrected by a transgene expressing an activated form of the Wnt downstream effector *Lef1* from the (Brachyury) *T* promoter (Young et al., 2009). This posterior Wnt gain of function rescued the morphogenesis of the hindgut and bladder outflow tracts in eight out of nine cases analyzed (89%) (e.g. Fig. 2A,B). Given this functional rescue of Cdx mutants by an activated *Lef1* transgene, we also analyzed the cloacal derivatives of *Wnt3a* mutant embryos. *Wnt3a* null embryos fail to generate tissues posterior to the forelimbs, which prevents such an analysis, but embryos

**Table 1. Genotype and phenotype of Cdx and Wnt mutants, and Hox13 transgenic embryos**

Genotypes	Posterior vertebral truncation* (level of the truncation)	Cloacal septation and ano-rectal defects <sup>†</sup>	Posterior neural tube dysmorphology <sup>‡</sup>
<i>Cdx1<sup>-/-</sup></i> ( $n>10$ )	–	–	–
<i>Cdx4<sup>-/-</sup></i> ( $n>10$ )	–	–	–
<i>Cdx2<sup>+/-</sup></i> ( $n>10$ )	+ (Tip of the tail)	–	–
<i>Cdx2<sup>+/-</sup> Cdx4<sup>-/-</sup></i> ( $n>10$ )	+ (Hindlimb level)	+	+
<i>Cdx2<sup>-/-</sup> Sox2Cre</i> ( $n=10$ )	+ (Forelimb level)	ng	+
<i>Cdx2<sup>-/-</sup> RosaCreER<sup>T2</sup> Tam E7.5</i> ( $n=3$ )	+ (Hindlimb level)	+	+
<i>Cdx2<sup>-/-</sup> Cdx4<sup>-/-</sup> Sox2Cre</i> ( $n=4$ )	+ (Forelimb level)	ng	+
<i>Cdx2<sup>+/-</sup> Cdx4<sup>-/-</sup> TPLef1 Tg</i> ( $n=7$ )	– (Largely rescued)	–	–
<i>Wnt3a<sup>-/-</sup></i> ( $n=10$ )	+ (Forelimb level)	ng	+
<i>Wnt3a<sup>hypo/hypo</sup></i> ( $n=10$ )	+ (Hindlimb level)	+	+
<i>Cdx2PHoxb13</i> homozygous ( $n=5$ )	+ (Caudal level)	+ <sup>§</sup>	+
<i>Cdx2PHoxc13</i> hemizygous ( $n=4$ )	+ (Sacral level)	+	+

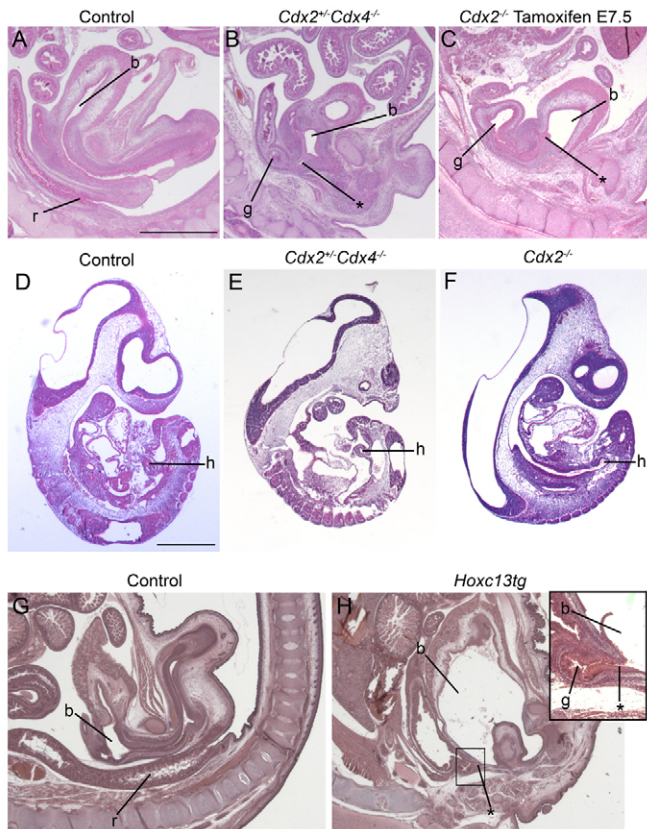
\*Analyzed at E10.5 or new born.

<sup>†</sup>Analyzed at E12.5, E14.5 or E18.5.

<sup>‡</sup>Analyzed at E10.5.

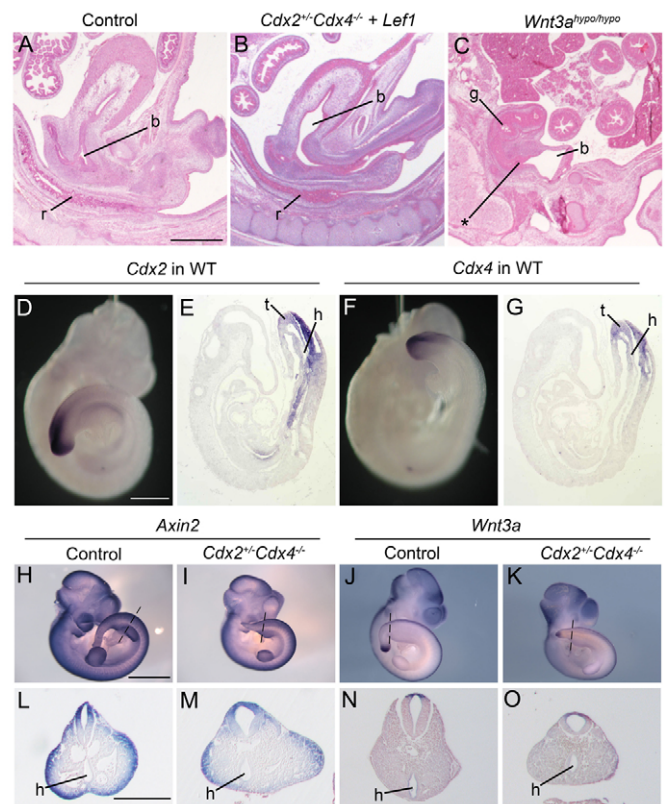
<sup>§</sup>Young et al. (Young et al., 2009).

ng, region not generated.



**Fig. 1. Phenotypes of *Cdx* mutants and *Hoxc13* transgenic embryos in the uro-rectal region.** (A–C) Sagittal sections of the uro-rectal region of a E15.5 embryos control (A), *Cdx2*<sup>+/+</sup>*Cdx4*<sup>-/-</sup> mutant (B) and *Cdx2*<sup>-/-</sup> (C); upon *Cdx2* inactivation at E7.5 by tamoxifen induction of *Rosa26* Cre ER<sup>T2</sup>. (D–F) E10.5 sagittal sections of a control (D), *Cdx2*<sup>+/+</sup>*Cdx4*<sup>-/-</sup> mutant (E) and a *Cdx2*<sup>-/-</sup> mutant (F), showing the blunt-ending hindgut. (G,H) Sagittal sections through the uro-rectal region of a E18.5 control (G) and transgenic fetus expressing *Hoxc13* from the *Cdx2* promoter (H). The inset in H is a higher magnification of part of an adjacent section showing the fistula between the bladder and the gut. h, hindgut; b, bladder; g, gut; r, rectum. Asterisks indicate communication between bladder and gut. Scale bars: in A, 1 mm for A–C; in D, 0.5 mm for D–F.

homozygous for an hypomorphic *Wnt3a* allele (Wansleben et al., 2011) exhibit a vertebral truncation phenotype of intermediate severity between the *Wnt3a*-null and the mild *Wnt3a* hypomorph *Vestigial tail (Vt)* mutants (Greco et al., 1996). All examined embryos ( $n=8$ ) homozygous for the new *Wnt3a* hypomorphic allele arrest their axis extension at sacral levels ( $n=8$ ), thereby resembling *Cdx2/4* compound mutants, and *Cdx2*-null embryos generated with the conditional *Cdx2* allele and Cre recombinase induction at E7.5. The severity of the phenotype of homozygous *Wnt3a* hypomorph mutants was variable, and some embryos displayed sirenomelia (fused hindlimbs). This latter phenotype, observed in one out of the four mutants, was the most severe and was accompanied by bladder agenesis. All the other mutants (75%) were deficient in cloacal development at E10.5, similar to *Cdx2/4* mutants. They failed to undergo complete septation of the urogenital and ano-rectal tracts (compare Fig. 2C with 2A).



**Fig. 2. Phenotypes of *Lef1* rescued *Cdx* mutant and *Wnt3a* mutants, and gene expression in the uro-rectal region.**

(A–C) Sagittal sections of the uro-rectal region of a E15.5 control (A), *Cdx2/4* rescued by the *TPLe1* transgene (B) and *Wnt3a*<sup>hyp/hyp</sup> (C). The section in C is not exactly mid-sagittal but shows the bladder-rectum fistula. (D–G) In situ hybridization of E9.5 whole-mount wild-type embryos with a *Cdx2* (D) and *Cdx4* (F) probes, and sagittal sections through E9.0 embryos hybridized with the same probes (E and G, respectively). (H,I,L,M) In situ hybridization with an *Axin2* probe on a whole-mount E10.5 control (H) and *Cdx2/4* embryo (I), and on cross-sections (L,M) of these whole mounts at the level indicated by the broken lines in H and I, respectively. (J,K,N,O) In situ hybridization with a *Wnt3a* probe on a whole-mount E10.5 control (J) and *Cdx2/4* mutant (K) embryos, and corresponding transverse sections (N,O). b, bladder; r, rectum; t, tailbud; g, gut; h, hindgut. Asterisk indicates communication between bladder and gut. Scale bars: 1 mm in A; 0.5 mm in D; 0.5 mm in H,L.

*Cdx2* and *Cdx4* are normally expressed in all three germ layers at the tail end of embryos, and in overlapping domains in the gut endoderm at E9.0 (Fig. 2D–G). *Cdx2* remains expressed in the endoderm at later stages, with a maximum in the para-cecal region, decreasing in both directions (Beck, 2002). It is, thus, expressed at a much lower level in the cloaca than more anteriorly at E12.5 (see Fig. S2 in the supplementary material). We analyzed the expression of *Wnt3a*, and of *Axin2*, an indicator of canonical Wnt signaling, during cloacal septation and ano-rectal morphogenesis in wild type and *Cdx* mutants. *Wnt3a* and *Axin2* are expressed in the posterior growth zone at the tail end of the embryo, but are not expressed in either the endodermal lining of the cloacal cavity, or in the mesoderm of the uro-rectal septum at E10.5 (Fig. 2H–O). The same restriction to tailbud tissues applies to the activity of the *T* promoter driving the rescuing *Lef1* transgene. This promoter is the ‘primitive streak’ promoter fragment shown previously to be active exclusively and

transiently in the mesoderm emerging from the primitive streak during gastrulation, and in the tailbud thereafter (Clements et al., 1996). The *TPLe1* transgene is thus not active in the anlage of ano-rectal and urethral tissues at E10.5. These data suggest that the defect in uro-rectal septation in *Wnt3a* mutants and the rescue effect of *TPLe1* in *Cdx* mutants must originate from the progenitors of cloacal structures at a time when they still resided in the posterior growth zone.

### Wnt pathway components in *Cdx* mutants

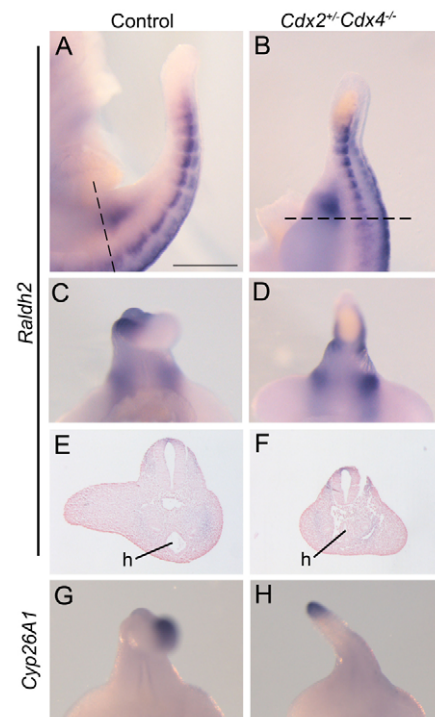
In searching for a mechanism that underlies the impaired development of posterior tissues in *Cdx* mutants, in particular with respect to Wnt signaling, attempts were made to identify *Cdx* transcriptional targets. Transcriptional analysis was performed in posterior tissues of *Cdx2*-null mutants versus controls. This analysis had to be performed at early somite stages (see Fig. S3 in the supplementary material) when *Cdx2* is active in the wild type, but before posterior morphogenesis is heavily altered in the mutants. Two microarray screens, performed at slightly different stages and in duplicate (see Table S1 in the supplementary material) did reveal a slight downregulation of *Wnt3* (by a factor of 1.43 at the stage of 7/8 somites), whereas no change in expression of *Wnt3a* and *Axin2* was detected. *Axin2*, an indicator of canonical Wnt signaling, was thus not downregulated in *Cdx2* mutants at early stages, when posterior tissues in the mutant can still be compared with wild-type counterparts. Given the rescue of the *Cdx2/4* mutant phenotype by *T* promoter-driven expression of an activated form of *Lef1* (*TPLe1*), we performed quantitative PCR analysis of *Axin2* expression in early *Cdx2/4* mutant transgenic for *TPLe1*, compared with non transgenic mutants. We found that *Axin2* was not significantly upregulated in the *TPLe1* transgenic embryos. These data suggest that the activity of canonical Wnt signaling is not modulated extensively, either in *Cdx* mutants or in their *TPLe1* rescued counterparts, at these early stages. It is possible that a subtle modulation of this pathway increases in amplitude with time, a possibility that cannot be cleanly investigated because the posterior tissue of *Cdx* mutants becomes increasingly affected.

Two other genes concerned with canonical Wnt signaling were affected in early *Cdx2* mutants. *Frzb1*, a Wnt antagonist at the level of ligand-receptor binding (Kawano and Kypta, 2003), which was also found to facilitate diffusion of Wnt ligands in *Xenopus* embryos (Mii and Taira, 2009), was found to be downregulated in both transcription screens, by a factor of 1.97 and 1.65, respectively (see Table S1 in the supplementary material). *Nkd1*, which encodes a protein that negatively interacts with Dishevelled (Dvl) (Wharton et al., 2001) was found to be upregulated in both arrays, by a factor of 1.50 and 1.77, respectively (see Table S1 in the supplementary material). Dvl is a central mediator for both the canonical and non canonical Wnt pathways. Altered transcription levels of *Frzb1* and *Nkd1* in our transcriptome analysis of E8.5 *Cdx2*-null mutant embryos were validated with RNA from an independent pool of embryos at the same stage. In situ hybridization revealed that these genes are expressed in caudal embryonic tissues but not in the cloacal area at the time of uro-rectal septum development (not shown). We conclude from the phenotypes of *Wnt3a*, and *TPLe1*-rescued *Cdx* mutants, and from the gene expression analysis, that *Cdx* genes and the canonical Wnt pathway are involved in the morphogenesis of cloacal tissues, and that they play their essential role in the posterior growth zone at the tail end of the embryo at the time this zone generates cell descendants that contribute to the uro-rectal septum mesoderm.

### Retinoic acid signaling in cloacal development of *Cdx* mutants

Exposure of E9.5 embryo to excess RA in utero (Bitoh et al., 2001; Iulianella et al., 1999; Sasaki et al., 2004) and inactivation of the RA-degrading enzyme *Cyp26a1* (Abu-Abed et al., 2001; Sakai et al., 2001) lead to caudal defects similar to *Cdx* and *Wnt* mutations, and to premature expression of *Hox13* genes. *Cyp26a1* is expressed exclusively in caudalmost tissues in the tailbud during trunk and tail axial extension, and *Cdx* loss-of-function mutants and transgenic embryos expressing *Hoxb13* or *Hoxc13* precociously were found to transcribe it at lower levels in these tissues (Savory et al., 2009; Young et al., 2009). *Cyp26a1*, which was shown to be a direct *Cdx* target (Savory et al., 2009), is not expressed at all in cloacal derivatives (Fig. 3G,H). The impact of inactivating *Cyp26a1* on cloacal septation must therefore occur early through the function of the gene in caudal progenitors of cloacal tissues that reside in the growth zone.

Transcription of the gene encoding the RA biosynthetic enzyme *Raldh2* takes place in the somites and in two lateral areas in the cloacal region at E10.5, the time of cloacal septation (Fig. 3A-F). *Raldh2* expression is absent in the cloacal tissues themselves but is localized in lateral ventral mesenchyme, probably associated with the anlagen of the genital tubercle that develops at a later stage. Ventral *Raldh2* expression was slightly increased in *Cdx2/4* mutants compared with controls (Fig. 3A-D). The transcriptome analysis of *Cdx2* mutants at early somite stages also revealed upregulation of *Raldh2* in posterior tissues at stages earlier than the first manifestation of the posterior axial defects (see Table S1 in the



**Fig. 3. Expression of genes of the biosynthesis and degradation of RA in control and *Cdx2/4* mutants.** (A-F) In situ hybridization with a *Raldh2* probe on a whole-mount E10.5 control (A,C,E) and *Cdx2/4* (B,D,F) viewed laterally (A,B) and from the ventral side (C,D), and transverse sections thereof (E,F). (G,H) Ventral views of a E10.5 control (G) and *Cdx2/4* (H) embryos hybridized with a *Cyp26a1* probe. h, hindgut. Broken lines in A and B indicate the level of sections E and F, respectively. Scale bar: 0.5 mm.

supplementary material). This potential increase of diffusing RA together with lower Cyp26a1 in the posterior growth zone from which cloacal descendants are generated may causally contribute to ano-rectal malformations in Cdx mutants.

### Cdx function in cloacal development is not mediated by *Shh* or 5' Hox genes

Disruption of *Shh* signaling has long been associated with the etiology of ano-rectal malformations (Cheng et al., 2008; Kang et al., 1997; Kohlhase et al., 1998). *Shh* signaling is required in different and successive phases of cloacal and genital development (Seifert et al., 2009). *Shh* is expressed in the notochord and floor plate of the neural tube, and in the gut endoderm (Echelard et al., 1993). At E12.5, *Shh* is expressed in the endoderm lining of the cloaca and its derivatives, but not in the mesenchyme of the uro-rectal septum (Seifert et al., 2009) (Fig. 4A,D,G). Transcriptome analysis of posterior tissues of early embryos suggested that *Shh* was slightly downregulated in *Cdx2* mutants (see Table S1 in the supplementary material). However, gene downregulation was not evident in the cloacal region of E10.5 *Cdx2/4* mutant embryos hybridized as whole mounts with *Shh* probes (Fig. 4A,B and data not shown,  $n=4$ ). We examined the expression of *Shh* in the cloacal region of E12.5 *Cdx2/4* mutants, which all exhibit incomplete septation, and could not identify any difference in expression level in the endoderm relative to control embryos (Fig. 4G,H). These experiments argue against a major direct impact of Cdx gene products on *Shh* expression in the cloacal region in this crucial E10.5-E12.5 window. *Shh* expression was not downregulated either in *Wnt3a* hypomorph mutants (compare Fig. 4C,F,I with 4A,D,G).

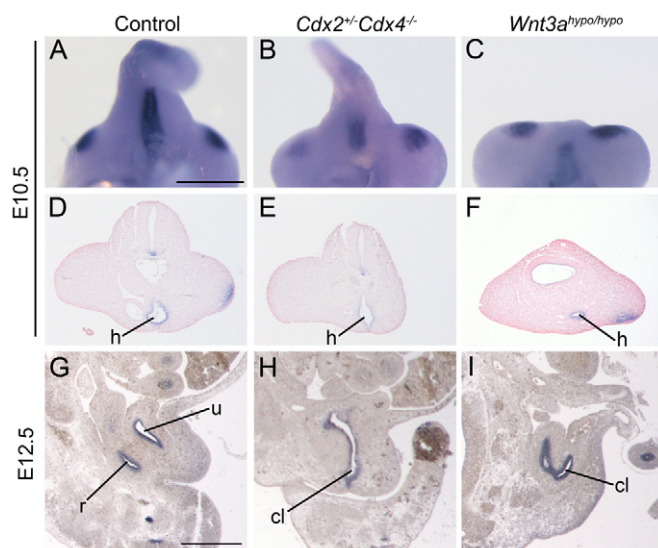
*Hoxa13* and *Hoxd13* are expressed in the endoderm and mesoderm of the cloacal tissues in both mice and chicks (Davis and Capocchi, 1996; de Santa Barbara and Roberts, 2002; Dolle et al., 1991b; Roberts et al., 1995; van der Hoeven et al., 1996). Double inactivation of these genes also leads to a defect in partition

between the urogenital and rectal outflow tracts (Warot et al., 1997). The expression of Hox13 genes was monitored in *Cdx2/4* mutants and controls at E10.5 and E12.5. *Hoxc13* and *Hoxb13* are not expressed in the cloacal region at E10.5. *Hoxa13* and *Hoxd13* are expressed in the cloacal area at this stage, but no difference was found in their expression levels between mutants and controls (Fig. 5A-H). In situ hybridization on sagittal sections of E12.5 embryos revealed that *Hoxa13*, *Hoxb13* and *Hoxd13* are expressed at the same level both in the cloacal endoderm and in the uro-rectal septum mesoderm in *Cdx2/4* embryos and in controls (Fig. 5I-P). We could not detect *Hoxc13* expression in these tissues, although the gene was expressed in the tail bud mesoderm and neurectoderm (not shown). We therefore conclude that Cdx mutants are not causing uro-rectal septum defects by downregulating Hox13 genes.

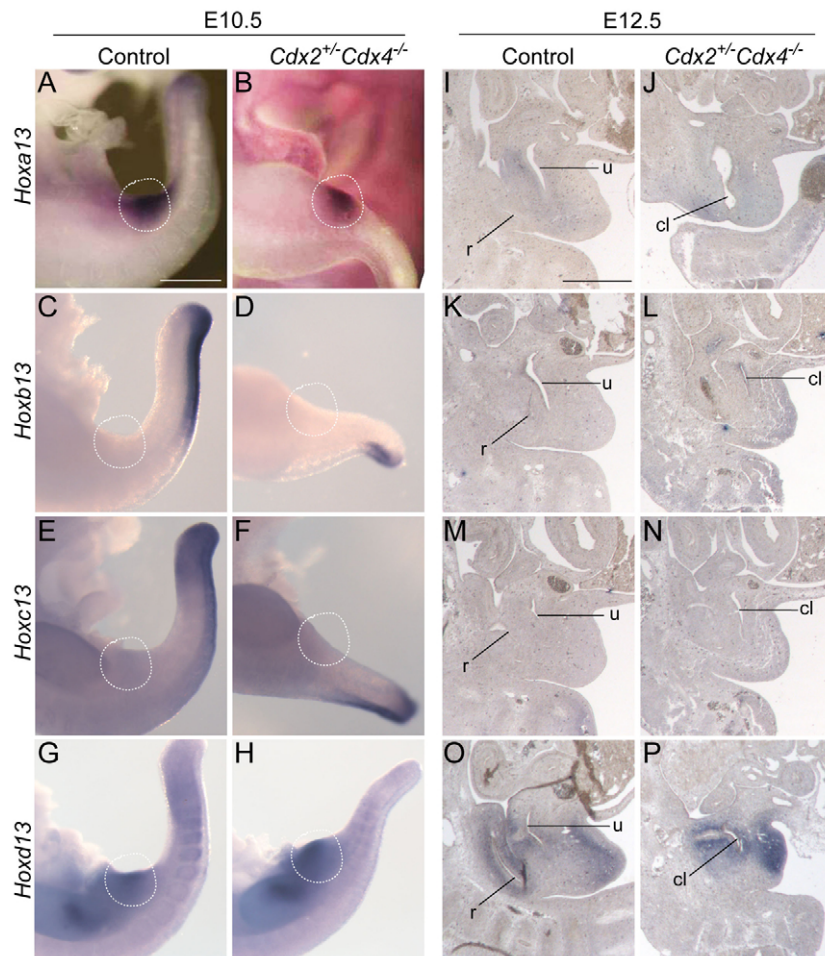
### Aberrant neurepithelial morphogenesis in Cdx and *Wnt3a* mutants and in embryos precociously expressing Hox13 genes

An additional phenotypic feature indicates the relevance of Cdx mutations to the human caudal regression syndrome. Not only do mouse and human conditions exhibit ano-rectal septation and posterior skeletal defects, but both also manifest abnormalities in the caudal neural tube. Transverse sections of *Cdx2/4* compound mutant embryos at axial levels cranial to the truncation revealed *Sox2*-positive ectopic tubular structures ventral to the neural tube, and irregularities in the cellular arrangement in the neurepithelium in all cases (Fig. 6A,D; see Fig. S4A,B in the supplementary material for the axial levels of these defects;  $n=6$ ). Ventral ectopic neural structures (ens) were also found in *Cdx2* null ( $n=4$ ) and in *Cdx2/4* double null ( $n=3$ ) mutant embryos obtained after epiblast-restricted inactivation of the *Cdx2* conditional allele (Fig. 6B,E and 6C,F, respectively). We characterized the posterior neurepithelium of the latter mutant embryos at E10.5 with a number of antibodies on transverse sections.  $\alpha 6$ -Integrin is normally expressed on cell membranes in the ventral neural tube and in the gut endoderm (Bajanca et al., 2004). It was either not expressed ( $n=1$ ) or expressed considerably less ( $n=2$ ) in the mutant neural tube (Fig. 6C,F), in spite of the fact that expression was observed in the mutant gut endoderm (not shown). The neural cell arrangement was disrupted in the mutant at these caudal levels, and the lumen of the neural tube was irregular in shape (Fig. 6F). Neural tube morphogenesis was analyzed in sections of *TPLef1*-rescued *Cdx2/4* mutants, and found to be similar to that in wild type even at posterior levels (not shown).

Transgenic embryos precociously expressing *Hoxb13* under the transcriptional control of the *Cdx2* promoter were examined for neural tube morphogenesis. These embryos were reported earlier to exhibit an axially truncated vertebral column resembling in that to embryos with decreased Cdx expression (Young et al., 2009). The truncation owing to homozygosity for the *Cdx2PHoxb13* transgene, though relatively mild (axial arrest after 25 sacral and caudal vertebrae, instead of the normal 35, see Fig. S4G,H in the supplementary material) was found to be more severe than in hemizygotes. Cross-sections of E10.5 embryos of this genotype revealed in all cases a disturbed cellular arrangement of the neurepithelium, and the presence of ectopic tubular structures expressing the neural marker *Sox2*, similar to those in Cdx mutants (Fig. 6G,J) ( $n=4$ ).  $\alpha 6$ -Integrin staining labeled the adherens junctions between cells of the ventral aspect of the neural tube in both *Hoxb13* transgenic and wild-type embryos, attesting to correct dorsoventral patterning of the mutant and transgenic neural tubes. Analysis of the posterior neural tube of embryos transgenic for *Cdx2PHoxc13* (see Fig. S4I,J



**Fig. 4. Expression of *Shh* in *Cdx2/4* mutants and *Wnt3a* hypomorph mutants.** (A-F) In situ hybridization with a *Shh* probe of a whole-mount E10.5 control (A), *Cdx2/4* (B) and *Wnt3a*<sup>hypohypo</sup> (C) embryos, and transverse sections thereof (D-F). (G-I) In situ hybridization on sagittal sections of the uro-rectal region of a E12.5 control (G), *Cdx2/4* (H) and *Wnt3a*<sup>hypohypo</sup> (I) mutant embryos. h, hindgut; r, rectum; u, urethra; cl, cloaca. Scale bars: 0.5 mm.



**Fig. 5. Expression of the four Hox13 genes in wild type and *Cdx2/4* mutants at E10.5 and E12.5.** (A-H) In situ hybridization with probes for *Hoxa13* (A,B), *Hoxb13* (C,D), *Hoxc13* (E,F) and *Hoxd13* (G,H) on whole-mount E10.5 control (A,C,E,G) and *Cdx2/4* mutant (B,D,F,H) embryos. (I-P) In situ hybridization of transverse sections of the uro-rectal region of a E12.5 control (I,K,M,O) and *Cdx2/4* mutant (J,L,N,P) embryos with probes for *Hoxa13* (I,J), *Hoxb13* (K,L), *Hoxc13* (M,N) and *Hoxd13* (O,P). Scale bars: 0.5 mm. u, urethra; r, rectum; cl, cloaca. Broken circles indicate the cloacal area in E10.5 embryos.

in the supplementary material) revealed similar ectopic neural structures at E10.5 (Fig. 6H,K), and a split in the neural tube posteriorly at E18.5 (Fig. 6I,L).

The ectopic neural structures of *Cdx* mutants and of transgenic embryos prematurely expressing *Hox13* genes are reminiscent of features reported in *Wnt3a*-null mutants (Takada et al., 1994; Yoshikawa et al., 1997) (Fig. 6N,P; see Fig. S4K,L in the supplementary material), and we analyzed transverse sections of *Wnt3a*-null and *Wnt3a* hypomorph homozygous embryos (Fig. 6M,O; see Fig. S4M,N in the supplementary material). These also revealed ectopic neural structures and neurepithelial irregularities of a severe type (Fig. 6O), resembling the *Cdx2PHoxc13* split neural tube.

The similarity in neurepithelial defects between *Wnt3a* and *Cdx* mutants, and transgenic embryos prematurely expressing *Hox13* genes, strengthens the hypothesis that *Cdx* transcription factors and canonical Wnt signaling belong to interacting genetic pathways underlying posterior morphogenesis in the three germ layers.

## DISCUSSION

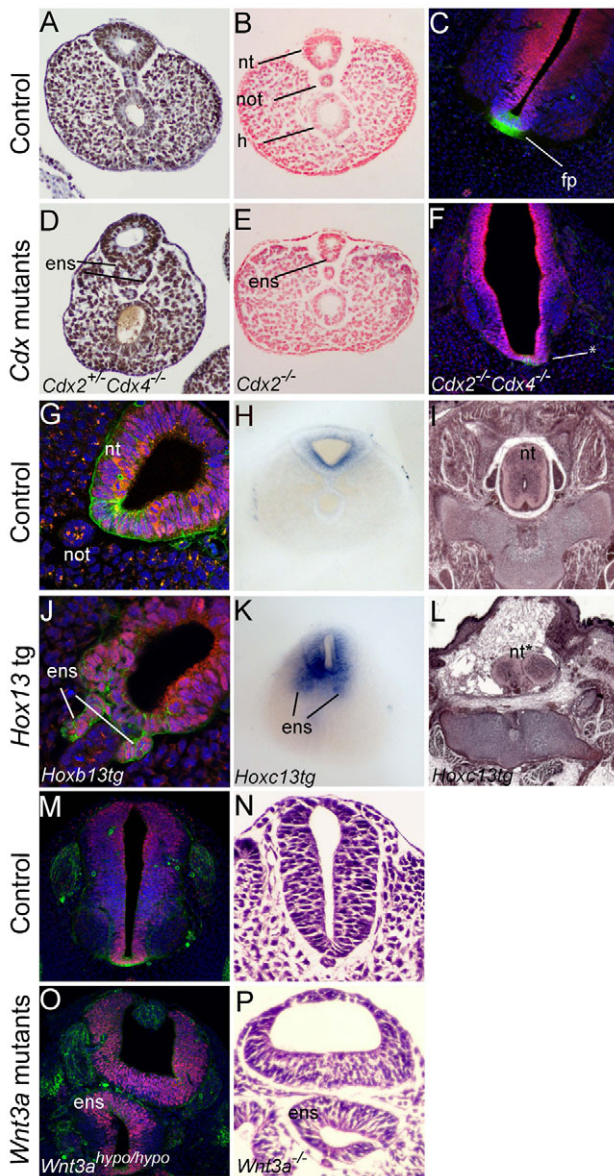
### **Cdx, Hox and Wnt and posterior morphogenesis**

The data in this work reveal that in addition to the extension of the vertebral axis, development of posterior neurepithelium and of cloacal derivatives also depends on the activity of *Cdx* genes, on precisely timed sequential *Hox* gene expression and on persisting canonical Wnt signaling in the posterior embryonic growth zone. Alteration of any of these parameters arrests the skeletal, neural and

cloacal development program and mimics human congenital caudal regression or caudal dysplasia. The involvement of a shortage of growth stimulation in the progenitor zone of posterior tissues at early stages in the tail bud therefore sheds a new light on the etiology of these syndromes, and possibly on that of ano-rectal malformations (ARMs) more generally.

Other genetic factors have been considered in the etiology of the Caudal Regression Syndrome, such as *Shh* and its proposed downstream signaling effectors *Wnt5a* and *Bmp4* (Mandhan et al., 2006; Nakata et al., 2009; Sasaki et al., 2004; Tai et al., 2009). *Shh* plays multiple essential roles during embryogenesis. Its inactivation in the mouse leads to agenesis of distal limbs and caudal axial structures in addition to midline patterning defects (Chiang et al., 1996). *Shh* has been shown to contribute to morphogenesis of uro-rectal structures by a specific function in the endodermal lining of the cloacal cavity, from where it signals onto the growing uro-rectal septum mesenchyme (Seifert et al., 2009). We did not observe a downregulation of *Shh* expression in posterior endoderm derivatives of *Cdx* mutants during the time window of uro-rectal septation. *Cdx* loss of function therefore does not appear to impair uro-rectal septum development by downregulating *Shh* in the endoderm. Furthermore, it is unlikely that *Cdx* involvement in ano-rectal development is exerted at the level of the endoderm. Grainger and colleagues (Grainger et al., 2010) report that endoderm-specific inactivation of *Cdx2* using the VillinP-Cre does not lead to imperforate anus. These data, together with our observations on the *Wnt3a* mutant phenotype, the *TPLe1* rescue of





**Fig. 6. Characterization of the posterior neural tube of wild type, *Cdx* mutants, *Wnt3a* mutants and transgenic embryos precociously expressing *Hox13* genes.**

The axial levels of the sections analyzed for the different genotypes are indicated in Fig. S4 in the supplementary material. (A-F) Controls and *Cdx* mutants. Transverse sections of a E10.5 control (A) and a *Cdx2/4* mutant (D) embryos immunostained for the proliferation marker Ki67; (B,E) Neutral Red stained histological section of a control (B) and a *Cdx2*-null (E) embryo; (C,F) immunofluorescence for  $\alpha 6$ -integrin (green) and Sox2 (red) on transverse sections at posterior levels of a control (C) and *Cdx2/4* double null mutant (F) embryo. Note the ectopic neural structures (ens) ventral to the neural tube in the mutants in D and E, and the arising ens on the left side (asterisk) of the very weak  $\alpha 6$ -integrin staining in F. (G-L) Controls and transgenic embryos expressing a *Cdx2PHox13* transgene. Immunofluorescence for  $\alpha 6$ -integrin (green) and Sox2 (red) on transverse sections of a control (G) and a *Cdx2PHoxb13* transgenic embryo (J). Cross-sections through the posterior part of a E10.5 control (H) and a *Cdx2PHoxc13* transgenic (K) embryo after hybridization with a *Sox2* probe. Cross-sections in the posterior region of a E18.5 wild-type (I) and *Cdx2PHoxc13* transgenic (L) embryo. (M-P) Controls and *Wnt3a* mutants. Staining of transversal sections of a E10.5 wild type (M) and a *Wnt3a*<sup>hypohypo</sup> mutant (O) with anti Sox2 (red) and anti  $\alpha 6$ -integrin (green) antibodies. Hematoxylin and Eosin stained sections through a E10.5 wild-type (N) and a *Wnt3a*-null embryo (P). h, hindgut, nt, neural tube; nt\*, split neural tube; not, notochord; \*, emerging ectopic neural structure.

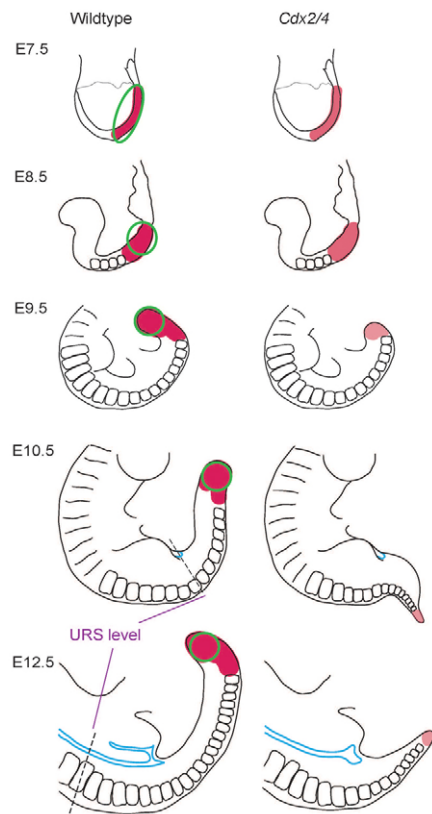
*Cdx* mutants and the growth zone-restricted expression of the canonical Wnt pathway components, emphasize an essential role of the *Cdx* and Wnt at the level of the posterior growth zone that supplies progenitors for the cloacal structures earlier than uro-rectal septum generation (see graph in Fig. 7).

### Timing of *Hox* expression is crucial for cloacal development

A posterior gain of function of the trunk *Hox* gene *Hoxb8*, rescues *Cdx2/4* mutant defects (Young et al., 2009), including uro-rectal septation failure. This rescue must occur early in the progenitor region in the tail bud as the promoter used on the rescuing transgene is the *Cdx2* promoter, which is not active in uro-rectal mesoderm. Although microarray screens had not revealed a downregulation of *Hoxb8* in early *Cdx2/4* mutants, transcriptome analysis in the severely impaired *Cdx2*-null mutants, revealed a downregulation of this *Hox* gene (2.75 fold at the 4/5 somite stage and 2.47 fold at the 7/8 somite stage, see Table S1 in the supplementary material). Phenotypical rescue of the *Cdx2/4*

mutation by posterior gain of *Hoxb8* function therefore probably results from the correction of a slight reduction of *Hoxb8* transcription, even though this slight reduction is not detectable at stages before tissue are visibly affected.

Expression of *Hox13* genes begins in the posterior growth zone of the embryo (remnants of the primitive streak) at around E9.5. The function of *Hox* genes during embryogenesis has been proven to be crucially dependent on a correct timing of their expression (Kondo and Duboule, 1999; Tschopp et al., 2009; Zakany et al., 1997; Zakany et al., 2004). *Hox13* genes normally control cloacal development after more anterior axial structures have been generated. Precocious expression of *Hox13* genes negatively interferes with the trunk developmental program, as shown for the axial skeleton (Tschopp and Duboule, 2011; Young et al., 2009). Precocious expression of *Hox13* genes also jeopardizes the development of cloacal mesoderm. Transgenic embryos expressing *Hox13* genes under the *Cdx2* promoter do generate the *Hox13* protein prematurely as the *Cdx2* promoter is active in posterior embryonic tissues at E7.2. *Cdx2PHoxb13* transgenic mice were found to manifest anal atresia in some of the cases, and transgenic fetuses expressing *Hoxc13* under the *Cdx2* promoter exhibit ano-rectal agenesia and abnormal communication between the bladder and the hindgut. These deleterious consequences of premature expression of *Hox13* genes prove that sequential temporal control of *Hox* gene expression is a prerequisite for balanced morphogenesis of uro-rectal tissues, in a similar manner to that indicated for axial skeletal structures (Young et al., 2009). Precociously expressed *Hox13* genes would functionally antagonize the action of earlier *Hox* genes, a phenomenon observed in different tissues and called posterior prevalence (Duboule, 1991; Duboule and Morata, 1994; Tschopp and Duboule, 2011; Young et al., 2009). The impairment of uro-rectal septation in *Hox13*



**Fig. 7. The morphology and gene expression in the posterior growth zone of wild type and *Cdx2/4* mutants.** The growth zone (circled in green) is along the primitive streak at E7.5 and E8.5, and in the tail bud region at E9.5, E10.5 and E12.5. The expression of *Cdx2*, *Wnt3a* and *Cyp26a1* is found in posterior tissues, including the growth zone of the wild type (red color) and of *Cdx2/4* (*Cdx2<sup>+/-</sup>Cdx4<sup>-/-</sup>*) mutants (less intense red or pink color), but is absent in the region where the uro-rectal septum (URS) region develops between E10.5 and E12.5. The situation in *Cdx2/4* mutants is the same as that in transgenic embryos precociously expressing *Hox13* genes and in *Wnt3a* hypomorph mutants. The cloacal endoderm (in blue) expresses *Cdx2* at low levels, but this is not shown here (not stained in red). The broken lines indicate the axial level where the uro-rectal septum mesoderm develops between E10.5 and E12.5.

transgenic embryos indicates that expression of these genes needs to be delayed in order not to interfere with earlier *Hox* genes in the control of cloacal progenitors in the posterior growth zone.

### Defects of the uro-rectal septum of *Cdx* and *Wnt* mutants must trace back to their impaired progenitors in the posterior growth zone

Expression of *Cdx* genes, activity of the canonical *Wnt* pathway and clearance of retinoic acid, which are all required for correct growth of the uro-rectal septum and ano-rectal development, are manifest in the embryonic posterior growth zone from early on, and are not seen in the septum during its development. Our hypothesis is therefore that these genes play their role in the progenitors of the septum at the time they will contribute descendants to the lateral plate mesoderm of the cloacal region.

Lateral plate mesoderm is generated in the gastrulating mouse embryo from the posterior one-third of the primitive streak, whereas somitic and midline mesoderm (notochord) emerge from

the anterior two-thirds and the anterior extremity of the streak, respectively (Cambray and Wilson, 2002; Cambray and Wilson, 2007; Tam and Beddington, 1987; Wilson and Beddington, 1996). At later stages, after the posterior neuropore closes, the anterior primitive streak, node-streak border and node region become internalized and form the chordoneural hinge (CNH) (Gont et al., 1993; Wilson et al., 2009), whereas the rest of the primitive streak is curved along the ventral outer surface of the tailbud, becoming the ventral ectodermal ridge (VER). Mesoderm emergence from the VER, regulated by BMP (Ohta et al., 2007; Zakin et al., 2005), contributes some cells to the posterior tailbud until E9.5 (Ohta et al., 2007; Wilson and Beddington, 1996) and has completely ceased to do by E10.5. The trunk lateral mesoderm at axial levels of the uro-rectal septum therefore must be laid down from progenitors that have emerged from the posterior growth zone earlier than when cloacal septation takes place.

### *Cdx*, *Hox*, *Wnt* and neurepithelium expansion and patterning

*Cdx* and *Wnt3a* loss-of-function mutations and precocious expression of *Hox13* genes impair elongation and morphogenesis of the caudal neurepithelium. Phenotypic similarity between the neural tube of *Cdx* mutants, of transgenic embryos precociously expressing *Hox13* genes and of *Wnt3a* and other *Wnt* pathway mutants (Galceran et al., 1999; Gregorieff et al., 2004; Yoshikawa et al., 1997) adds to the mutual resemblance of these mutants with respect to axial and lateral mesoderm. It consolidates the emerging concept that *Cdx* and *Hox* genes function in the same pathway as *Wnt* signaling in controlling generation and patterning of posterior tissues in the three germ layers.

Mild-tail truncation defects, such as those in transgenic embryos prematurely expressing *Hoxb13*, are accompanied by severe neurepithelium patterning defects at levels anterior to the truncation. This points to an intrinsic disturbance in the *Cdx/Hox* network during patterning of the caudal neurepithelium, rather than to a mere consequence of axial growth arrest. The abnormalities observed in the posterior neurepithelium arrangement in *Cdx* mutants and transgenic embryos precociously expressing *Hox13* genes are in some way reminiscent of the aberrant gut endoderm histology observed recurrently at posterior axial levels on sections of *Cdx2/4* mutants (van Nes, 2006) and in transgenic embryos that prematurely express *Hoxb13* (data not shown). Another study (Gao and Kaestner, 2010) also reported that *Cdx2*-null embryos exhibit multiple lumens, with disturbed apico-basal polarity in the endoderm epithelium. Abnormal apico-basal position of nuclei in *Cdx2* mutant intestinal epithelium has also been described (Grainger et al., 2010), which resembles the irregular polarization of the neurectoderm in *Cdx* mutants described here.

### *Cdx*, *Hox* and *Wnt*, and neurectoderm versus mesoderm generation from the stem zone

The similar neural defects in *Cdx* and *Wnt* mutants, and in transgenic embryos prematurely expressing *Hox13* genes point to common or at least interacting steps in their genetic program. The neurectoderm defects seen in E10.5 *Cdx* and *Wnt* mutants, and in embryos precociously expressing *Hox13* genes take place exclusively at caudal axial levels, in tissues preceding the truncation, that have already emerged from a progressively declining growth zone. This makes it likely that the neural patterning defects result from impairment of the *Wnt*-depending growth zone.

Another transcription factor expressed in the posterior growth zone is *T* (Brachyury). *T* mutants were the first mutants isolated from a mutagenesis screen in the mouse (Dobrovolskaia-Zavadskaia, 1927). The *T* transcription factor is required for embryonic axial elongation, and *T* was shown to be a direct target of Wnt3a (Yamaguchi et al., 1999), and to exert its activity on axial extension by the maintenance of canonical Wnt signaling (Martin and Kimelman, 2008; Martin and Kimelman, 2010; Rashbass et al., 1994). *T* is expressed in the epiblast and mesoderm of the primitive streak, and strongly in the notochord. Ectopic neural structures have also been observed in *T* mutants (Yamaguchi et al., 1999).

Mutants in another *T* box gene, *Tbx6*, form ectopic neural structures more severe in extent than Hox13 transgenics, and *Cdx*, *Wnt3a* and *T* mutants. Additional neural tubes form in this mutant at the expense of somitic mesoderm, as a result of upregulation of *Sox2* expression in descendants of posterior progenitors in the growth zone (Takemoto et al., 2011). The *Sox2* expression domain is also expanded to include regions outside the neural tube at posterior axial levels in *Cdx* mutants, and in transgenic embryos precociously expressing Hox13 genes, suggesting an overlap in the mechanistic impairment of the partition of mesoderm and neuroectoderm in these mutants.

In *Cdx*, *Wnt*, *T* and *Tbx6* mutants, and in transgenic embryos precociously expressing Hox13 genes, the ectopic neural structures form at the ventrolateral side of the neural tube. This area is normally colonized by descendants of the anterior primitive streak and later chordo-neural hinge (CNH) (Cambray and Wilson, 2002; Cambray and Wilson, 2007), making it reasonable to assume that these mutations affect the activity of the population of long-term neural/mesodermal progenitors (Tzouanacou et al., 2009).

### **Cdx, T and Wnt: central players in posterior morphogenesis in the three germ layers in the embryonic growth zone**

*T*-null mutants are posteriorly truncated in the three germ layers, and anal atresia was reported for heterozygotes for *T curtailed*, one of the *T* mutant alleles (Inman and Downs, 2006), suggesting that interfering with *T* function leads to abnormalities in the urorectum as well as to axial truncation. *T* remains strongly expressed in the embryonic tailbud during cloacal development, but it is not expressed in the uro-rectum septal mesoderm and endoderm (data not shown), again suggesting that its involvement in anal atresia originates in its earlier function in the progenitor area in the tail end of the embryo. *T* mutants thus exhibit a largely overlapping spectrum of posterior defects with the *Cdx* and *Wnt* mutants described here. The similarity in impact of *Cdx* and *T*, mediated in both cases by Wnt signaling, strengthens even further the hypothesis that the canonical Wnt pathway is the central player in the balanced morphogenesis of the derivatives of the posterior growth zone during emergence of tissues from the different germ layers. Our data so far do not establish whether or not *Cdx* and Hox genes operate independently of *T* to sustain growth in the posterior growth zone.

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#### **Competing interests statement**

The authors declare no competing financial interests.

#### **Supplementary material**

Supplementary material for this article is available at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.066118/-/DC1>

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