Role of *Cdx* and *Hox* genes in posterior axial extension in the mouse

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Role of *Cdx* and *Hox* genes in posterior axial extension in the mouse

Functie van *Cdx*- en *Hox*-genen in posterieure asverlenging in de muis

(met een samenvatting in het Nederlands)

Proefschrift

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Part I

Introduction

Chapter 1

Cdx genes and posterior body axis elongation

Early mouse development

After fertilization (E0), the single-celled mouse embryo undergoes a series of divisions, or cleavages. The zygote completes the first cleavage at E1.5 and the second by E2.5, before reaching the 8-cell stage. The 8-cell blastula then undergoes the process of compaction. After the compaction, the next round of cleavage produces a 16-cell morula that consists of a small group of internal cells enclosed by a larger group of external cells in ratio ranged from 2:14 to 7:9 (Fleming, 1987). The morula then begins the process of cavitation that results in the generation of the blastocyst at E3.5. The blastocyst is composed of two cell types, pluripotent cells from the inner cell mass (ICM) that give rise to the embryo, and the trophectoderm. The trophectoderm is extra-embryonic tissues that allows the survival of the conceptus in the uterine environment. The ICM and the abutting trophectoderm, form the embryonic pole of the blastocyst. The opposite end, the abembryonic pole, is composed of the mural trophectoderm and the blastocoele cavity. At E4.5, soon after the blastocyst hatches from the zona pelucida and implants, a group of ICM cells differentiate into primitive endoderm. Descendants of the primitive endoderm then spread over the inner surface of the mural trophectoderm to become the parietal endoderm. The parietal endoderm and the giant trophectoderm cells, a derivative of the mural trophectoderm, together with Reichert's membrane, form the parietal yolk sac. Cells from the primitive endoderm that remain in contact with the ICM eventually become the visceral endoderm. Once the primitive endoderm forms, the descendants of the ICM, now epiblast, begin to bulge into the blastocoele. At the same time, the polar trophectoderm expands to form the extra-embryonic ectoderm and the ectoplacental cone. This growth transforms the embryo into a cylindrical structure called the egg cylinder. By E5.0, the egg cylinder is composed of the ectoplacental cone, which anchors the egg cylinder into the uterine wall, the extra-embryonic ectoderm and the epiblast. The visceral endoderm envelops both the extra-embryonic ectoderm and the epiblast and all of these structures are themselves surrounded by the parietal yolk sac.

Establishment of the antero-posterior (A-P) axis

The blastocyst already possesses a bilateral symmetry, that prefigures the position of embryonic long axis. At approximately E6.5 the primitive streak develops on one side of the epiblast and marks the caudal end of the growing embryo. The appearance of the primitive streak is concomitant with the initiation of gastrulation that generates mesoderm and definitive endoderm. The appearance of the primitive streak is preceded by an important series of events within the primitive endoderm at E5.2: restricted gene expression and a directional movement of distalmost primitive endoderm cells give rise to the anterior visceral endoderm (AVE), a crucial signaling center for the entire gastrulation process. The AVE specifies the anterior pole of the embryo and it is involved in the specification of the primitive streak at the opposite end of the embryonic axis. During gastrulation, anterior structures are generated first and the generation of the trunk and tail follows. (Beddington and Robertson

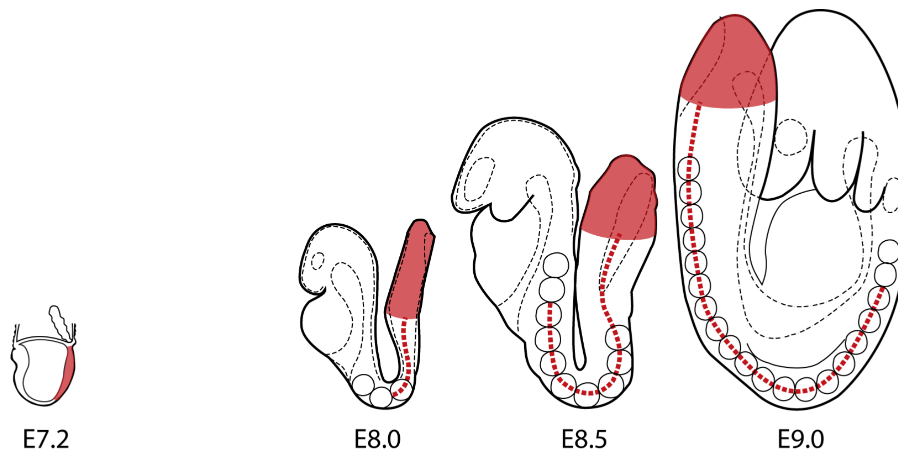

Figure 1.1. **Growth of the mouse embryo. During gastrulation the anterior structure of the mouse embryo that will become the head is already laid down, while the trunk and tail will be formed continuously by posterior tissue addition of cell progenitors located in the growth zone coloured in red. Dash line in red represents the trunk and tail tissues growth from rostral to caudal.**

1998; Rivera-Perez, 2007; Tam and Behringer 1997). Extention of the embryonic anteroposterior (AP) axis occurs according to several mechanisms that are growth of the axis within itself by cell proliferation, convergence extension and addition of tissue from a posterior growth zone (Cambray and Wilson, 2002; García-García *et al*., 2008; Mathis and Nicolas, 2000). It is on the AP axis extension during trunk and tail development by posterior addition of tissue that this thesis focuses (Figure 1.1).

Genetics of AP axis extension in the mouse

Several genes have been shown to be essential to ensure the continuation of tissue growth along the AP axis (Figure 1.2). Embryos without functional *Wnt3* gene develop to egg cylinder stage, do not form a primitive streak and therefore fail to gastrulate. Their AVE is formed and they therefore have an AP axis but do not extend it (Liu *et al*., 1999). A null mutation in *Wnt3a* causes axial truncation from a level caudal of the forelimb while anterior structures and chorioallantoic placenta are unaffected. The *Hox* genes expression domains are posteriorly shifted in *Wnt3a* mutants explaining the vertebral homeotic transformations observed (Takada *et al*., 1994). *Wnt5a* null mutant embryos die perinatally and are caudally truncated. In addition, structures such as limbs, snout, tongue, mandible and genital tubercle are also truncated in these embryos (Yamaguchi *et al*., 1999a). Homozygous mutant embryos in *T Brachyury* die around E10 and are truncated from a level caudal of the forelimb, similarly to *Wnt3a* null embryos. In *Wnt3a* homozygous mutant embryos,

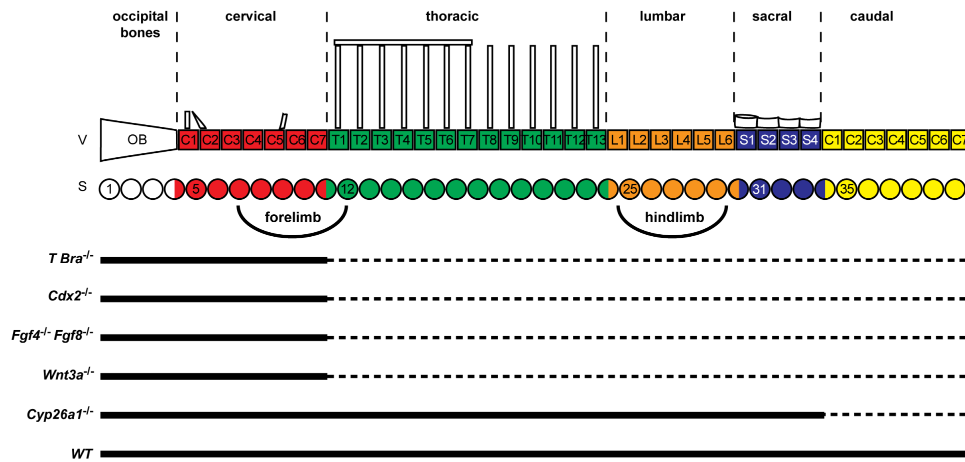

Figure 1.2. **Schematic representation of the axial skeleton vertebrae and their somitic origin of a wildtype mouse. From anterior to posterior, the occipital bones, the cervical, the thorcic, the lumbar, the sacral dan caudal vertebrae. The number of caudal vertebrae varies between from 32 to 36. Mutation in *Brachyury*, *Cdx2*, *Fgf4/Fgf8*, *and Wnt3a* cause posterior truncation from the level of forelimb, while loss of *Cyp26a1* produces caudalless mice. V is vertebrae, S is somite.**

the expression of *T* is downregulated and analysis of the *T* promoter has shown that *T* is a direct target of the canonical Wnt signaling (Yamaguchi *et al.*, 1999b).

Fgfr1 null mutant embryos appear growth retarded and cell migration through the primitive streak is impaired in these mutants (Deng *et al.*, 1994, Yamaguchi *et al.*, 1994). Chimeric embryo analysis revealed a cell autonomous function for *Fgfr1* in this process (Ciruna *et al.*, 1997; Deng *et al.*, 1997). The role of *Fgfr1* in AP axial extension at later stages was revealed by studying hypomorphic mutant alleles of *Fgfr1* that show axial truncation from the lumbrosacral level. In addition, anterior and posterior homeotic transformations of vertebral identity was also observed, accompanied by changes in the *Hox* gene expression patterns (Partanen *et al.*, 1998).

A Vitamin A derivative, retinoic acid (RA) is also involved in AP axis extension. At specific stages of embryonic development and depending on the RA dosage applied, excess RA causes trunk and tail truncation, homeotic transformations along the vertebral column and other defects (Padmanabhan, 1998). RA acts after binding to its nuclear receptors (*RAR*) that form heterodimer complexes with *retinoid X receptor* (*RXR*). These heterodimers then regulate RA target genes that contain RA response elements (*RARE*s) in their regulatory regions. Loss of *Cyp26a1*, the RA degrading enzyme that is expressed in the posterior end of the developing embryo causes abnormal morphogenesis reminiscent of the teratogenic effects of excess RA (Abu-Abed *et al.*, 2001; Sakai *et al.*, 2001). *Raldh2* is the major RA synthesizing enzyme in the developing trunk. Embryos lacking functional *Raldh2* have a shortened AP axis and other defects (Niederreither *et al.*, 1999), phenotypes that are similar to

the vitamin A deficiency syndrome. These data show that RA distribution in the developing embryos is finely controlled to ensure proper development.

The work in this thesis concerns the role of the *Cdx* genes (homeobox genes of the *ParaHox* family) and *Hox* genes during posterior axial extension in mice. The mouse genome contains thirteen paralogy groups of *Hox* genes and three *caudal* paralogous genes known as *Cdx1* (Duprey *et al.*, 1988), *Cdx2* (James and Kazenwadel, 1991) and *Cdx4* (Gamer and Wright, 1993). The expression of *Cdx1* and *Cdx4* is downregulated in *Wnt3a* null and *vestigial tail* (*Wnt3a* hypomorph) mouse embryos (Ikeya and Takada, 2001; Lickert *et al.*, 2000; Pilon *et al.*, 2006). It is not clear whether Fgf directly regulates *Cdx* in the mouse because the expression of *Cdx1* and *Cdx4* is not altered in *Fgfr1* hypomorphs mutants (Partanen *et al.*, 1998). Several *Hox* genes and *Cdx1* have been demonstrated to contain functional *RARE*s and to be under RA signal regulation (Deschamps and van Nes, 2005).

Developmental defects in *Cdx* mutant mice

Functional analysis of *Cdx1* in vivo has been assessed by knocking it out via homologous recombination. The homozygous *Cdx1* mouse mutant is fertile and shows axial skeletal abnormalities with anterior homeotic transformations affecting the region from upper cervical until the first lumbar vertebrae (Subramanian *et al.*, 1995, van den Akker *et al.*, 2002). The remaining posterior lumbar and caudal vertebrae are apparently normal. Heterozygous *Cdx1* mutants frequently show anterior transformation of the eighth thoracic and first lumbar vertebra. Anterior transformation of cervical and thoracic vertebrae are observed less frequently at the heterozygous state (van den Akker *et al.*, 2002). These transformations are accompanied by a posterior shift of the anterior boundary of the expression domain of several *Hox* genes in somites (Subramanian *et al.*, 1995; van den Akker *et al.*, 2002).

Cdx2 is first expressed extra-embryonically at E3.5 in the trophectoderm of blastocysts (Beck *et al.*, 1995). It is required for extraembryonic differentiation into trophoblast to enable blastocyst implantation into the uterus wall at E4.5 (Chawengsaksophak *et al.*, 1997; Chawengsaksophak *et al.*, 2004; Strumpf *et al.*, 2005). This explains why *Cdx2* homozygous null mutant embryos die at the peri-implantation stage (Chawengsaksophak *et al.*, 1997). To bypass the trophoblast defect affecting *Cdx2* null embryos, *Cdx2* null morulae or ES cells can be combined with wildtype tetraploid morulae. Following this "tetraploid rescue" protocol *Cdx2* null mutants implant and develop to the point when a chorio-allantoic placenta is required for survival. They then die because of underdevelopment of the allantois, which fails to fuse with the chorion and prevents the generation of the placental labyrinth. The *Cdx2* null mutant embryos are severely truncated posteriorly beyond the forelimb bud, and make a maximum of 17 somites. Somites posterior to s5 are smaller and irregular. This observation was confirmed by the expression of somitic molecular markers, such as *Mox1* and *paraxis* at E9. In addition, somites extend to almost the posterior tip of the embryos leaving only reduced amounts of unsegmented paraxial mesoderm (psm), as confirmed by *Tbx6* expression, a psm marker. Since the mesoderm, neurectoderm and endoderm formation in the posterior region of *Cdx2* null mutant is severely com-

promised leading to posterior truncation, *Cdx2* must be necessary for continuing tissue generation (Chawengsaksophak *et al.*, 2004). *Cdx2* heterozygous mutants survive and show anterior homeotic transformations from the sixth cervical to the eighth thoracic vertebrae (Chawengsaksophak *et al.*, 1997; van den Akker *et al.*, 2002) as well as kinky and variably shortened tail (Chawengsaksophak *et al.*, 1997). Combination of mutation in *Cdx1* and *Cdx2* leads to anterior transformations along the complete anterior-posterior (AP) axis, showing the synergistic effect of both genes compared to the single mutations. This combination of mutations also causes a more severe AP axis truncation compared to the Cdx2 heterozygous mutant alone (van den Akker *et al.*, 2002).

Cdx4 null mutant mice are healthy and fertile. Since *Cdx4* is *X*-linked, a male carrying the *Cdx4* null mutant allele is *Cdx4* null ($Cdx4^{-/0}$). The severity of the $Cdx1^{-/-}$/ $Cdx4^{-/0}$ compound mutant phenotype only increases slightly compared to that of $Cdx1^{-/-}$, while the combination of the *Cdx4* null mutation with a *Cdx2* null allele leads to anterior transformations of the eighth thoracic and first lumbar vertebrae. In terms of axial extension, $Cdx1^{-/-}$/$Cdx4^{-/0}$ mutants do not manifest any truncation but $Cdx2^{+/-}$/$Cdx4^{-/0}$ mutants are truncated more severely than $Cdx1^{-/-}$/$Cdx2^{+/-}$ mutants. This illustrates the synergistic effect of *Cdx2* and *Cdx4* in posterior tissue generation. The $Cdx2^{+/-}$/$Cdx4^{-/0}$ embryos are also growth retarded at E10.5 and most of them die in utero around that stage. The heartbeat, and embryonic as well as yolk sac blood circulation are normal at E9.5, but fail around E10.5. *Cdx2* and *Cdx4* are expressed in the anlage of the allantois during normal development (Deschamps and van Nes, 2005), and van Nes *et al.* (2006) found that $Cdx2^{+/-}$/$Cdx4^{-/0}$ embryos suffer from chorio-allantois placental defects. During normal development, chorioallantoic fusion occurs at E8.5 when the allantois has grown enough to fuse with the chorionic plate (Rossant and Cross, 2001). Measurement of $Cdx2^{+/-}$/$Cdx4^{-/0}$ allantoic length prior to fusion showed that this length is sufficient for the allantois to reach the chorion. Differentiation of the outer layer of the allantois was also shown to take place normally in $Cdx2^{+/-}$/$Cdx4^{-/0}$ mutants. It is therefore not clear why a proportion of $Cdx2^{+/-}$/$Cdx4^{-/0}$ mutant allantoises do not fuse. A significant proportion of $Cdx2^{+/-}$/$Cdx4^{-/0}$ embryos do undergo successful chorioallantoic fusion, but their placental labyrinth does not form the required network of blood vessels (van Nes *et al.*, 2006).

Beside the posterior truncation involving mesoderm and neuroectoderm, two other embryonic tissues are also affected in *Cdx* mutant. The first of these is the endoderm of the intestine. The adult intestinal epithelium expresses *Cdx2* along its rostrocaudal axis with the strongest expression around the caecum, between the distal midgut and proximal colon (James *et al.*, 1994). *Cdx2* heterozygous adult mice developed multiple intestinal polyp-like lesions, most frequently in this caecum area. These lesions do not express *Cdx2* and contain areas of squamous metaplasia in the form of keratinized stratified squamous epithelium, similar to that occurring in the mouse esophagus and forestomach. These lesions thus form heterotopic stomach and small intestinal mucosa (Beck *et al.*, 1999). Overexpressing *Cdx2* in the gastric epithelium induces the differentiation of intestinal-type cells with associated intestine specific gene expression (Mutoh *et al.*, 2002, Silberg *et al.*, 2002). These studies show that *Cdx2* is involved in the morphogenesis of intestinal epithelium, and plays

a homeostatic role in that tissue. Loss of *Cdx2* expression in *Cdx2*$^{+/-}$ mice transforms intestinal identity into more anterior identity and conversely gain of *Cdx2* function causes posterior transformations.

Another embryonic system affected by *Cdx* mutations is hematopoiesis. During mouse embryogenesis, the process of blood formation takes place in two different domains at two different time points. The first one (primitive hematopoiesis) takes place in the extraembryonic yolk sac at E7.25 and generates the primitive erythrocytes. Concomitantly with this, the vasculature begins to develop in closed proximity with the erythrocytes. This led to the hypothesis that both erythrocytes and endothelial cells share a common precursor called hemangioblasts. The second wave of (definitive) hematopoesis concerns the definitive hematopeietic stem cells (HSC) (Huber *et al.*, 2004) that are the source of hematopoietic cells throughout the life of the mouse. They reside intraembryonically at E10.5 in the aorta-gonad-mesonephros (AGM), and in the vitelline vessels, umbilical arteries and placenta (Dzierzak and Speck, 2008). These cells then migrate and populate the liver and subsequently (prior to birth) the bone marrow, sources of adult hematopoietic stem cells. The blood precursors in both the extra and intraembryonic tissues originate from the mesoderm layer emerged from the primitive streak (Dzierzak and Speck, 2008). At the early streak stage (E6.5), prospective mesoderm from the posterior primitive streak will form the yolk sac. It contains hematopoietic and endothelial progenitors. These populations have been shown to express *Brachyury* and *Flk1* (Huber *et al.*, 2004). At the late streak stage, lateral mesoderm that will become the AGM arises from more caudal region of streak (Kinder *et al.*, 1999). *Cdx* gene expression is detected intraembryonically in the primitive streak from the late streak on (Deschamps and van Nes, 2005), thus *Cdx* genes could potentially play a role in definitive hematopoeisis. No report mentions any hematopoetic defect in mice embryos lacking *Cdx* function. However in vitro work utilizing murine embryonic stem cells (ESC) has show that *Cdx*- deficient mouse ESCs are impaired in hematopoietic progenitor formation. These ESCs also exhibit altered *Hox* gene expression. Hematopoietic defect are more severe in compound *Cdx* mutants than upon loss of the function of any single *Cdx* gene. Reduced hematopoietic progenitor formation of ESCs deficient in multiple *Cdx* genes can be rescued by ectopic expression of *Cdx4* and this is accompanied by restored *Hox* gene expression (Wang *et al.*, 2008).

Cdx mutant phenotype in zebrafish

It is exclusively in zebrafish embryogenesis that *cdx* genes have been shown to be involved in hematopoesis in vivo. Like in other vertebrates, zebrafish hematopoiesis also occurs twice during embryonic development. The primitive hematopoiesis in zebrafish occurs at 10-12 hours post fertilization (hpf) in the intermediate cell mass which is located intraembryonically above the yolk tube between the notochord and endoderm of the trunk. Definitive blood formation takes place in the ventral wall of the dorsal aorta at 24-48 hpf. Later on, the definitive HSC colonize the kidney, thymus and pancreas, and from 13 days post fertilization (dpf), the kidney serves as the primary hematopoietic organ for the remainder lifespan of the fish (Hsia and Zon,

2005). A null mutation in *cdx4* (*kgg*) causes a severe deficiency in embryonic blood formation. This defect is accompanied by abnormal anteroposterior patterning and change in *Hox* expression domains. Overexpressing *hoxb7a* or *hoxa9a* rescued the hematopoiesis defect in *kgg* mutant (Davidson *et al.*, 2003). Zebrafish embryos deficient in both *cdxa1a* and *cdx4* fail to specify both intermediate cell mass (primitive hematopoietic) precursors and AGM-derived (definitive hematopoietic) HSCs. This phenotype was rescued by overexpressing *hoxa9a* (Davidson and Zon, 2006). These studies of *Cdx* genes in zebrafish embryos and murine ESCs show the involvement of *Cdx-Hox* in hematopoesis.

Another mesoderm derived organ system that is affected in zebrafish *Cdx* mutant is the kidney. In higher vertebrate such as mice and human, three different kidneys of increasing complexity arise sequentially during embryogenesis from intermediate mesoderm: the pronephros, the mesonephros and the metanephros. The pronephros and the mesonephros degenerate successively, whereas the metanephros serves as the adult kidney. Lower vertebrates such as fish and amphibians, develop a pronephros during embryogenesis, and then form a mesonephros that will be used throughout their adult life as a kidney. Each of these kidneys contains the nephron as its basic functional unit (Dressler, 2006; Vize *et al.*, 1997). Individual nephrons consist of a blood filter (glomerulus) followed by a series of segmented tubules, and finally terminate with a collecting duct. In zebrafish embryonic pronephros, Wingert *et al.* (2007) found that retinoic acid (RA) is required to induce the proximal segment fate and prevents the expansion of the distal segment fate. Zebrafish embryos lacking functional *cdx1a* and *cdx4* genes exhibit a posterior shift of the pronephros along the embryonic axis and form a truncated pronephros in which only the proximal but not the distal nephron segments are generated. *Cdx* genes act upstream of *raldh2* and *cyp26a1* to localize the source of RA along the AP axis, thereby determining the position of pronephros formation and, subsequently RA signaling acts to direct the segmentation of pronephros (Wingert *et al.*, 2007).

In zebrafish, *Cdx* were also reported to control the morphogenesis of several organs developing from the endoderm layer. *cdx4* together with *cdx1a* specify the number of pancreatic beta cells, direct beta cells midline conversion during early pancreatic islet formation and determine the anterior posterior location of the pancreas, liver and small intestine. Loss of *cdx4* and *cdx1a* causes increased beta cell number during early pancreatogenesis, delayed midline convergence of beta cells and posterior shift of pancreas, liver and intestine (Kinkel *et al.*, 2008), while morpholino mediated knock down of *cdx1b* causes hypoplastic development of these organs (Cheng *et al.*, 2008). RA treatment of wildtype embryos causes endoderm cells to express insulin throughout the anterior endoderm, ectopically to the normal expression domain. The same result was observed by overexpressing dominantly active RA receptors throughout the endoderm. However, in both cases the posterior endoderm cells never expressed insulin. When *cdx4* deficient embryos were treated with RA, ectopic insulin expressing cells are observed in posterior as well as in anterior endoderm cells. This suggests that normal *cdx4* expression in the posterior part of embryos functions to prevent posterior endoderm cells from responding to RA and developing into pancreas (Kinkel *et al.*, 2008).

Loss of *cdx1a* and *cdx4* activity in zebrafish also caused expansion of posterior

hindbrain at the expense of spinal cord. This expanded hindbrain is organized into ectopic rhombomeres arranged in a mirror-image duplicated pattern within the trunk region of the embryos. In these embryos, FGF and RA signaling activity overlap in the posterior body and display opposing gradients similar to those in the normal hindbrain region. These signaling activities are responsible for the generation of the ectopic structures. Overexpressing *Cdx* genes in the hindbrain induces spinal cord development. These results show that in the normal central nervous system development, *Cdx* suppresses the posterior hindbrain fate into spinal cord (Shimizu *et al.*, 2006; Skromne *et al.*, 2007).

A null mutation in zebrafish *cdx4* or *kgg* caused posterior axial truncation and shorter yolk extension. The same result was obtained with *cdx4* morphant embryos (Davidson *et al.*, 2003; Hammerschmidt *et al.*, 1996; Shimizu *et al.*, 2005). Zebrafish *cdx1a* morphant embryos are completely normal, but inhibition of both *cdx1a* and *cdx4* cause a phenotype more severe than in *cdx4* mutants, in which yolk extension and tail are completely absent, and only about 10 somites are made instead of 32-36. In these double morphants, the expression of *hoxa9a* and *hoxb7a* is absent. No significant reduction of cell proliferation was observed and only a slight increase in apoptosis was found in the psm of *cdx1a/4* morphants embryos. *wnt3a* morphant fish embryos do not exhibit any abnormality; however, inhibition of *wnt8* causes posterior truncation, and this phenotype becomes more severe in combination with *wnt3a* inhibition. The phenotype of *wnt3a/wnt8* double morphants is similar to that of embryos injected with large amounts of *dickkopf* mRNA which encodes an antagonist of Wnt signaling (Erter *et al.*, 2001; Lekven *et al.*, 2001; Shimizu *et al.*, 2005). *cdx1a* and *cdx4* are downregulated in the *wnt3a/wnt8* morphant embryos and upregulated in *wnt3a* overexpressing embryos. Posterior truncation with complete loss of the trunk and tail is also observed in zebrafish embryos without functional *fgf8* and *fgf24* or with overexpressed dominant negative *fgfr* (Draper *et al.*, 2003; Griffin *et al.*, 1995). Inhibition of FGF signaling by either treatment with SU5402 (a specific *FGFR* inhibitor) or overexpression of dominant negative *fgfr* leads to downregulation of *cdx1a* and *cdx4* (Shimizu *et al.*, 2005). In contrast, *cdx1a* and *cdx4* are upregulated in embryos overexpressing *fgf8*. Inhibiting both Wnt and FGF signaling further reduced *cdx1a* and *cdx4* expression. Posterior *Hox* genes (*hoxa9a* and *hoxb7a*) are upregulated in embryos overexpressing either *wnt3a*, *fgf8*, or *cdx1a*, and downregulated when Wnt or FGF signaling is disrupted. Embryos coinjected with *wnt3a* mRNA and *cdx1a/4* MO show absence of *Hox* genes expression and the same is seen in embryos coinjected with *fgf8* mRNA and *cdx1a/4* MO. These findings illustrate that *Hox* genes are under the regulation of these two signaling pathways and *cdx1a/4* are required to transduce these signals to *Hox* genes (Shimizu *et al.*, 2005).

Regulation of axial elongation and AP patterning by signaling pathways in frog and chicken

There are three *caudal* paralogous gene in *Xenopus*. Originally they were named *xcad2*, *xcad1* and *xcad3*, but in Keenan *et al.* (2006), they were renamed as *cdx1*, *cdx2* and *cdx4* respectively to adopt mouse and human *Cdx* nomenclature and to

respect orthology. Overexpression of *efgf* caused anterior truncation with reduced head structures and an enlarged proctodaeum. This phenotype is accompanied by anterior expansion of the expression of *xcad3/cdx4* and posterior *Hox* genes. A similar phenotype is also observed after overexpressing *cdx4* and posterior *Hox* (*hoxa7*) genes. In contrast, overexpression of dominant negative *fgfr* disrupts the expression of *cdx4* and posterior *Hox* genes and causes posterior truncation, while anterior structures develop normally (Northrop and Kimelman, 1994; Pownall *et al.*, 1996). *cdx1*, *cdx2* and *cdx4* are also down regulated in embryos overexpressing dominant negative *fgfr1* and *fgfr4* (Keenan *et al.*, 2006). Initiation of posterior *Hox* gene expression is dependent on Fgf signaling via *cdx4*, although its maintenance is independent of both Fgf and *cdx4* (Isaacs *et al.*, 1998; Pownall *et al.*, 1996; Pownall *et al.*, 1998). Cycloheximide-protein synthesis inhibitor assays in vitro on animal cap explants (Issacs *et al.*, 1998; Keenan *et al.*, 2006) showed that *cdx1*, *cdx2* and *cdx4* are direct targets of Fgf through MAP kinase signaling, but not *pi-3 kinase* (Keenan *et al.*, 2006). *cdx4* locus analysis revealed the presence of multiple Fgf response elements (FRE) within intronic sequences. These FRE lie in juxtaposition with Ets and *tcf/lef*-binding motifs, indicating the involvement of FGF and Wnt pathways in regulating *cdx4* expression (Haremaki *et al.*, 2003). Overexpression of *frzb* which is an antagonist of *wnt1*, *wnt8* and *wnt3a* causes posterior truncation in *Xenopus* embryos similar to the phenotype after overexpressing dominant negative *fgfr1*. Combination of these two treatments produced similar phenotypes. *Cdx* expression is also upregulated by overexpression of *wnt8*, and even much more when *wnt8* is combined with Fgf (Keenan *et al.*, 2006). By modulating the expression of the RA receptor *rarα2*, *cdx4* (not *cdx1* and *cdx2*) and posterior *Hox* were shown to be downstream targets of the RA pathway. Overexpression of a constitutively active *rar* (but not RA treatment) rescued the effect of Fgf loss of function on *cdx4* and posterior *Hox* expression. Because *rar* is also required for the correct *Fgf* expression, these experiments demonstrate a feedback loop between Fgf and *rar*. Posterior *Hox* genes are not rescued in embryos coinjected with *rar* MO and *cdx4* mRNA suggesting that a feedback loop exists between cdx4 and rar (Shiotsugu *et al.*, 2004).

In the anterior neural tube of the chicken (*Gallus gallus*) embryos anterior *Hox* genes (*hoxb1-5*, *hoxc5*) are under RA regulation, but do not depend on FGF. The posterior *Hox* genes (*hoxb6-9*) in the spinal cord and posterior stem zone depend on FGF, but do not depend of RA (Bel-Vialar *et al.*, 2002; Liu *et al.*, 2001). However, at later stages, more rostral domains of posterior *Hox* gene expression in the neural tube switch to dependence on RA (Muhr *et al.*, 1999) which is also observed in mice (Oosterveen *et al.*, 2003). The chicken genome also contains three *caudal* orthologs, *cdxA* (Frumkin *et al.*, 1991), *cdxB* (Morales *et al.*, 1996), and *cdxC* (Marom *et al.*, 1997). Sequence comparison with other vertebrate *Cdx* genes identifies *cdxA*, *cdxB*, and *cdxC* as *cdx1*, *cdx4* and *cdx2* respectively (Marom *et al.*, 1997). *cdxA* and *cdxB* depend on FGF signaling and regulate the *hoxb9* responses to FGF (Bel-Vialar *et al.*, 2002). FGF signaling is required in the stem zone (Akai *et al.*, 2005; Mathis *et al.*, 2001) to protect its resident cells from precocious differentiation. This protection occurs in part via inhibition by FGF of RA biosynthesis in the posterior paraxial mesoderm and posterior growth zone. As cells exit from this zone and become located more rostrally, where FGF signaling declines in neuroepithelium and psm, they are exposed to RA from the

somites that causes them to differentiate. *wnt8c* (canonical Wnt signaling) mediates this transition by promoting *raldh2* expression in the somites (Olivera-Martinez and Storey, 2007).

Cdx function in axial extension in short and intermediate germ band insects: an evolutionary conserved role of *Cdx* in axial extension

In long germ band embryos like those of the fruit fly, *Drosophila melanogaster*, body segments from anterior to posterior are generated simultaneously by compartmentalization of the whole syncytial blastoderm. In the short and intermediate germ band embryos like those of the brine shrime *Artemia franciscana*, of the beetle *Tribolium castaneum* and the cricket *Gryllus bimaculatus*, only the anterior structures arise from the syncytial blastoderm and the rest of the trunk segments are produced sequentially from a cellularized growth zone located at the posterior of the growing embryos (Davis and Patel, 2002). *caudal* is expressed maternally in the fruit fly oocyte and later, at the syncytial blastoderm stage its expression forms a gradient from posterior to anterior (Mlodzik *et al.*, 1985). Loss of *caudal* in the fruit fly was reported to transform the analia, the most posterior abdominal segment into an immediately anterior structure, the male genitalia in both male and female (Moreno and Morata, 1999). In the beetle, *caudal* is first expressed uniformly in the early blastoderm and as blastoderm matures, this expression changes into an anterior to posterior gradient that spans the anlagen of the gnathal and thoracic region, and of the growth zone. After the beginning of the germ-band expansion, *caudal* expression becomes restricted to the growth zone until the segmentation is completed. In *Artemia*, *caudal* is expressed in the growth zone until the last segment is formed. *caudal* expression in the cricket is similar to that of the beetle, and in these species, maternal contribution is reported. Embryos generated from RNAi knock down against *caudal* in the brine shrimp, the beetle and the cricket exhibit only anterior head tissues without thoracic, abdominal and posterior structures. In these short and intermediate germ band embryos, *caudal* disruption is accompanied by alteration of segmentation and *Hox* gene expression, indicating that *caudal* is upstream of those genes (Copf *et al.*, 2004; Shinmyo *et al.*, 2005). It was further demonstrated in cricket embryos that *caudal* expression in the growth zone is Wnt-dependent by knocking down *armadillo* (β*catenin* ortholog) which is an effector of the canonical Wnt signaling. Knocking down *armadillo* causes a truncated cricket embryos similar to the *caudal* less embryos. Nevertheless *wingless1* (*wnt1/wg*) knock down embryos developed normally. This could be explained by the redundancy between *wnt1* and other member of the *wingless* gene family in controling axial extension from the growth zone (Miyawaki *et al.*, 2004; Shinmyo *et al.*, 2005). In the beetle, knocking down *wnt1/wg* also does not produce truncated embryos, but knocking down *wntd/8* leads to a lack of posterior segments caudal to the second thoracic segment (Bolognesi *et al.*, 2008). Posterior axial truncation in the absence of *Cdx* expression in intermediate and short germ band embryos demonstrates the evolutionary conservation of the role of *Cdx* in axial elongation.

Aim and scope of this thesis

Previous works (Chawengsaksophak *et al*., 1997; Chawengsaksophak *et al*., 2004; van den Akker *et al*; 2002; van Nes *et al*., 2005) have revealed the role played by *Cdx* genes in posterior body extension. One goal of this thesis was to characterize the defects caused by *Cdx* mutations in paraxial mesodermal tissues, and its derivative-the vertebrae, in neuroectoderm, and in endodermal derived tissues especially the hindgut. Another aim was to understand why *Cdx* genes were involved in both tissue patterning along the AP axis like their close relatives the *Hox* genes and axial extension, whereas the *Hox* genes did not share the property of *Cdx* genes to control embryonic axial extension. Therefore I set out to investigate whether *Hox* genes are involved in posterior axial extension, in spite of the absence of posterior truncation phenotype in *Hox* mutants. The strategy I used was to overexpress several *Hox* genes under the regulation of a *Cdx2* promoter fragment in $Cdx2^{+/-}/Cdx4^{-/0}$ mutants. Another major purpose of this work was to understand the molecular genetic mechanism of the control of axial extension by *Cdx* gene product. We focused on studying the relationship of these homeodomain transcription factors with the canonical Wnt signaling pathway and with the clearance of retinoic acid in posterior tissues. Given the fact that mutations in *Cdx* genes impair tissue generation from progenitor cell populations located in the mouse primitive streak during gastrulation and later development, the possibility that *Cdx2* is involved in the establishment of the germ line, which originates at the base of allantois, was also investigated in this work. A *Cdx2* conditional knock out mouse strain was generated, allowing an easy generation of embryos devoid of *Cdx2*, the most dominant gene of the *Cdx* family required for embryo implantation. This conditional *Cdx2* knock-out mouse strain proved to be useful to study the impact of loss of *Cdx2* in the germ line, and should make it possible to study the role of the *Cdx2* in later developmental events such as gut endodermal differentiation and homeostasis, a role that could not be addressed before.

Chapter 2

Hox, *Cdx* and antero-posterior patterning in the mouse embryo

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Abstract

Cdx and *Hox* gene families descend from the same *ProtoHox* cluster, already present in the common ancestors of bilaterians and cnidarians, and thought to act by providing antero-posterior (A-P) positional identity to axial tissues in all bilaterians. Mouse *Cdx* and *Hox* genes still exhibit common features in their early expression and function. The initiation and early shaping of *Hox* and *Cdx* transcriptional domains in mouse embryos are very similar, in keeping with their common involvement in conveying A-P information to the nascent tissues during embryonic axial elongation. Considerations of the impact on axial patterning of the early expression phase of these genes that correlates with the temporally collinear expression of 3′-5′ *Hox* genes, suggest that it is concerned with the acquisition of anteroposterior (A-P) information by the three germ layers as the axis extends. This early A-P information acquired by all cells emerging from the primitive streak or tail bud and their neighbours in the caudal neural plate gets further modulated by the second phase of gene expression occurring later as the tissues mature and differentiate along the growing axis. We discuss the possibility that regulatory phase I, common to all *Cdx* and *Hox* genes is inherent to the concerted mechanism sequentially turning on 3′-5′ *Hox* genes at early stages, and keeping expression of the initiated genes subsequently in the new materials added posteriorly at the axis extends. The posterior *Hox* gene expression domain would be subsequently complemented by *Hox* regulatory phase II, consisting in a variety of gene specific, region specific and/or tissue specific gene expression controls. We also touch on the unanswered question whether vertebrate *Cdx* gene expression delivers A-P positional information in its own right, as *caudal* does in *Drosophila*, or whether it does so exclusively by upregulating *Hox* genes.

2.1. The *Hox* and *Cdx* gene family

A considerable extent of literature has recently solidly anchored the notion that *Hox* and *ParaHox* genes are close relatives that arose from common ancestors during the evolution of a unique *ProtoHox* cluster. This *ProtoHox* cluster would have comprised two or three genes, including one or two ancestors of anterior *Hox* genes and a precursor of posterior genes (the orthologs of *Drosophila AbdB*) (Brooke *et al*., 1998; Gauchat *et al*., 2000; Quiquand *et al*., 2009; Ryan *et al*., 2007), and might or might not have been concerned with antero-posterior axial patterning. The most widely supported hypothetical scenario assumes that a *cis*-transposition of an initial set of genes would have caused their tandem duplication generating the ancestor mega-cluster from which the *Hox*, *ParaHox* and *Nkx* genes would have derived after dissociation (Garcia-Fernandez, 2005; Quiquand *et al*., 2009; Ryan *et al*., 2007). After separation of the *Hox* and *ParaHox* clusters, the *ParaHox* genes would have kept their gene content, whereas additional duplications in *cis* would have led, in bilaterians to the larger *Hox* family known in protostomes and deuterostomes. According to a number of studies (Deutsch and Lopez, 2008; Garcia-Fernandez, 2005) a burst of *cis*-duplications of the anterior genes would have serially generated the five "central" *Hox* genes. Similarly, tandem duplication of the primitive (ancestral)

posterior *Hox* gene would have generated the series of six *AbdB*-like *Hox9-14* genes in invertebrate chordates (*Amphioxus*) and in the ancestors of jawed vertebrates. The lineage leading to teleost fishes, and the lineage leading to mammals have lost *Hox14* (Ferrier, 2004; Garcia-Fernandez, 2005). An interesting hypothesis was proposed to explain the intensive transposition activity in the *Hox* clusters in bilaterians: homology between the DNA binding domain of the homeodomain of the *Hox* transcription factors and that of DNA type II transposase suggested that an *ANTP* super transposon invaded the common ancestor of metazoans, generating the *Hox*-extended family that comprises the *Hox*, *ParaHox*, *Mox* and *Evx/Eve* genes (Deutsch and Lopez, 2008). The transposase activity would have later been reduced and lost, whereas DNA binding capability and a preexisting transcriptional modulation activity would have been maintained in the Hox homeodomain.

The regulation of the initial *ProtoHox* genes might have included some concerted transcriptional control. Be that as it may, the original 3′-5′ polarity of the linear arrangement of the *ProtoHox* genes has become associated with an early (3′) to late (5′) sequential gene expression of the *Hox* genes, a phenomenon called temporal colinearity (Duboule, 1992; Duboule, 1994). This phenomenon characterizes *Hox* gene expression in bilaterian species that develop according to a sequential, head to tail mode. Temporal colinearity is found exclusively for *Hox* genes organized in intact clusters, and thus in vertebrates where the *Hox* clusters have been consolidated (Duboule, 2007). Coordinated expression in space and in time of at least some of the *Hox* genes has been documented in evolutionary widely separated species such as the polychaete *Chaetopterus*, the beetle *Tribolium*, the cephalochordate *amphioxus* and the mouse (Reviewed by Deschamps, 2007). Some features of a spatial transcriptional colinearity are still visible in some of today's *ParaHox* genes (Brooke *et al*., 1998; Garcia-Fernandez, 2005), since vertebrates *Gsx*, *Pdx* and *Cdx* genes are expressed in domains with an anterior to posterior axial disposition. However, these genes are not expressed collinearly sequentially in time, since *Pdx* is initially expressed later than *Cdx*, and the most anteriorly expressed *Gsx* is initiated last (Ahlgren *et al*., 1996; Ohlsson *et al*., 1993; Szucsik *et al*., 1997; Valerius *et al*., 1995). Whether this means that temporally collinear expression is an exclusive acquisition of *Hox* genes, or whether extensive alterations of the *ParaHox* cluster have perturbed the temporal collinear expression of these genes at some time of their evolution, is not known. From a regulatory point of view, *ParaHox* genes form a less homogeneous gene family than the *Hox* genes. For instance, the transcriptional direction of *Cdx* is opposite to that of *Gsx* and *Pdx* (Ferrier *et al*., 2005). Even the tissue-specificity of expression differs between *ParaHox* cluster members: in mice, *Cdx* genes are expressed in posterior nascent tissues of the three germ layers similarly to their *Hox* counterparts (reviewed in Deschamps *et al*., 1999; and Deschamps and van Nes, 2005), before being restricted to the endoderm of the digestive tract. *Pdx* is first expressed in the embryonic endoderm and later in the brain as well (Perez-Villamil *et al*., 1999), and *Gsx* is expressed exclusively in the (neur)ectoderm (Corbin *et al*., 2000; Li *et al*., 1994; Szucsik *et al*., 1997; Valerius *et al*., 1995).

The objective in this review is to take a closer look at the relationship between mouse *Hox* and *ParaHox* genes (Figure 2.1) during patterning of the trunk and tail. We will mainly -but not exclusively- focus on the transcriptional regulation of "cen-

tral" (i.e. PG 8), and posterior (PG9-13) paralogy groups of *Hox* genes, and on the *ParaHox* genes *Cdx1*, *Cdx2* and *Cdx4*, distant paralogs of *Hox PG9* (Ferrier *et al.*, 2005; Quiquand *et al.*, 2009). All these genes are initially expressed during gastrulation and later, in the embryonic area where the progenitors of the posteriorly extending axial and paraxial structures of the trunk and tail are located. The expression and function of *Hox* and *Cdx* genes in the emerging tissues will also be compared.

2.2. Similarities and differences in the two expression phases of *Hox* and *Cdx* genes in the mouse embryo.

The spatio-temporal dynamics of the early expression of *Hox* genes and *Cdx* genes are very similar, presumably reflecting their close evolutionary relationship. In the mouse embryo, the earliest *Hox* genes are initially expressed at the boundary between extraembryonic and embryonic tissues, in the epiblast and overlying mesoderm at the posterior extremity of the primitive streak (Deschamps and Wijgerde, 1993; Forlani *et al.*, 2003). Their expression domains then spread anteriorly, progressively invading the progenitor region for the lateral plate, intermediate and paraxial mesoderm generated at successively more anterior levels of the primitive streak, and the epiblast containing the neural plate progenitors. The early *Cdx* expression follows the same dynamics (reviewed by Deschamps *et al.*, 1999, and Deschamps and van Nes, 2005) (Figure 2.2.A and 2.2.B, phase I, depicted in red). All mesoderm and all neurectoderm of the trunk and tail are generated from epiblast progenitors that have experienced *Hox* and *Cdx* expression during their early history. Because anterior *Hox* genes and the *Cdx* genes remain expressed in the posterior embryonic region from early to later stages, anterior trunk tissues have experienced expression of the anterior and central *Hox* genes and of *Cdx* genes when they were in the posterior growth zone, whereas posterior trunk and tail express in addition more and more posterior *Hox* genes initiated later during axial elongation. It is this sequential expression of more and more posterior *Hox* genes that is translated into more posterior axial identity of the newly added tissue. The particularity of *Cdx2* expression in the early (E3.5) trophectoderm where it plays a unique, non *Hox*-related function (Strumpf *et al.*, 2005) will not be discussed here.

After the initially similar expression of *Hox* and *Cdx* genes in the primitive streak, the expansion of the expression domains of these genes rostrally to the node region differs significantly from gene to gene. For example, the expression of *Cdx2* and *Cdx4* in the mesoderm and neurectoderm never expands into the trunk region, but gets downregulated soon after cells are carried away from the node region anteriorwards, as a result of continuing tissue addition during axial extension. *Cdx2* and *Cdx4* transcription in mesoderm and neurectoderm thus remain confined to the immature posterior of the embryo. Their transcripts decay in the presomitic mesoderm (PSM) and neural tube, creating a gradient decreasing rostrally (Gaunt *et al.*, 2003; Gaunt *et al.*, 2004; Gaunt *et al.*, 2005) (Figure 2.2.A). In contrast to this, expression of other genes including the *Hox* genes and *Cdx1* is maintained in mesoderm and neurectoderm cells carried away from the node region as the axis extends (although the rostral expression boundaries still will change slightly in the neurectoderm and

Mouse

Hox

3' Anterior 5' Posterior

1 2 3 4 5 6 7 8 9 10 11 12 13

A

B

C

D

ParaHox

Gsx2 *Gsx1* *Pdx1* *Cdx2*

Cdx1

Cdx4

Figure 2.1. **Schematic representation (not to scale) of the *Hox* and *ParaHox* genes in the mouse. The four *Hox* clusters are depicted (A to D), with the paralogy group (PG) numbers above the genes. The color blue stands for *Hox* and *ParaHox* genes derived from anterior *ProtoHox* genes; green stands for the derived "central" *Hox* genes, and yellow stands for genes derived from the "posterior" *ProtoHox* gene. The transcriptional direction of the genes is indicated by an arrow. See text for references.**

in the mesoderm in some cases, see below). The final rostral expression boundaries differ in the neurectoderm and the mesoderm, and are more anterior for 3′ than for 5′ genes (Figure 2.2.B and 2.2.C show the situation for *Hoxb2* and *Hoxb9*) (reviewed by Deschamps and van Nes, 2005). In describing the dynamics of the expanding *Hox* and *Cdx* expression patterns in the terms of the earlier model of the two *Hox* expression phases (Deschamps and Wijgerde, 1993), we note that all *Hox* and *Cdx* genes behave similarly during phase I of expression, when transcripts first appear in the posterior primitive streak and spread anteriorly along the streak in a way clearly at odds with the movements of cell descendants, thus relying on inductive processes (Deschamps and Wijgerde, 1993; Forlani *et al*., 2003). Posterior *Hox* genes lag behind 3′ *Hox* genes in this initial phase I (Figure 2.2.B and 2.2.C*). Cdx2* and *Cdx4* in the mesoderm and neurectoderm remain in phase I during the rest of their expression time while axial extension progresses. *Hox* gene transcription subsequently enters phase II, during which expression in the maturing mesoderm is maintained for most genes and regresses for some (Chen and Capecchi, 1997; Forlani *et al*., 2003; Iimura and Pourquie, 2006), whereas expression in the neurectoderm expands further anteriorly in many cases, in a way that is not clonally supported (Forlani *et al*., 2003). The second phase of regulation (phase II) thus also critically depends on inductive processes. The cells have not acquired their definitive *Hox* code in the node region (Forlani *et al*., 2003), and both the temporally collinear phase I and the later phase II contribute to the final *Hox* code of axial and paraxial tisues.

The pioneer model of Ed Lewis in *Drosophila* proposed a mechanism of action of the homeotic gene family, whereby embryonic cells on both sides of a *Hox* expression boundary assume a different positional identity as a result of expressing or not expressing a given gene. Given the complex elaboration of the *Hox* expression domains during mouse embryogenesis described above, one can wonder which of these boundaries (phase I, phase II or intermediate states) is functionally relevant for the acquisition of tissue identity during mouse embryonic development. We will address that issue for the mesoderm, neurectoderm and endoderm in the following paragraphs.

2.3. *Hox* and *Cdx* gene expression and A-P patterning

2.3.1. Patterning of the mesoderm

Hox and *Cdx* genes are expressed all along the primitive streak and its remnant in the tailbud. This comprises the epiblast, which contains progenitors of all types of mesoderm (extraembryonic, lateral plate, intermediate and paraxial) except the axial mesoderm of the notochord. The extraembryonic mesoderm is not considered here.

2.3.1.1. Paraxial mesoderm (presomitic and somitic):

Functional inactivation of *Hox* and *Cdx* genes have proven that these genes are required for correct patterning of embryonic structures along the antero-posterior (A-P) axis. This function has been most extensively documented for paraxial meso-

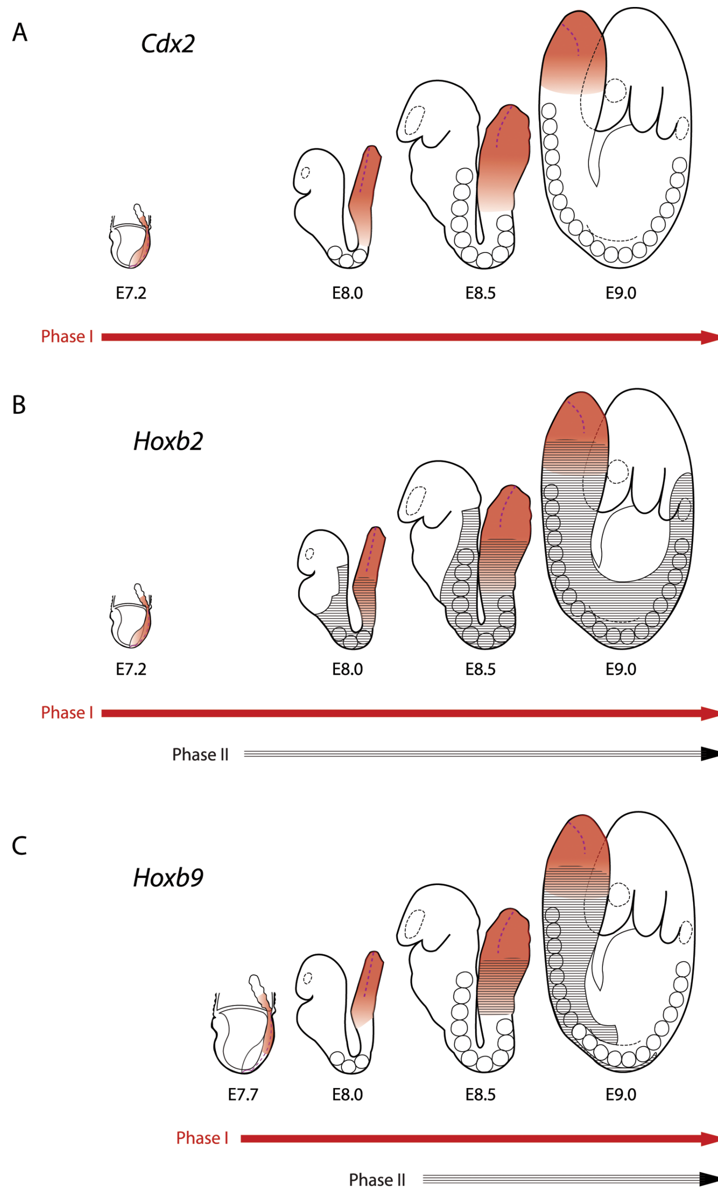

Figure 2.2. **(A-B) Representation of phases I and II of expression of a typical *Cdx* gene (A, *Cdx2*), and one of the *Hox* genes (B, *Hoxb2*). The expression of the two genes is shown at four developmental stages. Expression starts for both genes in the posterior part of the primitive streak at the late primitive streak stage, and the transcript domains expand anteriorwards (early headfold stage represented here). At the early somite stage, expression has passed the node and anterior streak, ...continue to the next page...**

Legend of Figure 2.2 continue here ... and reached the posterior presomitic mesoderm (PSM) and overlying caudal neural plate. This expression, shown in red in all schemes, corresponds to expression phase I. At E8.5 and E9.0, the posterior part of the embryos is still in *Hox* expression phase I, since the genes are turned on in the new materials continuously generated in the streak and its continuation in the tailbud (at later stages not shown here). Phase I is therefore represented underneath the schemes by a continuous red line, which would stop at the end of posterior tissue addition. For *Hoxb2* (B), at a stage between E7.2 and E8.0, expression continues rostrally to the node region under regulation phase II, shown here with horizontal shading. Expression in the maturing mesoderm is maintained for this gene, while new mesoderm is continuously produced from the streak with time, resulting in an expanding expression domain with a rostral expression boundary farther and farther from the posterior end (B). *Hoxb2* transcript domain in the neurectoderm is not only maintained, but expands further anteriorly in a way that is not clonally supported (Forlani *et al.*, 2003), until reaching the rostralmost expression boundaries (rhombomere R2/R3 boundary), before E 8.0 already. Phase II is represented by a time line underneath the scheme for *Hoxb2*. Concerning the mesoderm and neurectoderm, all *Hox* genes and *Cdx1* would undergo a phase I and phase II type of regulation, whereas *Cdx2* and *Cdx4* would only undergo phase I. The situation in the endoderm (not shown here, since data on *Hox* genes in the endoderm throughout development have not been exhaustively reported) is different, since *Cdx* genes are expressed in overlapping patterns in the gut endoderm until late embryonic stages, suggesting that they are all submitted to a type phase I and II regulation in that tissue. It should be stressed that the categories of genes illustrated here with *Hoxb2* (phase I + phase II) and *Cdx2* (only phase I) do not match with the *Hox* versus *Cdx* gene classes since one of the *Cdx* genes, *Cdx1*, behaves like the *Hox* genes and has a phase II regulation in neurectoderm and mesoderm. *Cdx1* expression is then downregulated rapidly from anterior tissues. The downregulation of *Cdx1* is similar to the anterior downregulation of the most 3′ gene of *HoxB*, *Hoxb1* (with the exception that *Hoxb1* expression in R4 is specifically reinforced by a molecular interaction at the level of one of the enhancers). These similarities between *Cdx1* and the *Hox* genes emphasize even more the similarities between *Hox* and *Cdx* families of genes. Expression of the *Hox* genes other than *Hoxb1* generally remains longer active in their entire expression domain in neurectoderm and mesoderm along the axis as shown here for *Hoxb2* phase II (see text for references). (B-C) Comparison of the phases I and II of expression of a 3′ *Hox* gene (B, *Hoxb2*) and a more 5′ gene (C, *Hoxb9*) during development. The evolution of the expression of *Hoxb2* has been described in the legend of (B). Expression of *Hoxb9* in phase I starts in the posterior part of the streak at a later stage than *Hoxb2* (temporal colinearity). Therefore, the transcription domain would not have passed the streak/node region before E8.0, after which phase II would start. Transcription of *Hoxb9* under phase II would be maintained in the maturing mesoderm, although its expression boundary regresses somewhat caudally subsequently (Chen and Capecchi, 1997), whereas expression in the neurectoderm is maintained (see text for more references). Expression in the endoderm is not indicated in the schemes, since it has not been documented systematically for these *Hox* genes at the stages represented here. Dark red dashed lines in all schemes represent the primitive streak.

derm which gives rise to the somites and later to the prevertebrae (pvs). Because it is possible to distinguish individual rostral and caudal pvs in mice, the patterning defects resulting from inactivation of single, paralogous and contiguous *Hox* genes

could be determined. This aspect of the *Hox* function is more exhaustively addressed by D. Wellik in this issue. Work in many laboratories over 2½ decades has led to the notion that *Hox* genes pattern the vertebral column in a roughly collinear way, 3′ genes playing a role at relatively anterior (occipital and cervical) levels, whereas more 5′ members affect the identity of trunk vertebrae (thoracic, sacral and caudal) (reviewed by Wellik, 2007).

The stages of vertebral development at which *Hox* and *Cdx* expression exerts an instructive function seem to vary, depending on the genes considered, and possibly the axial level. The work of Iimura and Pourquié in the chicken embryo showed that overexpression of *Hox* genes in the epiblast before its ingression in the primitive streak can alter the level at which the cells contribute to the mesoderm (Iimura and Pourquié 2006; see chapter in this issue). In the mouse, it was shown that the level of expression of *Hoxa10* in the PSM exclusively had a deep impact on vertebral morphogenesis (Carapuço *et al*., 2005) whereas it had no impact when the gene was overexpressed in formed somites. On the other hand, other experiments have shown that the expression of particular *Hox* genes, such as *Hoxa11*, in the already formed somites is important for correct morphogenesis of the caudal part of the axis. Similarly to the stage of action of some *Hox* genes in the PSM, a decrease in *Cdx2* expression in the PSM causes pattern abnormalities of vertebrae at their ultimate upper thoracic level (van den Akker *et al*., 2002), although the gene is not expressed at all in formed somites. The PSM is therefore a phase in vertebral morphogenesis when *Hox* and *Cdx* genes impose positional information on the paraxial mesoderm.

Because *Cdx* gene products are able to upregulate some at least of the *Hox* genes after binding sites in their regulatory regions (Charité *et al*., 1998; Subramanian *et al*., 1995; Tabariès *et al*., 2005; Taylor *et al*., 1997), and since *Cdx* and many *Hox* genes are co-expressed in the posterior region of the embryo including the PSM, *Cdx* genes may activate *Hox* genes in the PSM, and thereby modulate axial identity. In spite of the fact that all three *Cdx* genes are paralogs of each other (and distant paralogs of *Hox 9-13*), the impact on vertebral patterning of functionally impairing each of the *Cdx* genes is unequal. *Cdx1* null mice mainly exhibit abnormalities in the cervical and thoracic region, some of which obey the rules defined for anterior transformations (van den Akker *et al*., 2002 and references therein). *Cdx2* and *Cdx4*, both exclusively expressed in the PSM, give rise to transformations of vertebrae located in the thoracic region exclusively, with a severity and a penetrance that are much lower for *Cdx4* than for *Cdx2* (van Nes *et al*., 2006). Analysis of compound *Cdx* mutants has revealed that *Cdx* genes act on vertebral patterning in a redundant way (T.Y., J. van Nes, W. de Graaff and JD, unpublished).

Altogether these data suggest that the *Hox* gene family might affect the positional identity of paraxial mesoderm during their expression phase I (*Cdx* genes and some *Hox* genes) as well as in phase II (certain *Hox* genes).

2.3.1.2. Lateral Plate mesoderm:

Studies of vertebral phenotypes of mutants in “Central *Hox* genes”, (PG6, 7 and 8) have illustrated the patterning function of these genes in the lateral plate mesoderm contributing to the thoracic region of the axial skeleton (Nowicki and Burke, 2000), and shown that it is regulated independently from that in the paraxial mesoderm

(McIntyre *et al.*, 2007). *Cdx* genes are likely also to affect the contribution of the lateral plate mesoderm since their loss of function phenotypes include sternal rib defects (van den Akker *et al.*, 2002).

Patterning of the limbs is another manifestation of the role of the *Hox* genes in lateral plate mesoderm derived structures. Positioning of the limb field along the axis is thought to be relatively independent from patterning of the axial skeleton, since limbs developed at the normal axial position in *Hox* mutants with highly deregionalized axial structure and a transformed sacrum (Wellik and Capecchi, 2003). But the early limb buds themselves are patterned by the *Hox* genes along their antero-posterior axis concomitantly with A-P patterning along the main axis (Tarchini and Duboule, 2006; Zakany *et al.*, 2004). This early A-P polarization of the limb buds results from a restriction of 5′ *HoxD* gene expression to the posterior side of the bud, causing subsequent activation of *Shh* on the posterior limb margin. *Shh* activation is essential for the second phase of *HoxD* gene expression in the distal limb area leading to the development and patterning of the digits (Kmita *et al.*, 2005; Zakany and Duboule, 2007). The 5′ members of *Hox* clusters A and D (*Abdb*-like) are the only *Hox* genes that activate *Shh* on the posterior side of the limb bud (Tarchini *et al.*, 2006). Non-*Abdb* *Hox* genes, such as *Hoxb8*, had also been found to contribute posterior information to limb bud cells when overexpressed in the anterior limb bud tissue (Charité *et al.*, 1994). However, subsequent studies indicated that only particularly strong transgenic *Hoxb8* overexpression in anterior limb tissue, causing upregulation of 5′ *HoxD* genes, could evoke anterior *Shh* expression and elicit development of posterior digits at the limb anterior side (J. van Nes and JD, unpublished).

In addition to playing a role in A-P patterning of the limbs, *Hox* genes also instruct proximo-distal morphogenesis of the growing limbs. This was evidenced by the absence of distal limb segments in mice lacking *Hox* clusters A and D (Kmita *et al.*, 2005). Previous work by the Chambon, Capecchi and Duboule laboratories had shown that *AbdB* related *Hox* genes of paralogy groups 9 to 13 pattern the limbs along the proximo-distal axis in a gene dependent, colinear way.

Cdx genes, as distant paralogs of *Hox9-13* genes might theoretically be expected to exhibit some patterning activity in the early limb buds. However, no abnormal limb phenotype (except in one incidental instance, see van den Akker *et al.*, 2002) was observed so far in mice with decreased *Cdx* function. This situation may result from the fact that the expression of *Cdx* genes is absent (*Cdx2* and *Cdx4*) or in any case not maintained long enough (*Cdx1*) in the lateral plate mesoderm to play a role in limb bud development. The observation that *Hox* genes are involved in limb development while *Cdx* are not makes it likely that *Hox* expression phase II is essential for early and later limb bud patterning.

Another lateral mesoderm tissue that requires *Cdx* and *Hox* genes is the hematopoietic progenitor cell population. Work on mouse *Hox* regulatory mutants affected in the *Mll* (*trithorax*-like) gene (Ernst *et al.*, 2004a; Ernst *et al.*, 2004b) and alteration of *Hox* and *Cdx* expression in mouse ES cells (Lengerke *et al.*, 2008) suggest that *Hox* and *Cdx* genes play a role in early hematopoietic development. Investigations in zebrafish *Cdx* loss of function mutants has proven that *Cdx* and *Hox* genes are required to specify blood (Davidson and Zon, 2006), and analysis of primitive hematopoiesis in ES cell derived embryoid bodies and early mouse *Cdx* mutant embryos (Wang *et*

al., 2008) indicate that this function is at work as well in the mouse. The action of *Hox* and *Cdx* genes on primitive hematopoietic progenitors would take place during expression phase I, common for *Cdx* and *Hox* genes.

2.3.1.3. Intermediate mesoderm

Some at least of the mouse *Hox* genes are and remain expressed in the derivatives of the mesonephric duct and in the matanephric kidney mesenchyme. Posterior *Hox* genes have been shown to be involved in the ontogenesis of the metanephros (Mugford *et al.*, 2008; Wellik *et al.*, 2002). Recent work in the Duboule laboratory revealed that two overlapping sets of *HoxD* genes are expressed in the epithelial (anterior genes *Hoxd1-9*) versus mesenchymal (posterior genes *Hoxd9-12*) cells of the metanephros, and function respectively in maintaining the integrity of the renal tubular epithelia (*Hoxd8* and *Hoxd9*) and in regulating metanephric mesenchyme ureteric bud interactions (*Hoxd11* to *Hoxd13*) (Di-Poi *et al.*, 2007). *Cdx* genes are involved in kidney patterning in the zebrafish, as shown by Wingert *et al.* (2007), who reported that *Cdx* loss of function causes a posterior shift in the axial position of the pronephos. The situation in the mouse has not been examined in the most severe *Cdx* loss of function allelic combination possible, *Cdx* null embryos.

2.3.2. *Hox* and *Cdx* genes in the neurectoderm

The regulation of *Hox* gene expression takes place at both ends of the neural territory patterned by the *Hox* genes, the forming hindbrain at the rostral end, and the posteriorly extending neural tube on the caudal side.

At the caudal end of the axis, *Hox* genes are regulated within the posteriorly elongating neural plate, encompassing the area where neural progenitors reside around the node region (Diez del Corral and Storey, 2004; Delfino-Machin *et al.*, 2005; Mathis *et al.*, 2001). The sequential expression of 3'-5' *Hox* genes and of *Cdx* genes during phase I of their regulation confers positional identity to the nascent neural tissue at the caudal end of the extending axis. The regulation of *Hox* gene expression by *Fgf* and RA (Bel-Vialar *et al.*, 2002; Olivera-Martinez and Storey, 2007) modulates neuronal cell fate specification in the ventral spinal cord (Dasen *et al.*, 2003; Liu et al., 2001). Nordstrom and colleagues (Nordstrom *et al.*, 2006) showed that differential *Cdx* and *Hox* expression profiles exert a rostro-caudal patterning role on the hindbrain and spinal cord progenitors in response to early Wnt signals. Experiments in zebrafish *Cdx* mutants revealed that *Cdx* expression contributes to posterior neural identity. Loss of *Cdx* function causes posterior expansion of the hindbrain at the expense of trunk and tail (Shimizu *et al.*, 2006; Skromme *et al.*, 2007). The early role of *Hox* and *Cdx* expression during the acquisition of early axial identity of motorneurons occurring in regulation phase I must be followed by a later function of these genes during motorneuron pool diversification and topographic organization of target muscle connectivity (Dasen *et al.*, 2005) presumed to happen during expression phase II. The evaluation of the functional impact of this regulation of the *Hox* genes on the neural tube in mouse *Hox* mutants has mainly been assessed at later stages, after morphological and functional maturation of the neural tube occuring around and after birth. Gait and walking defects of some of the *Hox* mutant mice have indi-

cated central and posterior *Hox* genes of the four clusters as playing patterning roles in the trunk motoneurons (Tarchini *et al.*, 2005; Tiret *et al.*, 1998; Wu *et al.*, 2008). The role of *Hox* genes in the sensory nervous system has also been studied in the mouse. Recently a function of "central" *Hox* genes in the elaboration of the sensory nervous system in the mouse spinal cord was described. *Hoxb8* was shown to be required for correct projection of sensory afferents from the lower lumbar area to the dorsal horn (Holstege *et al.*, 2008). *Hoxb8* null mice exhibit an altered response to temperature and nociceptive stimuli at the level of the hindlimbs, and have abnormal secondary neuron organization in the dorsalmost laminae of the lumbar neural tube (Holstege *et al.*, 2008). The data show an A-P specificity in the axial location of the defects, compatible with a role of the *Hox* gene during early regulation phase I. However, the defect in *Hoxb8* null mice can also be seen as affecting the specific dorso-ventral confinement of the *Hox* expressing cells in the dorsal horn, possibly linked to expression phase II. Motor and sensory neural defects have not been described yet in *Cdx* mouse mutants, and awaits the availability of a *Cdx* null genotypic situation.

On the anterior side of the early neural tube, the expression of the *Hox* genes 3′ to PG8 is subjected to a specific regulation establishing the definitive *Hox* rostral expression boundaries in the hindbrain. The extreme rostral expression boundary of 3′ (*Hoxb1* to *Hoxb4*) and "central" *Hox* genes (*Hoxb5* to *Hoxb8*) in the hindbrain has been shown to be achieved in a temporally sequential manner, and to depend on endogenous retinoic acid (RA) signaling from the flanking paraxial mesoderm. A collinear window of RA response of these latter genes in the hindbrain was shown to exist between E8.5 (for *Hoxb1* and *Hoxb2*) and E10.5 (for *Hoxb8*). Retinoic acid responsive elements (RAREs) have been identified and functionally characterized for the 3′ most genes, (*Hoxb1*-*Hoxb2*), and for *Hoxb4*. An additional RARE was discovered between *Hoxb4* and *Hoxb5*, and shown to function on *Hoxb5* and *Hoxb8* reporter transgenes in vivo (Oosterveen *et al.*, 2003). However, inactivation of this RARE element by gene targeting did not affect the expression boundaries of any 3′ or central *Hoxb* gene, suggesting functional redundancy of this RARE with the 3′ RAREs of the *HoxB* cluster (W. de Graaff and JD, unpublished). This temporally collinear regulation of 3′ and "central" *Hox* genes by RA in the hindbrain, superimposed to the early *Hox* expression pattern, would take place during expression phase II, and reflect the important patterning role of the *Hox* genes in this part of the central nervous system (reviewed by Trainor and Krumlauf, 2000). A specific function of 3′ *Hox* genes was documented by the study of Goddard *et al.* (1996), describing the absence of the somatic motor component of the facial nerve and subsequent facial paralysis of *Hoxb1* mutant mice. Gaufo and colleagues (Gaufo *et al.*, 2004) studied the contribution of *Hox* genes to the diversity of the hindbrain sensory system. An essential function was documented for *Hoxa2* in the innervation map of the sensory nervous system corresponding to the whiskers (Oury *et al.*, 2006) (see chapter by F. Rijli in this issue). Mouse *Cdx2* and *Cdx4* are never expressed in the hindbrain and upper neural tube, and *Cdx1* is only expressed there very early and transiently. These genes do not play a patterning role in the differentiating hindbrain.

2.3.3. *Hox* and *Cdx* and the endoderm of the digestive system

Trunk endoderm is generated differently from the mesoderm. While trunk mesoderm is produced continuously from epiblast through the primitive streak and its continuation in the tailbud, only fore- and anterior midgut endoderm arises from epiblast ingressing through the anteriormost part of the streak. Caudal midgut and hindgut endoderm are formed from expansion of the endoderm produced earlier (Franklin *et al.*, 2008; Tam, *et al.*, 2007). The posterior endoderm of the early-somite stage embryo harbors the progenitors of the full length of the gut from the level of the forelimb bud to the end of the embryonic gut (Franklin *et al.*, 2008).

The early endoderm is patterned from early stages on along the A-P axis by diffusible growth factors, among which *Fgf* (Wells and Melton, 2000). Since Fgf signaling is known to regulate *Cdx* expression in vertebrates (Keenan *et al.*, 2006; Lohnes, 2003), it is likely that the Fgf/Cdx pathway plays an early patterning role in the endoderm (Stringer *et al.*, 2008). Work on zebrafish documents early functions of *Cdx* genes in endoderm patterning. *cdx1b* plays a very early role in endoderm formation (Cheng *et al.*, 2008), and loss of function of *cdx4* and *cdx1a* leads to a caudal shift of the foregut-derived pancreas and liver, and patterning defects in these foregut derivatives (Kinkel *et al.*, 2008). It has not been reported so far whether any *Cdx* loss of function mice affects foregut organs.

The expression and function of mouse *Cdx* genes has mostly been studied at midgestation. *Cdx* genes are expressed in gene-specific patterns in the endoderm (Beck *et al.*, 2000; Sekimoto *et al.*, 1998). Expression of *Cdx1* is detected after E12 in the gut endoderm, at levels decreasing from the rostral midgut till the end of the hindgut (Duprey *et al.*, 1988). *Cdx2* is the most anteriorly expressed *Cdx* gene in mouse endoderm. It is expressed in the posterior foregut down to the hindgut (Beck *et al.*, 1995). *Cdx4* is exclusively expressed in the hindgut endoderm (Gamer *et al.*, 1993; T.Y. and J.D., unpublished). Homozygote null mutants for both *Cdx1* and *Cdx4* are healthy, reflecting the lack of functional gut dysmorphogenesis. A-P patterning defects in the endoderm of *Cdx* loss of function mutants was exclusively observed in the case of *Cdx2*. Chimerism of mice with *Cdx2* null cells, or haploinsufficiency of *Cdx2* in *Cdx2* heterozygote mice lead to areas of heteroplasia around the ceacum where the epithelium has adopted a stomach-like identity (Beck *et al.*, 1999; Chwengsaksophak *et al.*, 1997). Ectopic expression of *Cdx2* in the stomach on the other hand causes the appearance of areas of intestine epithelium (Mutoh *et al.*, 2002; Silberg *et al.*, 2002). These anterior and posterior transformations, respectively, of epithelium identity in *Cdx2* loss or gain of function fit into the category of homeotic-like transformations. Recent work reveals that endodermal expression of *Cdx2* is the initiating step for gut histodifferentiation, and that subsequent endoderm/mesoderm cross-talk involving *Barx1* in the mesenchyme lead to the postnatal gut phenotype (Stringer *et al.*, 2008). Absence of *Cdx2* expression in the endoderm of the gut is associated with the expression of *Barx1* in the underlying mesoderm and the subsequent histodifferentiation of stomach mucosa (Stringer *et al.*, 2008).

The majority of intestinal *Hox* genes are expressed in the mesoderm apart from the terminal hindgut endoderm expressing *Hoxd13* (reviewed by Beck *et al.*, 2000). *Hox* mutations have been shown to give rise, in several cases, to specific defects at

morphological boundaries in the digestive tract, such as the ceacum (Zacchetti *et al.*, 2007) and the anal sphincters (Kondo *et al.*, 1996).

Since initial emergence of definitive endoderm from the anterior primitive streak is relatively early and shortlasting, phase I of *Hox* and *Cdx* expression is probably short in the endoderm, and subsequent expression of the genes during expansion of the posterior endoderm to form the caudal midgut to the hindgut, and during histodifferentiation and patterning of the digestive tract would correspond to gene expression phase II (endoderm expression is not shown in Figure 2.2).

2.4. Conclusion

The close evolutionary relationship between *Hox* and *Cdx* genes, the similarities between the regulation of their early expression domains in nascent tissue and the functional involvement of both gene families in patterning mesoderm (lateral, intermediate and paraxial), neurectoderm and endoderm in vertebrate embryos are consistent with the view that *Cdx* and *Hox* genes act as closely related homeotic-like family members. They both participate in patterning the PSM of the extending trunk, the nascent neural tissue and the early endoderm. This collaboration would include a *Cdx-Hox* cross regulatory link during phase I, at a time when the genes are co-expressed in the primitive streak, PSM and nascent neural and endoderm tissue.

Cdx loss of function mutations affect central *Hox* gene expression (van den Akker *et al.*, 2002; TY and JD, unpublished). *Cdx* genes may therefore contribute some additional posteriorizing information to that of these *Hox* genes. Whether *Cdx* genes exert homeotic patterning functions in their own right, in addition to upregulating *Hox* genes is difficult to test in the absence of a *Hox* null mutation, but the truly homeotic function of *caudal* in *Drosophila* (Moreno and Morata, 1999) proves the potential of a homeotic role for vertebrate Cdx proteins.

Hox and *Cdx* genes appear to share the early phase (phase I, Figure 2.2) of their transcriptional regulation in the embryo proper. *Cdx* and 3′ *Hox* genes may respond to the same initiating signals during gastrulation, in a way inherently linked to the inductive events in the early embryo. Wnt signals seem to be the earliest modulators of rostro-caudal identity in the neural progenitors during gastrulation (Nordstrom *et al.*, 2006) and *Wnt* is a candidate to initially induce *Hox* genes in the primitive streak (Forlani *et al.*, 2003).

The beginning of phase I of *Hox* expression, common to all *Cdx* and *Hox* genes would be inherent to the concerted mechanism sequentially turning on more and more 5′ *Hox* genes in nascent tissues from the three germ layers as the axis extends. It may thus underlie temporal colinearity of sequentially induced 3′-5′ *Hox* genes, and it is likely to be ancestral and to have been evolutionary conserved in bilaterians that extend their body by posterior additions. Alterations in phase I *Hox* and *Cdx* expression would cause early modifications in axial identity of mesoderm and neuron progenitors.

Phase II of *Hox*/*Cdx* expression constitutes a second and later regulatory phase. For each gene, expression phase II would be engaged after its domain of early expression has encompassed the whole primitive streak. It would concern events occurring

later than the initial setting of A-P instructions to the emerging caudal tissues. *Hox* expression phase II would account for gene-specific, region specific and sometimes tissue specific expression and function of the different genes, and therefore consist in a sum of gene transcription modulation events, including auto- and cross- regulations in some cases. It would contribute to morphogenesis of the axial skeleton, and control processes such as late segmental differentiation of hindbrain neurons, proper connectivity of spinal motorneurons, dorso-ventral organization of the dorsal horn, proximo-distal development of the limbs and rostro-caudal histogenesis of gut endoderm. This phase II, which follows the temporally collinear phase I, would end with the setting of the *Hox* expression boundaries in a spatially collinear way along the axis in the different germ layers. The relative independence of phase I and II has recently got support from work in the Duboule laboratory, showing that temporal and spatial regulation of mouse HoxD genes can be uncoupled (Tschopp *et al.*, 2009).

Phase I expression of *Hox* and *Cdx* genes has an impact on A-P identity of cellular precursors of trunk tissues, but does not impose a definitive *Hox* code on these precursors (Forlani *et al.*, 2003) because this *Hox* code will still be modified in most cases subsequently during phase II, in a tissue-specific and axial position-dependent way. In the end, a likely answer to the question asked above regarding the most relevant *Hox* and *Cdx* expression phase for the acquisition of tissue identity during mouse embryonic development, is that every stage of expression of these genes during the history of the tissues must be important. *Hox* and *Cdx* expression are likely to impact on successive, sometimes transient states of cell populations interacting with their environment, more than representing discrete definitive "*Hox* identity codes" for individual cells at a given moment.

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Part II

Experiments

Chapter 3

Cdx and *Hox* genes differentially regulate posterior axial growth in mammalian embryos

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Submitted

Abstract

Hox and *Cdx* genes are phylogenetically related transcription factor-encoding genes that control positional tissue identity during embryonic development. In addition, mutations impairing *Cdx* activity in mice elicit posterior body truncations, affecting the axial skeleton, the neuraxis and caudal uro-rectal structures. This phenotype of precocious axial termination has not been reported for *Hox* loss of function mutations. We show that trunk *Hox* genes stimulate axial extension as they are able to largely rescue the caudal truncations seen in *Cdx* loss of function embryos. Paralogous group 13 *Hox* genes on the contrary cause premature arrest of posterior axial growth when precociously expressed. Our data suggest that the time-related shift between expression of trunk and posterior *Hox* genes successively regulates construction and termination of axial structures in the mouse embryo. Thus, *Hox* genes seem to differentially orchestrate the complex process of posterior expansion of embryonic tissues during axial morphogenesis as an integrated part of their function in specifying head to tail identity. In addition, we present evidence for a mechanism whereby the *Cdx* and *Hox* transcription factors exert their axial extension control by differentially maintaining Wnt signaling in the posterior region during trunk and tail elongation. Moreover, these transcription factors protect the posterior growth zone from retinoic acid during trunk elongation by stimulating *Cyp26a1* expression, until they are down regulated past the trunk-tail transition.

Key words: Vertebrate axial elongation, Mouse *Hox* and *Cdx* genes, posterior embryonic morphogenesis, Wnt signaling, Retinoic acid inhibition of axial extension.

3.1. Introduction

The *Hox* and *ParaHox* gene families are believed to be derived from a common *ProtoHo*x gene cluster that existed before the cnidarian and bilaterian clades diverged from each other (Chourrout *et al.*, 2006; Garcia-Fernandez, 2005). This archetype *Hox* cluster acquired additional genes in *cis*, and it was then duplicated twice as part of the genome-wide duplications in vertebrates. In contrast, the ancestral *ParaHox* cluster apparently never acquired more than three or four members. One of these is the *caudal*-related gene *cad* or *Cdx*. Sequence analysis supports the view that vertebrate gene duplications of the *ParaHox* cluster together with gene loss have resulted in a single remaining mammalian *ParaHox* cluster carrying *Cdx2*, and two isolated *Cdx* genes on different chromosomes named *Cdx1* and *Cdx4* (Ferrier *et al.*, 2005). The mouse genome thus has three *Cdx* genes that are believed to be paralogs of a non-anterior ancestral *Hox* gene (Chourrout *et al.*, 2006).

It is widely accepted that *Hox* genes regulate anteroposterior specification in bilaterians (reviewed by Holland and Garcia-Fernandez, 1996; Kmita and Duboule, 2003; McGinnis and Krumlauf, 1992). In addition, *Cdx* genes are required to correctly pattern the head to tail axis. *caudal* was shown to be a posterior homeotic gene in flies (Moreno and Morata, 1999), and the zebrafish *Cdx* genes instruct A-P patterning of the neural tube (Shimizu *et al.*, 2006; Skromme *et al.*, 2007), pronephros (Wingert *et al.*, 2007) and gut endoderm (Kinkel *et al.*, 2008). Mouse *Cdx* genes modulate

antero-posterior vertebral patterning, at least in part by regulating the expression of particular *Hox* genes (Subramanian *et al*., 1995; van den Akker *et al*., 2002). Analyses in *Xenopus laevis* and *Gallus gallus* embryos have shown that *Cdx* gene products act downstream of Fgf signaling to regulate 5′ *Hox* genes in the posterior part of the embryo (Bel-Vialar *et al*., 2002; Isaacs *et al*., 1998; Pownall *et al*., 1996; Pownall *et al*., 1998) during establishment of anteroposterior identity. Wnt signaling acts in concert with Fgfs to generate combined patterns of *Hox* and *Cdx* profiles characteristic of anterior versus more posterior motor neuron identities (Nordstrom *et al*., 2006).

Most of the work on *Hox* and *Cdx* genes during axial development has focused on analyzing the role of these genes in antero posterior patterning of nascent tissues. More recently, *Cdx* genes have been found to be essential to posterior tissue expansion during embryogenesis in mice (Chawengsaksophak *et al*., 2004; van den Akker *et al*., 2002), in zebrafish (Davidson and Zon, 2006; Shimizu *et al*., 2005) and in a short germ band insect, an intermediate germ band cricket and arthropods (Copf *et al*., 2004; Shimnyo *et al*., 2005). These studies revealed the evolutionary conservation of an essential function of *Cdx* genes in axis growth and strengthened the evidence that vertebrates share a basal strategy of head to tail development with invertebrates that extend their long axis by posterior addition of tissues, the ancestral mode of axial development in bilaterians.

Loss of function mutations in mouse *Cdx* genes cause premature termination of axial extension, meaning that these genes are involved in tissue generation. Mutations causing loss of *Hox* function have not been found so far to cause posterior axial truncations, in spite of the common ancestral origin and the similarity in early expression of *Cdx* and *Hox* genes. We now provide experimental evidence that gain of function of *Hox* genes belonging to the "central" group significantly rescues the truncation of the posterior axis in *Cdx* mutants. These results demonstrate that central *Hox* genes stimulate trunk tissue expansion during posterior axial growth. Strikingly, a *Hox* gene of the most 5′ paralogy group, *Hoxb13*, had been shown to inhibit axial extension since its loss of function caused an overgrowth of caudal spinal cord and tail vertebrae (Economides *et al*., 2003). We show here that paralogous group 13 (PG13) *Hox* genes control axial elongation in that their precocious expression arrests the posterior addition of tissues prematurely. We propose that the time-dependent balance between the expression of *Cdx* and trunk *Hox* genes on the one hand, and late expressed posterior most *Hox* genes on the other hand, is essential to successively promote and arrest axial elongation. The *Hox* genes therefore would integrate the processes of tissue generation and A-P patterning, ensuring coordinated axial morphogenesis. We also present evidence that posterior activation of the canonical Wnt signaling pathway in the *Cdx* mutants rescues their axial truncation, suggesting that *Cdx* transcription factors are maintaining active posterior Wnt signaling needed to sustain tissue generation during trunk axial extension. *Cdx* and trunk *Hox* genes would also ensure posterior RA clearance in the "posterior growth zone" (term adopted from the terminology used in short and intermediate germ band animals, Copf *et al*., 2004), during trunk elongation, possibly acting as a support to ensure the maintenance of tissue expansion from the posterior progenitors.

3.2. Materials and methods

3.2.1. Generation of transgenic constructs and mice

To construct the *Cdx2PHoxb8*, *Cdx2PHoxa5*, *Cdx2PHoxa13*, and *Cdx2PHoxb13* transgenes, we used the expression vector *p301* kindly provided by I. Rodriguez (Geneva). The *IRES-EGFP* marker present in the construct appeared not to lead to detectable *GFP* expression, but was used for genotyping the transgenic embryos and mice. Full length cDNAs from *Hoxb8* (W. de G. and J.D., unpublished), *Hoxa5* (a kind gift of L. Jeannotte, Quebec), *Hoxa13* (kind gift of J. Innis), *Hoxb13* (kind gift of D. Wellik) were inserted into *p301* between the rabbit *ß-globin* intron and *IRES-EGFP*, upstream of *ß-globin* polyA. Finally the 9.4kb *Cdx2* promoter (Benahmed *et al.*, 2008) fragment was inserted upstream of the *ß-globin* intron. A *BraPHoxa13* or *TPHoxa13* construct was generated using the *T Brachyury* promoter fragment cloned by Clements *et al.* (1996). The *BraP-CatC-Lef1* construct was described in Galceran *et al.* (2001). The constructs were linearized, gel purified and microinjected into the male pronucleus of zygotes recovered from C57Bl/6J X CBA F1 crosses, following the standard procedure. Founder mice were recovered for *Cdx2PHoxb8*, *Cdx2PHoxa5*, *Cdx2PHoxb-13* and *BraP-CatC-Lef1*. Two or three transgenic lines were established for each transgene. Embryos from the *Cdx2PHoxb8*, *Cdx2PHoxa5* and *Cdx2PHoxb13* lines were found to express the transgenes at levels comparable to that of the endogenous *Cdx2*. These transgenic lines were bred with $Cdx2^{+/-}$ and $Cdx4^{-/-}$ mice to generate $Cdx2^{+/-}/Cdx4^{-/0}$ embryos carrying the *Hox* transgenes (van Nes *et al.*, 2006). Transgenic mice were maintained under standard husbandry conditions, according to the Dutch regulation for laboratory animal work. To generate the *Hoxc13* transgene, a cDNA for this gene (IMAGE 6171228) was cloned into an expression cassette containing the *Dll1* promoter (Beckers *et al.*, 2000), and behind the *Cdx2* promoter described above. These constructs were injected in pronulei of FVB zygotes to generate transgenic embryos. Because founders did not survive to adulthood, embryos were isolated at different stages for analysis.

3.2.2. Genotyping

Genotyping conditions for the wildtype and mutant alleles of *Cdx2* and *Cdx4* were described earlier (van Nes *et al.*, 2006). For *Hox* transgenic lines, genomic DNA was isolated from yolk sac or ear cuts after lysis with a solution containing 100mM TrisHCl pH 8.5, 5mM EDTA, 0.2% SDS, 200mM NaCl, 100µg/mL proteinase K at 55°C overnight, isopropanol precipitation and dissolution in 500µL TE buffer overnight at 55°C. *EGFP* primer sequences were AGA ACG GCA TCA AGG TGA AC (forward) and CTT GTA CAG CTC GTC CAT GC (reverse), generating a 242 bp specific *EGFP* fragment. The reaction conditions were 95°C for 30 sec, 60°C for 60 sec and 72°C for 30 seconds for 35 cycles in 10µL mixture that contained 0.5µM of each primers, 0.2mM of each dNTP, 1.5mM $MgCl_2$ and 1x PCR Buffer (Promega 5x Flexi Green GoTaq Buffer M891A and GoTaq M830B). Genotyping *BraP-CatC-Lef1* transgenic mice were done by PCR as described in Galceran *et al.* (2001). *Hoxc13* transgenic embryos were genotyped by PCR on DNA isolated from yolk sacs (E10.5

embryos) or from the intestines (E18.5 embryos) using primers ACT TCG CTG CTC CTG CAT CCA (forward) and CAG CTG CAC CTT AGT GTA GGG (reverse).

3.2.3. Skeletal analyses

Staining of embryonic E15.5, E18.5 and postnatal day P2 skeletons was according to van den Akker *et al*. (2002). Vertebral formulae were determined as described in Tables 3.1 and 3.2.

3.2.4. In situ hybridization

In situ hybridization on whole mount embryos using riboprobes was according to van Nes *et al*. (2006).

3.2.5. Histological analysis

Specimens were fixed in 4% paraformaldehyde in phosphate-buffer saline, embedded in paraffin, sectioned at 10μm, stained with hematoxylin & eosin in standard conditions before inspection and photography.

3.2.6. Genome-wide transcriptome analysis of *Cdx* mutants versus controls

Posterior part of 13 somite embryos were dissected at a level halfway in the PSM. Posterior tissues from 7 wildtype, and from 7 *Cdx2*$^{+/-}$/*Cdx4*$^{-/-}$ mutant embryos were pooled and stored in RNAlater (Ambion, cat# AM1924). RNA was purified using a kit from Qiagen (cat# 74104). cDNA was generated and cDNA samples hybridized in duplicate on 44K Agilent transcriptome arrays (Agilent cat# 5184-3523), according to the manufacturer's instructions. The arrays were scanned by the "DNA micro-array scanner" from Agilent, and processed by ArrayAssist. Genes with an expression value higher than twice the control value were considered as candidate *Cdx2*$^{+/-}$/*Cdx4*$^{-/-}$ targets.

3.2.7. Quantitative RT-PCR analysis

Tail tips were dissected at the level of the last formed somite of E10.5 wildtype and *Cdx2PHoxc13* transgenic embryos. RNA was extracted from individual tips using TRIzol method (Invitrogen, UK) and quantified using nanodrop analysis (Thermoscientific, USA) according to the manufacturer's instructions. Exactly 800ng of template from each tail RNA preparation was reverse transcribed to cDNA using Superscript II kit (Invitrogen, UK). For analysis of gene expression in tail tips, real-time PCRs were performed in duplicate in 4 samples of each genotype, with 1μL of amplified cDNA per reaction using the QuantiFast SYBR Green PCR System according to manufacturers instructions (Qiagen). Real-time PCR was carried out using a Light Cycler loaded with capillary tubes (Roche diagnostics) carrying a 10μL reaction mix for each replicate. Expression of mouse *Gapdh* was used as the endogenous control.

Relative gene expression was calculated by the comparative 2-DDCT method. Statistical analysis was performed using the Mann Whitney test (see legend of Figure 3.14). Primers used:

Gapdh: foward ACC ACA GTC CAT GCC ATC AC, reverse TCC ACC ACC CTG TTG CCA TCA C (452bp product)

Cyp26a1: foward TCG CAC AAG CAG CGA AAG AAG G, reverse ATG TGG GTA GAG TCC TAG GTA AGT (569bp product)

Wnt3a: foward ATT GAA TTT GGA GGA ATG GT, reverse CTT GAA GTA CGT GTA ACG TG (317bp product)

Hoxc13: foward TCT AGG GCC AAG GAG TTC G, reverse CGT GGG TTC GGT TAT GGT A (557bp product)

Gdf11: foward ACC ACC GAG ACG GTC ATA AG, reverse CAA AGG CGT TGA TCT CGA TT (376bp product)

3.2.8. Statistical Analysis

Statistical analysis of vertebral counts is described in Table 3.4. The Mann-Whitney test was used to analyze the significance of the difference between the sacro-caudal vertebral columns of *Cdx2*$^{+/-}$/*Cdx4*$^{-/-}$ mutants and *Cdx2*$^{+/-}$/*Cdx4*$^{-/-}$ mutants expressing the *Hox* transgenes. The non-parametric Mann-Whitney test was chosen because the data sets for each genotype were not normally distributed (Z values obtained from the Kolmogorov Smirnov test for each genotype was higher than 0.05). Statistical analysis of the real time PCR data was performed using the Mann-Whitney test as well.

3.3. Results

3.3.1. *Hox* and *Cdx* genes are expressed in progenitor areas for the trunk and tail

During mouse embryogenesis, *Hox* genes are first transcribed in a sequential manner in and along the primitive streak and node region at the late primitive streak stage (Deschamps and van Nes, 2005; Forlani *et al*., 2003). These areas contain the progenitors of the future trunk axial and paraxial tissues in the three germ layers (endoderm: Fanklin *et al*., 2008; Tam *et al*., 2007; mesoderm and neurectoderm: Cambray and Wilson, 2007; Kinder *et al*., 1999; Tam and Beddington, 1987; Wilson and Beddington, 1996). Like *Hox* genes, *Cdx* genes are first expressed in the primitive streak at the late streak stage (Deschamps and Van Nes, 2005). This expression in progenitor regions that contribute descendants during body axis extension is maintained at later stages as the embryo generates its trunk and tail tissues from the older primitive streak, and later the tailbud (Figure 3.1).

3.3.2. *Cdx* partial loss of function leads to a premature arrest in the generation of nascent mesoderm and neurectoderm, and in endoderm expansion.

Single or combined inactivation of *Cdx1* and *Cdx4* does not compromise axial elongation (Subramanian *et al*., 1995; van Nes *et al*., 2006). *Cdx2* null embryos do not

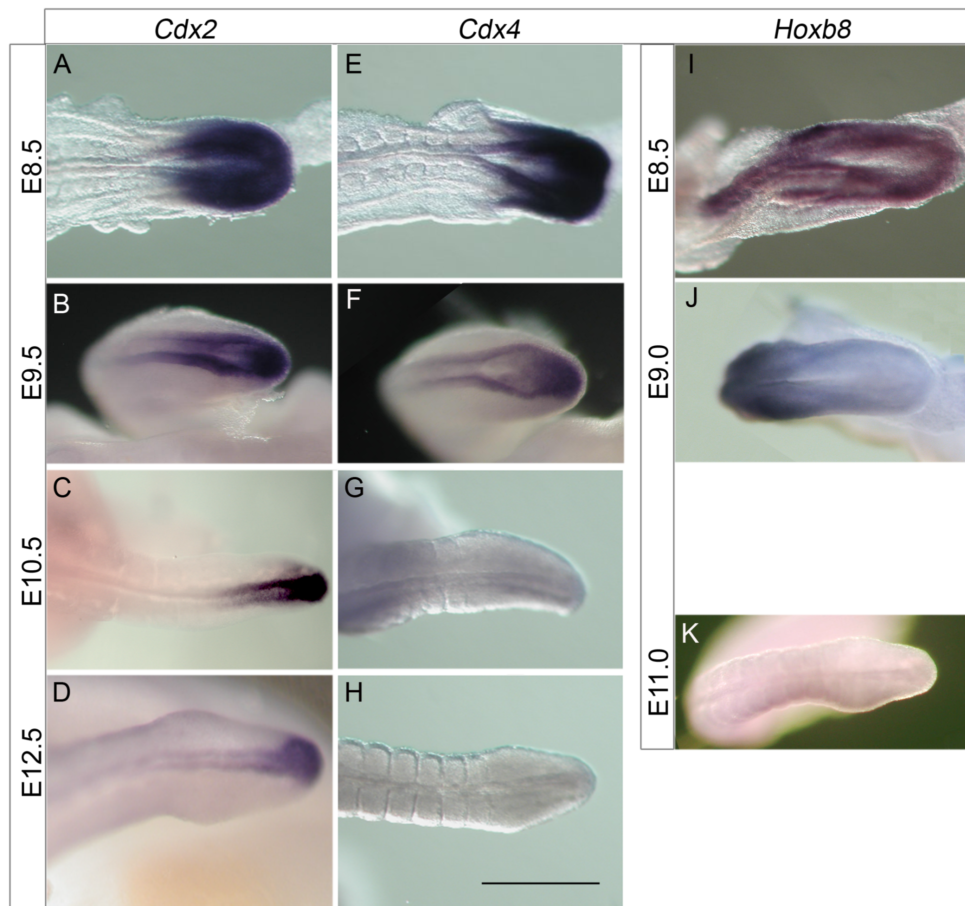

Figure 3.1. **Expression of *Cdx2* and *Cdx4*, and of *Hoxb8* (a "central" or trunk *Hox* gene) revealed by in situ hybridization are shown in posterior tissues from E8.5 to E12.5 in wild-type embryos. These results and data not shown indicate that the expression of *Cdx2* in the posterior "growth zone" ends after E12.5, that of *Cdx4* before E10.5, and that of *Hoxb8* around E9.5. Scale bar=0.5mm**

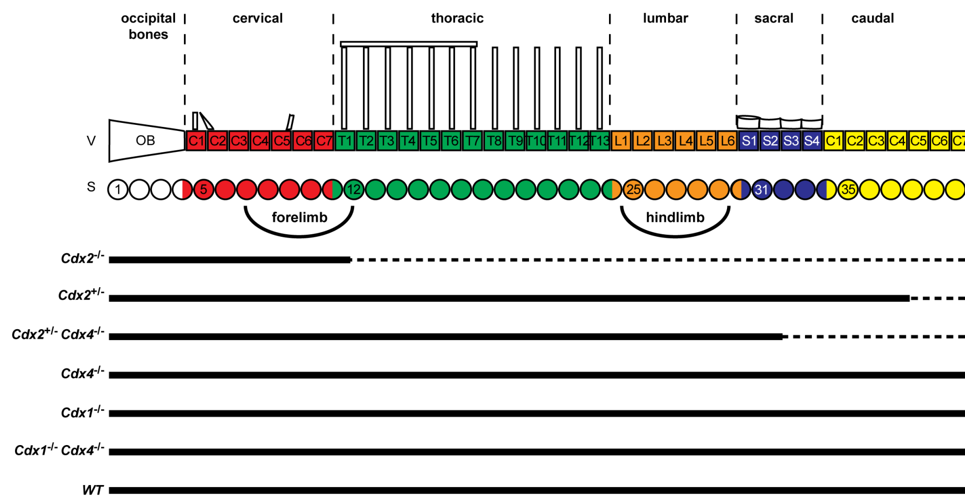

Figure 3.2. **Schematic representation of the axial extension phenotypes of wildtype and *Cdx* mutant embryos around E10.5. Only *Cdx2* mutants, or compound *Cdx* mutants carrying a mutated *Cdx2* allele exhibit premature arrest of the embryonic body axis. *Cdx2* null embryos can not be obtained by natural breeding of *Cdx2* heterozygote mutant parents since they do not implant due to trophectoderm defects (see text). The *Cdx* mutants studied the most extensively in this work are the compound *Cdx2*$^{+/-}$/*Cdx4*$^{-/-}$ embryos.**

implant due to trophectoderm defects (Chawengsaksophak *et al.*, 1997; Niwa *et al.*, 2005) but tetraploid rescue bypasses this early block and generates embryos with severe posterior axial truncations (Chawengsaksophak *et al.*, 2004). *Cdx2* heterozygotes miss the very last tail vertebrae (Chawengsaksophak *et al.*, 1997). When introduced in the *Cdx4* null background, *Cdx2* heterozygosity leads to a strong increase in the severity of the premature arrest of axial extension of *Cdx2* heterozygotes (Figure 3.2). The *Cdx2*$^{+/-}$/*Cdx4*$^{-/-}$ embryos are axially severely compromised. In addition, most of them die around E10.5 due to deficiencies in the placental labyrinth, the vascular part of which derives from posterior mesoderm expressing *Cdx* genes at earlier stages (van Nes *et al.*, 2006). Examination of the *Cdx2*$^{+/-}$/*Cdx4*$^{-/-}$ mutant embryos at E9.5 revealed deficiencies in posterior axial and paraxial tissues (Figure 3.3.A-B). At E15.5, the surviving mutants were developmentally retarded and their axial skeleton was truncated at sacro-caudal levels (Figure 3.3.C-E). Prevertebrae in the *Cdx2*$^{+/-}$/*Cdx4*$^{-/-}$ mutants appeared thinner from lumbar levels onwards, and vertebral fusions and dysmorphologies were observed, mostly at lumbar and sacral levels (not shown in detail here). In addition, homeotic-like anterior transformations were detected at the thoraco-lumbar transition (Table 3.1). Using mutant alleles for the three *Cdx* genes, we found that *Cdx2* is the main player in ensuring completion and patterning of the posteriorly extending embryonic axis (Figure 3.2, and data not shown).

The first manifestation of the axial phenotype of *Cdx2*$^{+/-}$/*Cdx4*$^{-/-}$ mutants is a decrease in size of the presomitic mesoderm (PSM) compared with that in age-matched wildtype littermates. This is first apparent in mutant embryos at the 7 to 9 somite-sta-

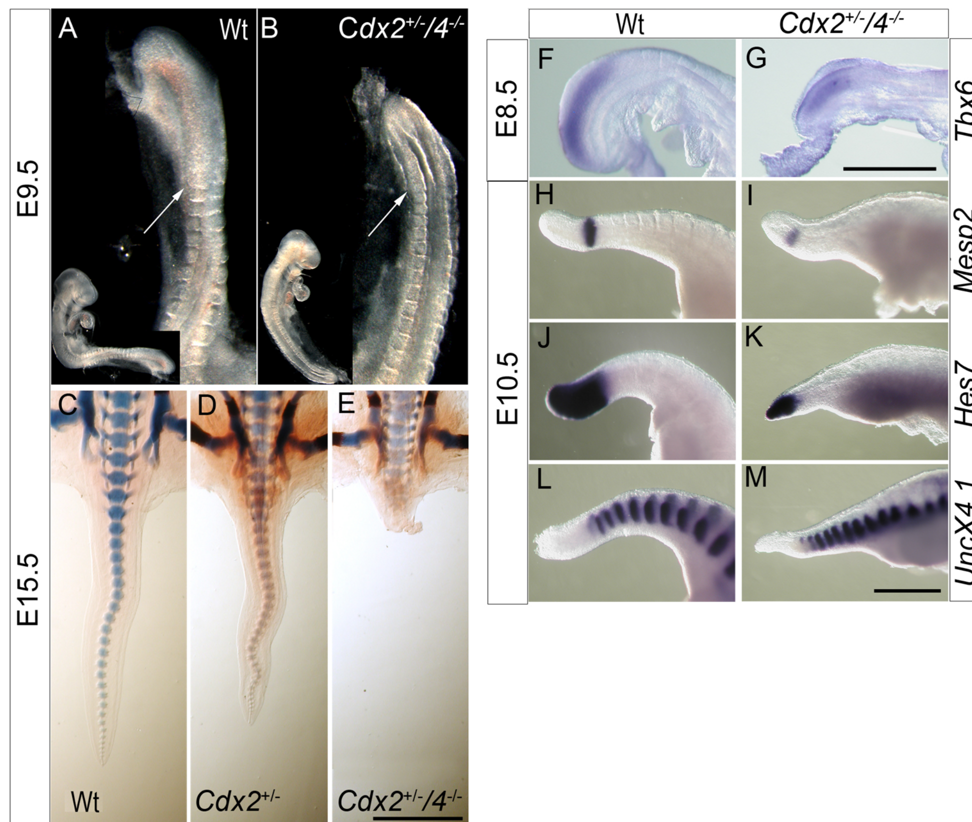

Figure 3.3. **Phenotype and gene expression in the posterior region of wildtype and *Cdx* mutant embryos. (A-B) Photograph of whole mounts (insert) and posterior parts of a 20 somite wildtype (A) and *Cdx2*$^{+/-}$/*Cdx4*$^{-/-}$ mutant (B). (C-E) Skeletal preparations of E15.5 wildtype, *Cdx2*$^{+/-}$/*Cdx4*$^{+/-}$ and *Cdx2*$^{+/-}$/*Cdx4*$^{-/-}$ mutant embryos. (F-M) Whole mount in situ hybridization of E8.5 wildtype and *Cdx2*$^{+/-}$/*Cdx4*$^{-/-}$ mutant embryos with *Tbx6* (F-G), and E10.5 wildtype and *Cdx2*$^{+/-}$/*Cdx4*$^{-/-}$ mutant embryos with *Mesp2* (H-I), *Hes7* (J-K), and *UncX4.1* (L-M). Arrows in (A) and (B) indicate the last formed somite. Anterior is to the top in (C-E), and to the right for (F-M). Scale bars in (C-E) is 1.5 mm, (F-M) is 0.5 mm.**

	Wildtype	*Cdx2*$^{+/-}$/*Cdx4*$^{-/-}$
Abnormality of cervical vertebrae	0/6	0/6*
First ribs on vertebra 8	6/6	6/6
Most caudal vertebra bearing ribs at position 20	6/6	1/6
Most caudal vertebra bearing ribs at position 21	0/6	5/6
Rib fusions	0/6	6/6
Lumbar fusions	0/6	6/6
Number of distinct caudal vertebrae	25-28	2-9
Fusion of caudal vertebrae	0/6	6/6

* anterior tuberculi (normally on vertebrae 6) are not visible at this stage

Table 3.1. Skeletal patterning in E15.5 widltype and *Cdx2*$^{+/-}$/*Cdx4*$^{-/-}$

ge, a stage when the PSM corresponds to presumptive cervical prevertebrae. Observation of freshly isolated embryos and detection of transcripts of genes expressed in the PSM (*Tbx6*, Chapman *et al.*, 1996) and in segmenting mesoderm (*Mesp2*, Saga *et al.*, 1997; *Hes7*, Bessho *et al.*, 2001; and *UncX4.1*, Rovescalli *et al.*, 1996) confirmed that the PSM is shortened in mutant embryos and that the last formed somite is located closer to the distal tip of the axis (Figure 3.3.A-B and F-M). These observations point to an imbalance between the generation of mesoderm and continuing somitogenesis in mutant embryos. The decrease in mesoderm production becomes more severe with time, eventually leading to exhaustion of the PSM shortly after E10.5.

The impaired posterior elongation in *Cdx* mutants was not confined to the paraxial mesoderm but affected all germ layers, as documented by the truncation of the neural tube, posterior lateral mesoderm and caudal hindgut endoderm. In particular, rectal agenesis was observed in mutant embryos at E15.5, leading to enlarged fluid filled bladder and hindgut in fetuses and newborns (an example is shown in Figure 3.6.E).

3.3.3. Trunk *Hox* genes rescue posterior truncation defects in *Cdx* mutants

Given the evolutionary relatedness of *Cdx* and *Hox* genes, and the similarities of their early expression in the posterior part of the embryo at the site of axial extension, we tested if *Hox* genes are also involved in tissue expansion during body axis elongation. *Cdx* genes regulate at least some of the *Hox* genes, such as *Hoxa5* and *Hoxb8* (Charité *et al.*, 1998; Gaunt *et al.*, 2008; Subramanian *et al.*, 1995; Tabariès *et al.*, 2005; van den Akker *et al.*, 2002). This suggests that *Hox* genes might be targets and mediators of *Cdx* function.

We tested *Hoxb8* and *Hoxa5*, which are regulated by Cdx proteins via Cdx binding sites in their regulatory region (Charité *et al.*, 1998; Tabariès *et al.*, 2005; and Figure 3.4.A-D), for their potential to rescue the posterior axis truncation of *Cdx2*$^{+/-}$/*Cdx4*$^{-/-}$ mutant embryos. These trunk *Hox* genes belong to the "central" group of *Hox* genes (Ogishima and Tanaka, 2007). We expressed these genes within the *Cdx2* spatio-temporal window using a *Cdx2* promoter fragment which recapitulates the embryonic

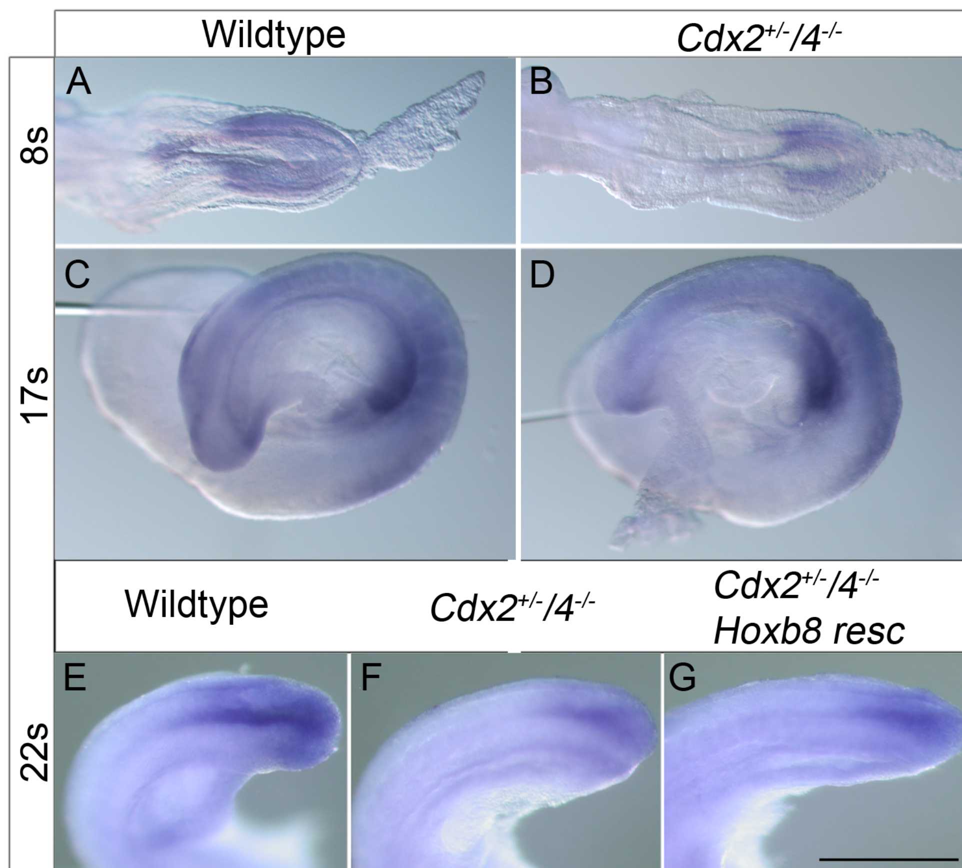

Figure 3.4. **(A-B) 8 somite wildtype and *Cdx2*$^{+/-}$/*Cdx4*$^{-/-}$ embryos were hybridized with a *Hoxb8* probes. (C-D) 17 somite wildtype and *Cdx2*$^{+/-}$/*Cdx4*$^{-/-}$ embryos were hybridized with a *Hoxa5* probe. (E-G) 22 somite wildtype, *Cdx2*$^{+/-}$/*Cdx4*$^{-/-}$ mutant and *Hoxb8* rescued *Cdx2*$^{+/-}$/*Cdx4*$^{-/-}$ mutant were hybridized with a *Cdx2* probe.**

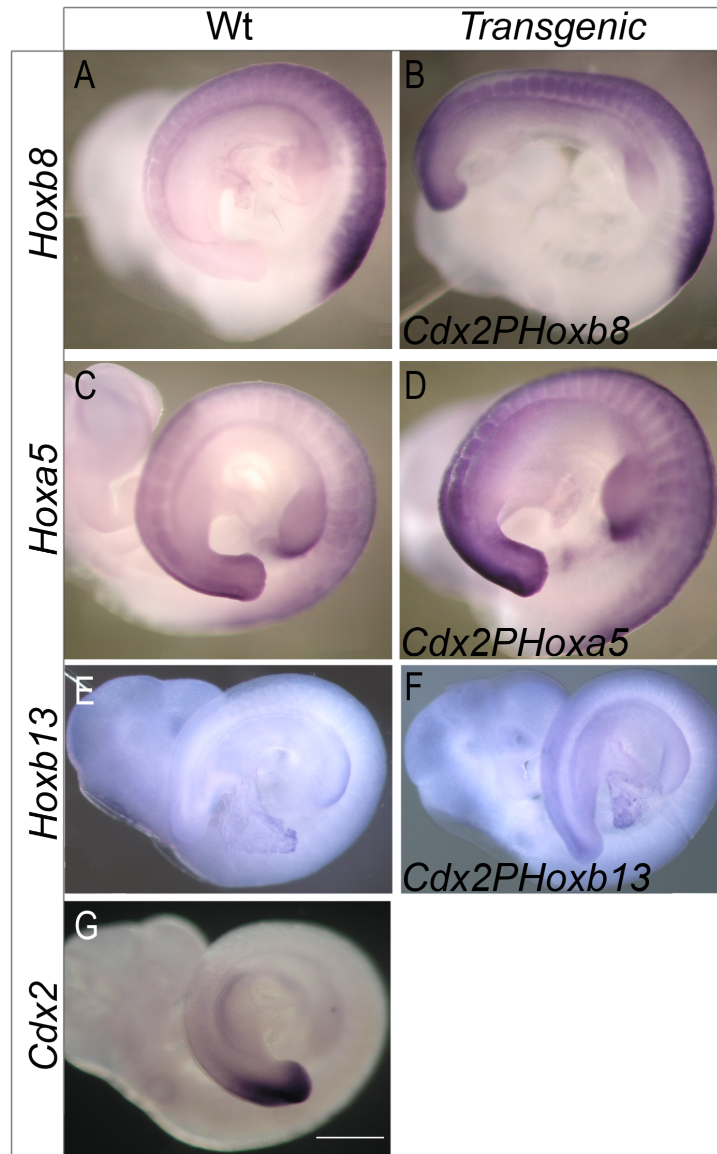

Figure 3.5. **Expression of *Hoxb8*, *Hoxa5* and *Hoxb13* in *Hox* expressing transgenic embryos. (A-F) Expression of *Hoxb8*, *Hoxa5* and *Hoxb13* in wildtype (A,C,E) and *Hox*-expressing transgenic embryos (B,D,F) at E9.5. Shown here are embryos from the one among the transgenic lines made for each construct, which has been used the most in the functional experiments. (G) Expression of *Cdx2* in an E9.5 wildtype embryo. Anterior is to the left.**

expression pattern of the gene in the posterior structures (Benahmed *et al.*, 2008). Embryos from the transgenic lines expressed the *Hox* transgenes in a domain and at a level similar to endogenous *Cdx2*, in addition to their endogenous expression (Figure 3.5.A-D and G). Newborn *Cdx2PHoxa5* and *Cdx2PHoxb8* transgenic mice in the wildtype background exhibited no axial extension abnormalities (data not shown). In the *Cdx2*$^{+/-}$/*Cdx4*$^{-/-}$ mutant background, gain of *Hoxb8* expression from the transgenes compensated for the loss of *Cdx* alleles and significantly restored the axial skeleton morphology of the trunk and tail (Figure 3.6.A-K and Table 3.2). The placental deficiency which causes in utero lethality of many *Cdx2*$^{+/-}$/*Cdx4*$^{-/-}$ mutants (van Nes *et al.*, 2006) was also rescued by the *Hoxb8* transgene. This was indicated by a significantly higher number of newborns with the *Cdx2*$^{+/-}$/*Cdx4*$^{-/-}$ mutant genotype that carry the *Hoxb8* transgene, compared with *Cdx2*$^{+/-}$/*Cdx4*$^{-/-}$ mutants (Table 3.3), and by histological analysis of the placental labyrinth of the *Hoxb8* transgenic *Cdx2*$^{+/-}$/*Cdx4*$^{-/-}$ mutants at E10.5 (not shown). Analysis of mutant and rescued animals at postnatal day 2 (P2) showed that the uro-rectal function is restored as well in most cases (Figure 3.6.F versus 3.6.E, and data not shown; Table 3.3). Besides *Hoxb8*, *Hoxa5* was also able to rescue the posterior truncation of *Cdx2*$^{+/-}$/*Cdx4*$^{-/-}$ mutants, although to a lesser extent than *Hoxb8* (Figure 3.7 and Table 3.2). Thus at least two trunk *Hox* genes (members of different paralogy groups) can partly compensate for *Cdx* deficiency in posterior axial elongation.

These data reveal the capacity of *Hox* genes to correct *Cdx* deficiency in the generation and expansion of posterior axial tissues. The *Hox* transgenes did not act by upregulating the intact *Cdx2* allele in the *Cdx2*$^{+/-}$/*Cdx4*$^{-/-}$ mutants (Figure 3.4.E-G and data not shown). "Central" *Hox* genes might rescue the posterior truncation seen in *Cdx2*$^{+/-}$/ *Cdx4*$^{-/-}$ mutants by virtue of their acting downstream of *Cdx* genes. A *lacZ* reporter driven by a promoter containing a series of functional Cdx binding sites (Charite *et al.*, 1998) indeed did not respond to the rescuing *Hoxb8* transgene in *Cdx2*$^{+/-}$/ *Cdx4*$^{-/-}$ mutant embryos (data not shown). *Cdx* gene products thus must act hierarchically higher than the trunk *Hox* genes in body axis elongation.

This work thus provides evidence that *Hox* genes have the capacity to control both the generation of well-organized new axial tissue in the three germ layers, as well as providing it with A-P positional instruction. The rescue of *Cdx2*$^{+/-}$/*Cdx4*$^{-/-}$ axial truncations by the *Hoxb8* or *Hoxa5* transgenes is not complete, but considering the extent of improvements brought about by a single member of the 39 *Hox* gene family members, expressed in a physiological range (Figure 3.5), the case for the *Hox* genes as regulators of posterior axial tissue expansion is quite compelling.

3.3.4. Paralogous 13 *Hox* genes prematurely arrest axial extension when precociously expressed in wildtype embryos

Hoxb13 (Zeltser *et al.*, 1996) is unique among the 39 mouse *Hox* genes in that its loss of function results in a phenotype with a longer axis (Economides *et al.*, 2003), suggesting that this latter *Hox* gene functions differently from central *Hoxa5* and *Hoxb8* during axial extension. To look further into this difference, we wanted to compare the effect of *Hoxb13* and other paralogy group (PG) 13 *Hox* genes with the effect of "central" *Hox* genes on the *Cdx2*$^{+/-}$/*Cdx4*$^{-/-}$ truncated mutants, in conditions

Animals at P2:	WT							*Cdx2*+/- *Cdx4*-/-							Rescued by *Hoxb8*							Rescued by *Hoxa5*							*Hoxc13 tg*			*Hoxb13 tg**			
	1	2	3	4	5	6	7	M1	M2	M3	M4	M5	M6	M7	R1	R2	R3	R4	R5	R6	R7	R8	R9	R10	R11	R12	R13	R14	D	C1	C2	C3	C4	C5	C6
Normal cervical vertebra 1 (Atlas)	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Normal cervical vertebra 2 (Axis)	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
First vertebra with ribs: V8	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	8/9	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
First vertebra with ribs attached to the sternum: V8	X	X	X	X	X	X	X	X	X	X	X	X	X	X								X	X	X	X	X	X	X	X	X	X	X	X	X	X
V8+V9 unilaterally																	X			X															
V9 bilaterally															X	X		X	X		X														
Most caudal V with sternal ribs: V14	X	X	X	X	X	X	X																									X	X	X	X
V13																													X	X	X				
V15								X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X							
Most caudal vertebra carrying ribs: V20	X	X	X	X	X	X	X	X						X	X	X			X	X	X	X	X	X	X	X	X	X	X						
V19																														X	X	X	X	X	X
V21 partial ribs										X		X						X																	
V21 complete ribs									X		X		X				X																		
Total rib number left	13	13	13	13	13	13	13	13	14	14	14	13	14	13	13	13	14	14	12	13	13	13	13	13	13	13	13	13	13	12	12	12	12	12	12
Total rib number right	13	13	13	13	13	13	13	13	14	14	14	14	14	13	13	13	14	13	13	13	13	13	13	13	13	13	13	13	13	12	12	12	12	12	12
Rib fusions																																			
first and second ribs								X	X	X	X	X	X	X			X			X			X		X	X		X							
ninth and tenth ribs																															X				
eleventh and twelfth ribs																															X				
Fusion of lumbar vertebrae								X	X	X	X	X	X	X	X							X	X	X	X	X					X				
Number of lumbar vertebrae	6	6	6	6	6	6	6	5	6	6	6	6	6	7	6	6	6	5	6	6	6	6	7	7	7	6	6	6	3	4	5	5	5	5	5
First sacral vertebra V27	X	X	X	X	X	X	X								X	X		X	X	X	X	X				X	X	X							
V25																																X	X	X	X
V28									X	X	X	X	X	X			X						X	X	X										
Number of sacral vertebrae	4	4	4	4	4	4	4	4	4	4	5	5	5	4	6	6	6	5	6	6	6	6	5	5	5	5	4	6	0	**	**	5	5	5	5
Number of caudal vertebrae	28	28	28	27	25	27	27	0	2	4	7	8	6	5	12	13	20	15	15	16	17	9	7	12	4	14	9	10	0	f	f	9	9	9	9
Total number of visible vertebrae	58	58	58	57	55	57	57	29	33	35	39	40	38	36	46	47	53	45	49	50	51	43	39	44	36	45	39	42	23	23	24	38	38	38	38

Footnote: *: Cdx4-/- background; **: abnormal sacral vertebrae; f:fused

Table 3.2. **Vertebral phenotype of wildtype, mutants, *Hox*-rescued mutants and *Hox13* transgenic newborns**

	Cdx2$^{+/-}$ X *Cdx4*$^{-/-}$ mutant crosses	*Cdx2*$^{+/-}$ X *Cdx4*$^{-/-}$ crosses with *Hoxb8*
Number of *Cdx2*$^{+/-}$/*Cdx4*$^{-/-}$ recovered	13/30	17/15
Cdx2$^{+/-}$/*Cdx4*$^{-/-}$ with very short/no tail	13/13	0/17
Cdx2$^{+/-}$/*Cdx4*$^{-/-}$ with dilated bladder and no anus	13/13	2/17
Cdx2$^{+/-}$/*Cdx4*$^{-/-}$ with normal bladder and anus	0/13	15/17
Cdx2$^{+/-}$/*Cdx4*$^{+/-}$ with very short/no tail	7/23	0/14
Cdx2$^{+/-}$/*Cdx4*$^{+/-}$ with dilated bladder	7/23	0/14
Cdx2$^{+/-}$/*Cdx4*$^{+/-}$ with fully normal phenotype	16/23	14/14

Table 3.3. **Skeletal and uro-rectal defects of *Cdx2*$^{+/-}$/*Cdx4*$^{-/-}$ mutants and *Cdx2*$^{+/-}$/*Cdx4*$^{+/-}$ with or without *Hoxb8* transgene at P2**

Descriptive statistics: number of PGCs

	N	Min.	Max.	Mean	St. Error	St. Deviation
Wildtype	7	29	32	30.71	0.52	1.38
Cdx2$^{+/-}$/*Cdx4*$^{-/-}$ mutant	7	4	13	8.86	1.18	3.13
Cdx2$^{+/-}$/*Cdx4*$^{-/-}$ mutant *Hoxb8* resc	7	18	26	21.43	0.99	2.64
Cdx2$^{+/-}$/*Cdx4*$^{-/-}$ mutant *Hoxa5* resc	7	9	19	14.43	1.27	3.36

Kolmogorov Smirnov test

	Z-value
Wildtype	0.78
Cdx2$^{+/-}$/*Cdx4*$^{-/-}$ mutant	0.48
Cdx2$^{+/-}$/*Cdx4*$^{-/-}$ mutant *Hoxb8* resc	0.40
Cdx2$^{+/-}$/*Cdx4*$^{-/-}$ mutant *Hoxa5* resc	0.37

Mann-Whitney test

	p-value
Cdx2$^{+/-}$/*Cdx4*$^{-/-}$ mutant vs *Cdx2*$^{+/-}$/*Cdx4*$^{-/-}$ mutant *Hoxb8* resc	0.001
Cdx2$^{+/-}$/*Cdx4*$^{-/-}$ mutant vs *Cdx2*$^{+/-}$/*Cdx4*$^{-/-}$ mutant *Hoxa5* resc	0.011
Cdx2$^{+/-}$/*Cdx4*$^{-/-}$ mutant *Hoxb8* resc vs *Cdx2*$^{+/-}$/*Cdx4*$^{-/-}$ mutant *Hoxa5* resc	0.001

Table 3.4. **Statistical analysis of vertebral phenotypes**

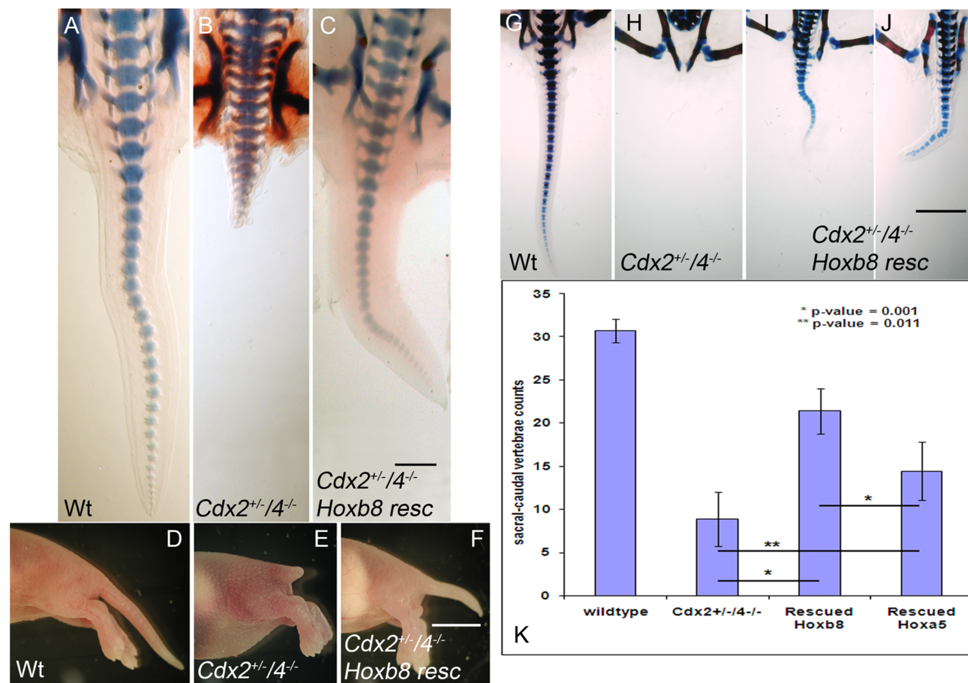

Figure 3.6. **Rescue of posterior axial extension and of uro-rectal defective function in *Cdx* mutant embryos and newborns expressing transgenic *Hoxb8*. (A-C) Skeletal preparation of the sacro-caudal region of a wildtype (A), *Cdx2*$^{+/-}$/*Cdx4*$^{-/-}$ (B), and *Cdx2*$^{+/-}$/*Cdx4*$^{-/-}$ carrying the *Hoxb8* expressing transgene (C). (D-F) External lateral view of the posterior body of a P2 wildtype (D), a *Cdx2*$^{+/-}$/*Cdx4*$^{-/-}$ mutant (E), and a *Cdx2*$^{+/-}$/*Cdx4*$^{-/-}$ mutant carrying the *Hoxb8* transgene (F); note the dilated bladder/gut visible on the ventral side of the mutant pup in (E), but not in (D) and (F); also note the longer tail of the rescued mutant in (F) versus (E). (G-J) Skeletal preparation of the sacro-caudal region of a P2 wildtype (G), *Cdx2*$^{+/-}$/*Cdx4*$^{-/-}$ mutant (H), and 2 different *Cdx2*$^{+/-}$/*Cdx4*$^{-/-}$ mutants carrying the *Hoxb8* transgene (I and J). (K) Graph showing the posterior vertebral counts at P2 and statistical significance of the *Hoxb8* and *Hoxa5* rescue of the *Cdx2*$^{+/-}$/*Cdx4*$^{-/-}$ truncation phenotype. Anterior is to the top for (A-C) and (G-J), and to the left for (D-F). Bars in (K) represent the standard deviation. n=7 for each genotype. p values are indicated in the graph. Scale bars are 1 mm for (A-C), 5 mm for (D-F), and 2 mm for (G-J).**

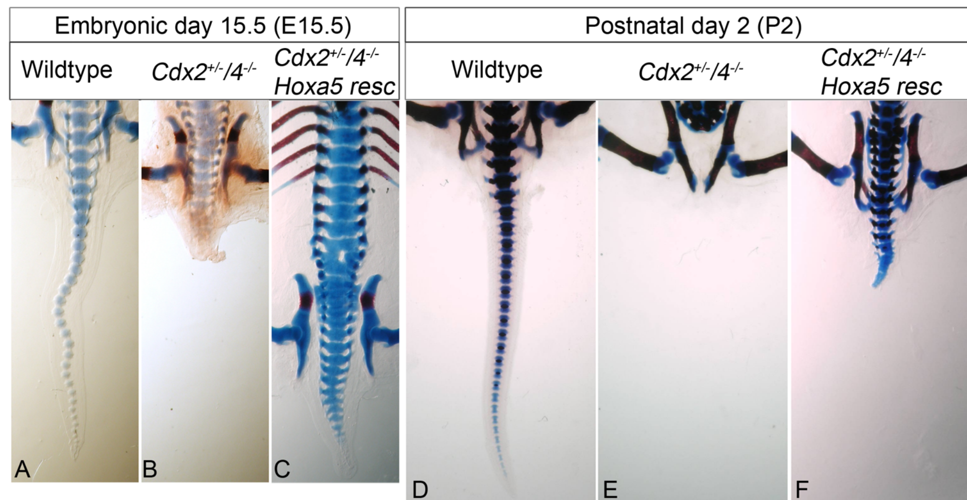

Figure 3.7. ***Hoxa5* driven by the *Cdx2* promoter rescues axial elongation of *Cdx2*$^{+/-}$/*Cdx4*$^{-/-}$ mutants. The axial skeleton of *Cdx2*$^{+/-}$/*Cdx4*$^{-/-}$ mutants rescued by the *Hoxa5* transgene exhibits significantly improved morphogenesis of the sacro-caudal vertebral column at E15.5 (A-C) and P2 (D-E).**

where they are expressed in the *Cdx2* spatio-temporal window. Loss of function mutations in *Hoxa13*, *Hoxc13* and *Hoxd13*, do not affect the elongation of the vertebral and neural axis (Dollé *et al*., 1993; Fromental-Ramain *et al*., 1996; Goodwin and Capecchi, 1998). PG 13 *Hox* genes are first expressed around E10.0-E10.5 in embryonic axial tissues that form caudally to the presumptive trunk/tail transition. This time point is at the end of the window of the *Cdx2*$^{+/-}$/*Cdx4*$^{-/-}$ mutant truncation phenotype (Figure 3.8). This means that PG13 *Hox* genes are normally not involved in axial extension of the trunk. We reasoned that they might be involved in slowing down axial extension after the trunk-tail transition.

We designed transgenic constructs precociously expressing a *Hoxa13* cDNA in the posterior growth zone of the embryo using the *Cdx2* (Benahmed *et al*., 2008) and the *T Brachyury* (Clements *et al*., 1996) promoters. No founder mouse expressing any of the transgenes survived to term. Unexpectedly, transgenic embryos at E10.5 were truncated in a way similar to the *Cdx2*$^{+/-}$/*Cdx4*$^{-/-}$ loss of function mutants (Figure 3.9). Transgenic mice expressing *Hoxc13* precociously from either the PSM-restricted *Dll1* promoter (Beckers *et al*., 2000), or from the posterior encompassing *Cdx2* promoter described above were generated but died at birth. Skeletal analysis of *Dll1PHoxc13* and *Cdx2PHoxc13* E18.5 fetuses revealed that they were missing most caudal vertebrae, had thinner, irregular and partially fused sacral vertebrae, and sometimes abnormalities at lumbar levels (Figure 3.9). Expression of a *Hoxb13* transgene from the *Cdx2* promoter was compatible with survival of the transgenic animals, and did not cause posterior truncations (not shown). It did however cause truncations in the presence of the *Cdx4* null mutation, which by itself does not affect axial extension. The *Cdx2PHoxb13* transgene thus acts similarly to the *Cdx*2 heterozygous

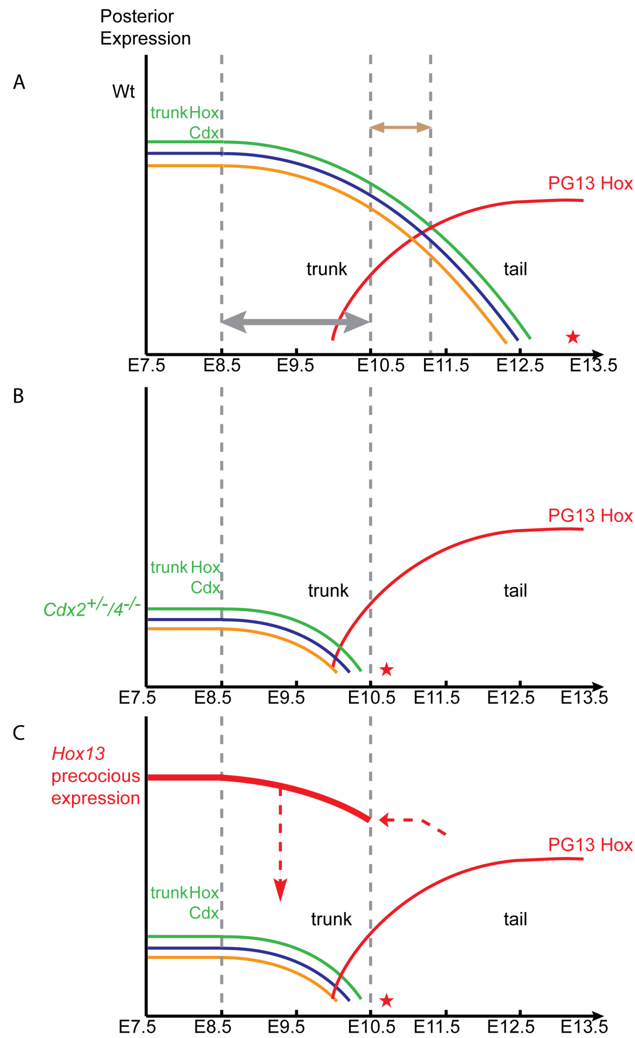

Figure 3.8. **Schematic model for the differential involvement of *Cdx/Hox* genes, and Wnt/RA signaling in posterior embryonic body axis elongation. (A) Dynamics of the expression of *Cdx* and trunk *Hox* genes (green) in the posterior part of growing embryos (developmental stages along the X axis) paralleled by the activity of canonical Wnt (blue) and *Cyp26a1* expression (orange). PG13 *Hox* genes are expressed with an opposite dynamics, starting around E10.0, shortly before the trunk-tail transition (brown double arrow). Red asterix indicates the end of axial extension by posterior addition of tissues. (B) Situation after the decrease in *Cdx* activity in *Cdx2*$^{+/-}$/*Cdx4*$^{-/-}$ mutants, leading to a decrease in Wnt activity and *Cyp26a1* expression in the trunk. (C) Situation after precocious expression of PG13 *Hox* genes, leading to a decrease in the function of *Cdx*/trunk *Hox* genes and in canonical Wnt activity and *Cyp26a1* expression in the trunk. Red asterixes in (B) and (C) indicate premature arrest of axial extension.**

loss of function allele when combined with the *Cdx4* null alleles (see scheme in Figure 3.2). The truncations of the *Cdx2PHoxb13* compound mutants in the *Cdx4* null background were less severe than the truncations of *Cdx2PHoxa13* and *Cdx2PHoxc13* transgenic animals, but the phenotypes of all these *Hox13* gain of function mice were reminiscent of the posterior defects of *Cdx2*$^{+/-}$/*Cdx4*$^{-/-}$ mutants. In addition, one of the *Hoxb13* transgenic founders, and the only *Hoxc13* founder that survived after birth, both succumbed after a few weeks and were found to exhibit hindgut and bladder abnormalities of the type discovered in *Cdx2*$^{+/-}$/*Cdx4*$^{-/-}$ mutants (not shown). Given this similarity between the phenotype of loss of *Cdx* versus precocious gain of function of PG13 *Hox* genes, we tested whether the expression of PG13 *Hox* gene is not precociously activated in *Cdx2*$^{+/-}$/*Cdx4*$^{-/-}$ mutants before and at the time they start manifesting an overt truncated phenotype. Like in wildtype at E9.5/E10.0, no expression of any PG13 gene was detected in *Cdx2*$^{+/-}$/*Cdx4*$^{-/-}$ mutants, revealing that the mechanism of action of *Cdx* gene products is not mediated by PG13 *Hox* genes (Figure 3.10.A-H).

We conclude that precocious expression of *Hox* genes of the last paralogy group interferes negatively with posterior extension of the embryonic axis with a phenotypic result similar to that of loss of function of *Cdx* genes. In agreement with this conclusion, we observed that *Cdx2PHoxb8* transgenic expression, shown above to rescue the *Cdx2*$^{+/-}$/*Cdx4*$^{-/-}$ mutant defects, also corrects the posterior truncation due to premature expression of *Hoxb13* (Figure 3.9.J).

We tested whether the transcription of *Cdx* genes was impaired by the early *Cdx2PHoxc13* gain of function in transgenic embryos. We observed that the extent of the expression domain of *Cdx2* was reduced at E9.5, but that the transcriptional level of the gene in the expressing tissue did not seem to be significantly reduced (Figure 3.10.I-J and data not shown). It is possible that precocious expression of the posterior PG13 *Hox* genes interferes with the action of *Cdx* and trunk *Hox* genes at a post-transcriptional level, a situation reminiscent of the "posterior prevalence" phenomenon described in mouse limb development, based on a mechanism of interference called "phenotypic suppression" in *Drosophila* (reviewed by Duboule and Morata, 1994).

3.3.5. *Cdx* and *Hox* transcription factors act upstream of Wnt signaling to maintain trunk axial elongation

Wnt signaling has been shown to act upstream of *Cdx* and *Hox* gene expression during early A-P patterning (Forlani *et al.*, 2003; Ikeya and Takada, 2001; Pilon *et al.*, 2006). In addition, *Wnt3a* has been reported to be essential for posterior axial elongation (Ikeya and Takada, 2001; Takada *et al.*, 1994; Yoshikawa *et al.*, 1997). The dynamics of expression of *Wnt3a* in posterior axial tissues follows that of *Cdx* and central *Hox* genes (Figure 3.1 and 3.11, and Figure 3.8). The end of axial elongation in wildtype embryos is preceded by the decrease of *Cdx*/central *Hox* transcription in posterior tissues from E10.5 on (Figures 3.1), and by the arrest of *Wnt3a* expression around E12.5 (Cambray and Wilson, 2007; Figure 3.11). *Cdx2*$^{+/-}$/*Cdx4*$^{-/-}$ loss of function is accompanied by a decrease in *Wnt3a* expression at E9.0 already, an earlier time point than in wildtype (Figure 3.12.A-D), anticipating an earlier end of axial

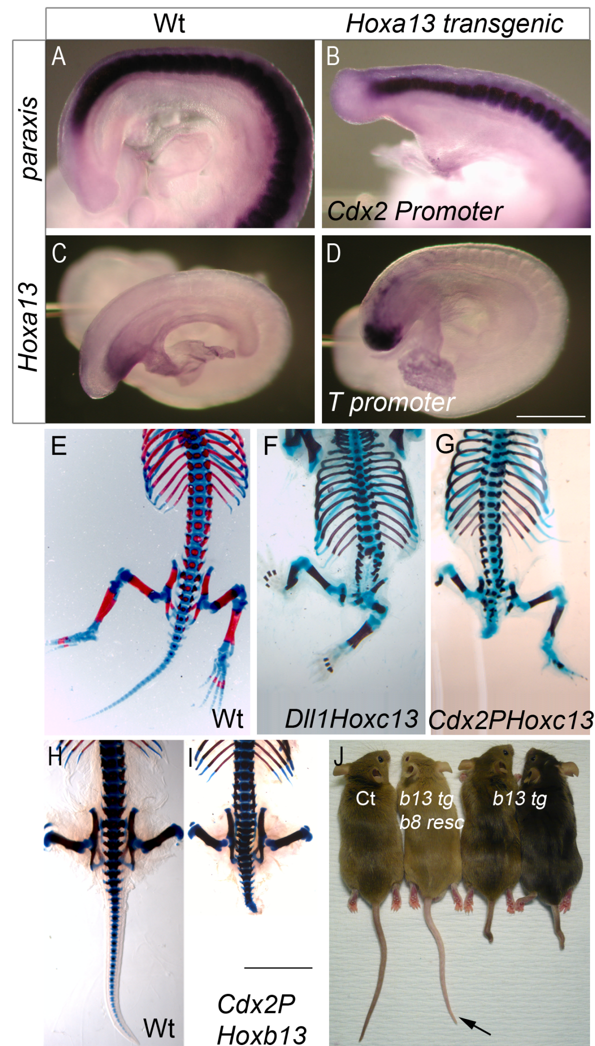

Figure 3.9. **Posterior truncation phenotype of embryos precociously expressing *Hox13* genes. (A-D) E9.5 embryos wildtype (A,C) or transgenic for *Cdx2PHoxa13* (B) or *TPHoxa13* (D), hybridized with a *paraxis* (A-B) or *Hoxa13* (C-D) probe. (E-G) Dorsal views of skeletal preparations of E18.5 wildtype (E), *Dll1PHoxc13* (F) and *Cdx2PHoxc13* (G). (H-I) Dorsal views of skeletal praparations of a P2 control and a *Cdx2PHoxb13* transgenic pup, both null for *Cdx4* (see text for explanations). (J) Dorsal photographs of adult mice, respectively (from right to left) transgenic for *Cdx2PHoxb13* (2 animals), double transgenic for *Cdx2PHoxc13* and *Cdx2PHoxb8*, and control. All four animals carry the *Cdx4* null mutation (see text). Note the virtually complete rescue of the *Hoxb13*-related posterior truncation by the *Hoxb8* transgene (arrow). Anterior is to the left for (A-D) and to the top for (E-J).**

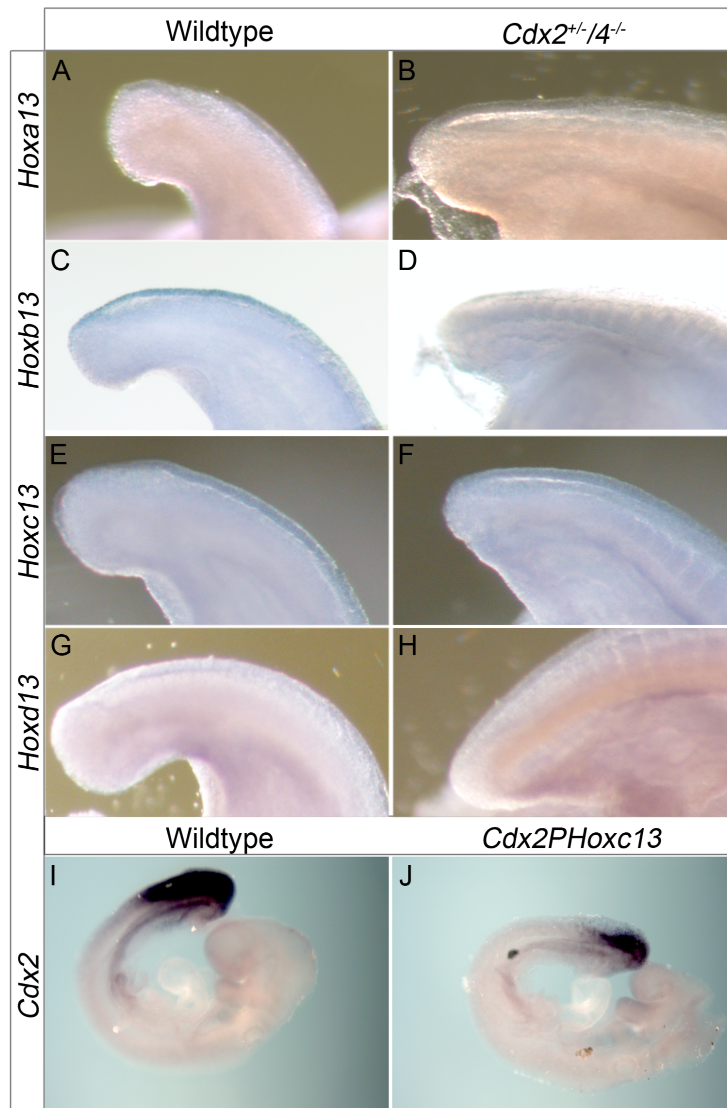

Figure 3.10. **(A-H) *Hox* 13 genes are not upregulated in the trunk and tail in *Cdx2*$^{+/-}$/*Cdx4*$^{-/-}$ mutants at E9.5/E10.0, which is the time point just before their first expression in axial tissues. Mutant and control embryos were hybridized with probes for *Hoxa13* (A-B), *Hoxb13* (C-D), *Hoxc13* (E-F), and *Hoxd13* (G-H). E11.5 and E12.5 embryos were hybridized in the same experiments and served as positive controls. (I-J) E9.5 *Cdx2PHoxc13* transgenic embryos show a decrease in the expression domain of *Cdx2* in the growth zone compared to that in wildtype. The expression level of *Cdx2*, the most critical *Cdx* gene for axial elongation, is still high in the expressing cells of the *Hoxc13* transgenic embryo.**

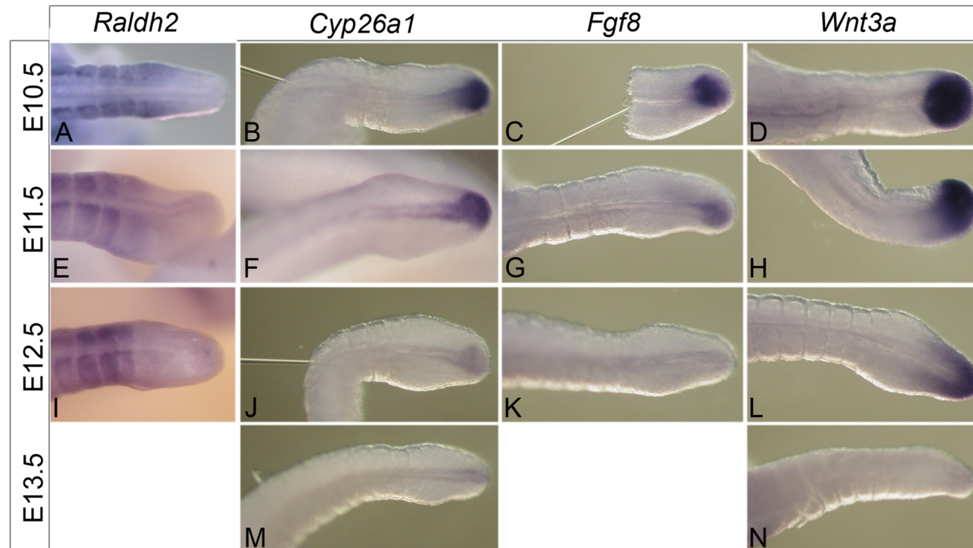

Figure 3.11. **Wildtype embryos at E10.5, E11.5, E12.5 and E13.5 were hybridized with probes for *Raldh2* (A,E,I), *Cyp26a1* (B,F,J,M), *Fgf8* (C,G,K), and *Wnt3a* (D,H,L,N). Only the tail tips are shown here.**

elongation around E10.5 in the mutants. Similarly, in line with the close resemblance between the phenotypes of *Cdx2*$^{+/-}$/*Cdx4*$^{-/-}$ loss of function mutants and mice prematurely expressing PG13 Hox genes, *Wnt3a* expression was lower in the posterior region of *Cdx2PHoxc13* embryos than in controls at E9.5 (Figure 3.12.E-F), and was lost at E10.5 (not shown).

To investigate whether the decrease in *Wnt3a* expression between the 6/7 somite stage and E9.5 was causally involved in the slowing down of axial extension in *Cdx2*$^{+/-}$/*Cdx4*$^{-/-}$ mutants, we asked whether a gain of canonical Wnt signaling reached by expressing an activated form of *Lef1* (Galceran *et al.*, 2001) would rescue the mutant truncation phenotype. The activated *Lef1* transgene did not affect embryonic growth and patterning in wildtype (data not shown on embryos from three *BraP-CatC-Lef1* transgenic lines). Embryos carrying the *Cdx2*$^{+/-}$/ *Cdx4*$^{-/-}$ mutations together with the activated *Lef1* construct were found to be largely rescued for their axial truncation defects, and cured for their uro-digestive pathology (n=4 out of 4) (Figure 3.13 and data not shown). *Cdx* is thus probably acting on axial extension by positively controlling Wnt signaling activity. Considering the fact that initial *Cdx* transcription is *Wnt*-dependent in the mouse embryo (Ikeya and Takada, 2001; Gaunt *et al.*, 2003; Pilon *et al.*, 2006; Prinos *et al.*, 2001), the molecular genetic control of posterior tissue addition appears to utilize a positive feed-back loop of *Cdx* gene products on Wnt signaling to maintain active axial extension. *Wnt3a* (Figure 3.14) and *Wnt* target genes (not shown) were also downregulated in *Cdx2PHoxc13* embryos.

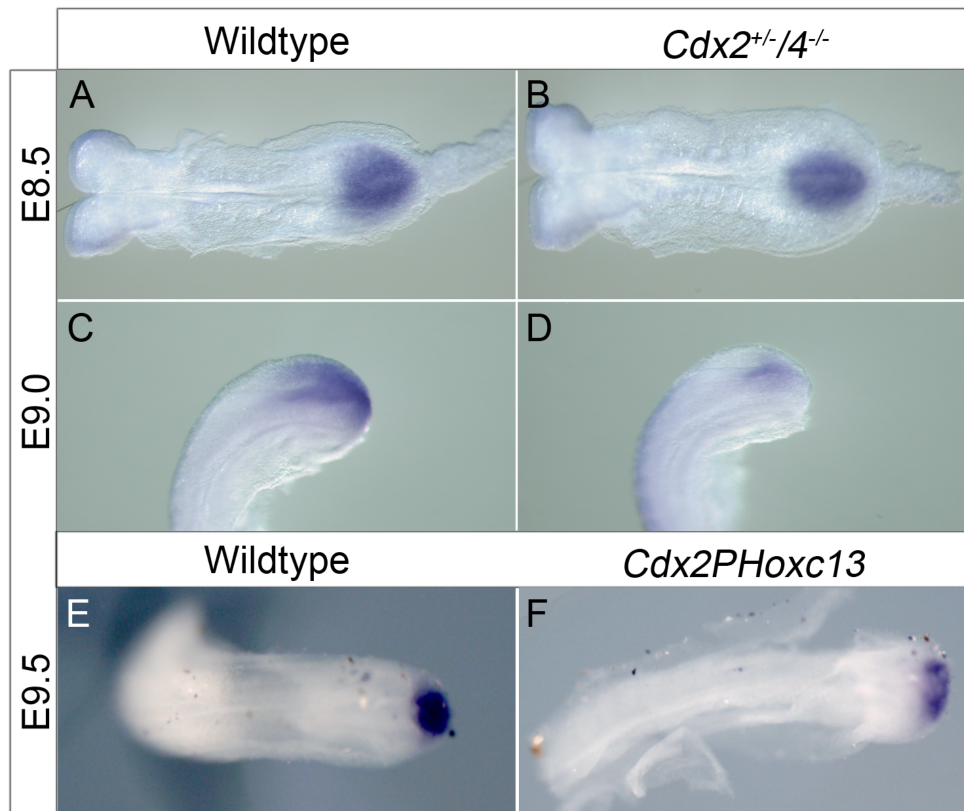

Figure 3.12. **(A-B) E8.5 (7-somite), and (C-D) E9.0 (14 somite) wildtype and *Cdx2*$^{+/-}$/*Cdx4*$^{-/-}$ mutant embryos were hybridized with a *Wnt3a* probe. (E-F) E9.5 wildtype and *Cdx2PHoxc13* transgenic embryos were hybridized with a *Wnt3a* probe.**

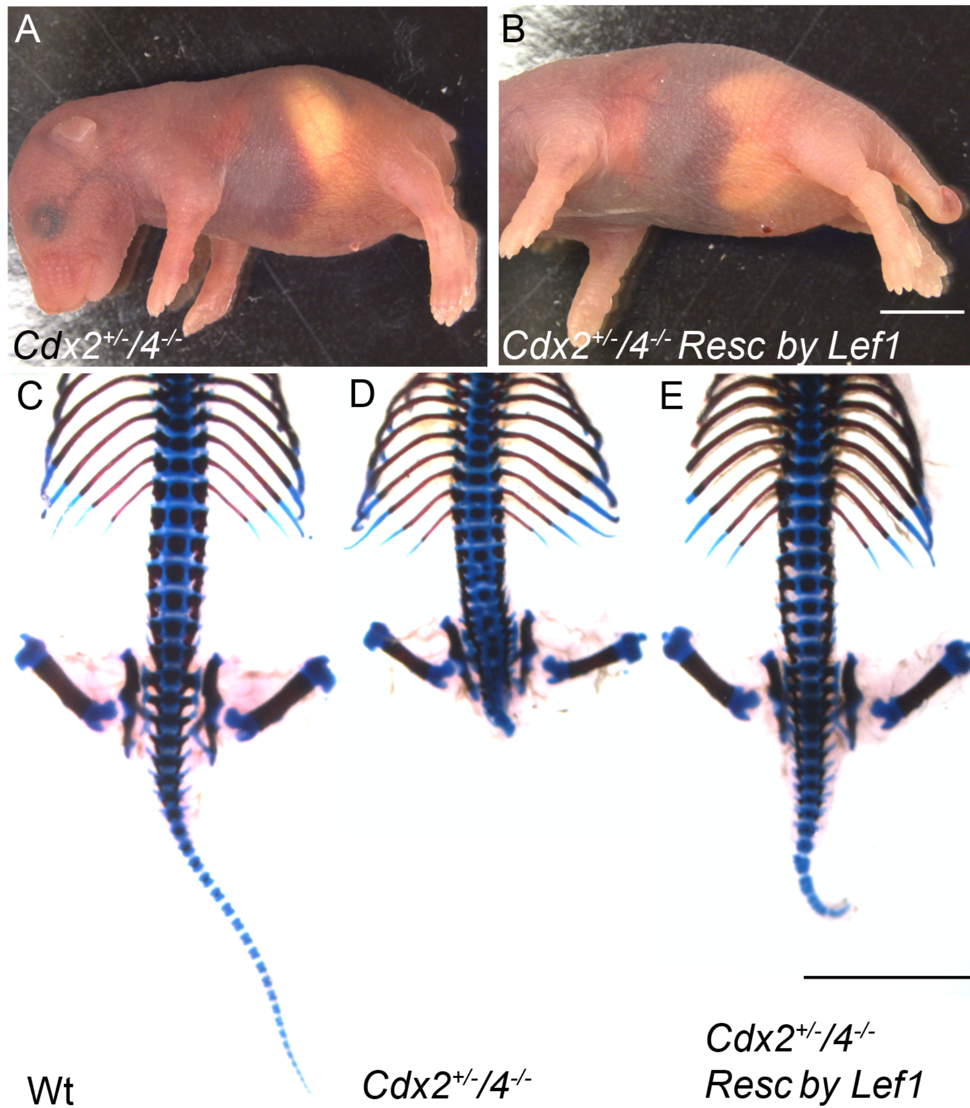

Figure 3.13. **Rescue of the posterior truncation phenotype of *Cdx2*$^{+/-}$/*Cdx4*$^{-/-}$ mutants by gain of function of the canonical Wnt pathway. Photographs (A-B) and skeletal preparations (D-E) of *Cdx2*$^{+/-}$/*Cdx4*$^{-/-}$ mutants (A,D) and *Cdx2*$^{+/-}$/*Cdx4*$^{-/-}$ mutants transgenic for the *BraP-CatC-Lef1* construct constitutively activating the canonical Wnt pathway (B,E). Anterior is to the left for (A-B) and to the top for (C-E).**

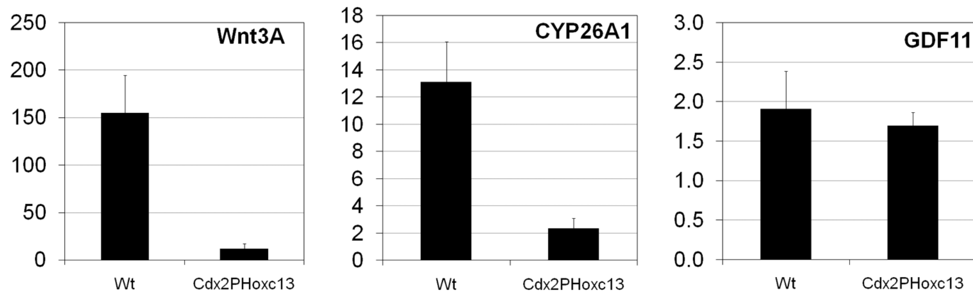

Figure 3.14. **Transcription of *Wnt3a*, *Cyp26a1* and *Gdf11* was quantitatively measured by real-time PCR in dissected posterior tissues of E10.5 wildtype embryos, and in embryos transgenic for *Cdx2PHoxc13*. *Gapdh* was used as endogenous control (see Materials and methods). Each measurement represents the outcome of duplicate PCR reactions on each of 4 RNA samples isolated from 4 individual embryos. Transcription of *Gdf11* is not significantly decreased (p=0.773) in the transgenics, whereas *Wnt3a* and *Cyp26a1* are significantly lower (p=0.021 in both cases).**

3.3.6. *Cdx* and *Hox* genes instruct posterior RA clearance by stimulating *Cyp26a1* expression in the posterior growth zone during trunk elongation

Transcription profiling experiments aimed at comparing gene expression in the posterior tissues of $Cdx2^{+/-}/Cdx4^{-/-}$ mutants and wildtype were performed at two embryonic stages, before (5-6 somite) and just after (13 somite) the decrease in PSM length became clearly detectable in the mutants. One of the genes found and confirmed to be downregulated (1.6 times and 2.3 times respectively) was the gene encoding the RA-degrading enzyme *Cyp26a1*. Confirmation of the expression decrease in mutants by quantitative PCR (not shown), and by in situ hybridization made it clear that the gene is expressed at a level lower than in wildtypes at the 5 to 15 somite stages (n=9, Figure 3.15) and later. Localization of RA using the *RARβ-lacZ* reporter transgene in $Cdx2^{+/-}/Cdx4^{-/-}$ mutant embryos indicated that RA was present closer to the posterior growth zone in the mutants at early somite stages (Figure 3.15). This zone comprises the progenitor populations for trunk tissues (Cambray and Wilson, 2002; Cambray and Wilson, 2007), and the newly emerged, still undifferentiated progenitors of ectoderm and mesoderm, that will undergo differentiation into neural tissue (Mathis *et al.*, 2001; Olivera-Martinez and Storey, 2007; both studies in chick embryos) and somitic mesoderm (Déqueant *et al.*, 2008; study in mouse embryos) after the axis extends further. An attempt to compensate for the impaired axial elongation of *Cdx* mutants by lowering RA biosynthesis through introduction of a null allele of *Raldh2* (Niederreither *et al.*, 2002) did not produce any significant rescue (not shown).

Strikingly, *Cdx2PHoxc13* transgenic embryos also manifest a decrease in the expression of *Cyp26a1* (Figure 3.14), in line with the hypothesis that failure to clear RA from the posterior embryonic structures contributes to the truncation phenotype in this case as well as in the *Cdx* loss of function mutants. Precocious expression of

PG13 *Hox* genes and decrease in *Cdx*/trunk *Hox* gene expression, which cause similar posterior truncation phenotypes, thus probably operate by the same mechanisms.

3.4. Discussion

3.4.1. *Cdx*, *Hox* and axial elongation in the mouse embryo

Cdx and central *Hox* genes are expressed during embryogenesis in the posterior progenitor areas delivering descendants to axial and paraxial structures of the trunk (Cambray and Wilson, 2007; Fanklin *et al*., 2008; Kinder *et al*., 1999; Tam *et al*., 2007; Tam and Beddington, 1987; Wilson and Beddington, 1996). The mutant phenotypes indicate a function of these genes in the maintenance of tissue generation from these progenitors. A role for *Cdx* genes in expanding the hematopoietic progenitors, located within the *Cdx* expressing domain was previously established in the mouse and zebrafish (Davidson and Zon, 2006; Ernst *et al*., 2004; Lengerke *et al*., 2008; Wang *et al*., 2008). Our present study shows that *Cdx* and *Hox* genes, as an integrated part of their function in A-P patterning differentially ensure the formation and expansion of the axial and paraxial mesoderm and neurectoderm emerging from progenitors in the primitive streak and its remnant in the tail bud.

Cdx mutations modulate axial elongation by affecting the production of posterior embryonic tissues. In wildtypes, termination of axial extension occurs after paraxial mesoderm has generated about 58 presumptive prevertebra. It is preceded by a period during which the developmental rate slows down, causing the PSM to gradually shrink until it is exhausted and no further somites form. In the mouse, this slowing down occurs at an axial level between the presumptive prevertebra 21 and 31, and roughly corresponds to the trunk-tail transition (Gomez *et al*., 2008). In the *Cdx2*$^{+/-}$/*Cdx4*$^{-/-}$ mutants, the earliest visible defect is the decrease in length of the PSM domain, first observable at the 7 to 9 somite-stage (presumptive lower cervical vertebrae), thus much earlier than in the normal situation (Figure 3.8). The decrease in PSM length in *Cdx* mutants becomes more severe with time, until axial extension terminates at about the sacral level shortly after E10.5 (Figure 3.8). Mutations in *Cdx* genes thus start to exhaust the presomitic mesoderm around the 7 somite stage, leading to precocious shortening of the PSM and ends with the precocious termination of axis elongation around E10.5 (around 30 instead of 58 vertebrae at birth). The rescue of these defects by a *Hox* transgene indicates that *Hox* genes participate in this axial elongation process as mediators or as collaborators of *Cdx*.

Anterior (PG1-4) *Hox* genes have not been examined in this study because their expression largely precedes in time, and is anterior in space relatively to the temporal and spatial impairment of the posterior growth in *Cdx* mutants. Anterior *Hox* genes pattern structures at hindbrain levels, that are unaffected by the *Cdx* mutations.

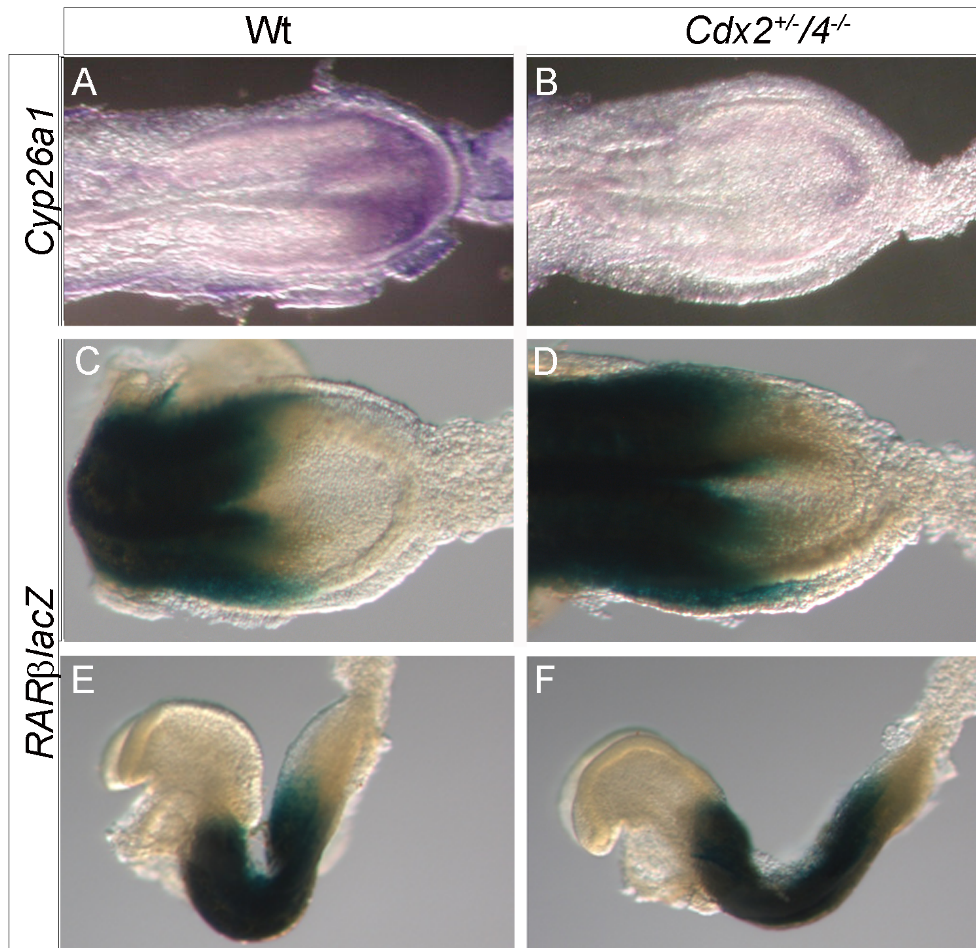

Figure 3.15. **(A-B) In situ hybridization of *Cyp26a1* transcripts in wildtype (A) and *Cdx2*$^{+/-}$/*Cdx4*$^{-/-}$ mutant (B) at E8.5 (7-somite). (C-F) Xgal staining of the *RaR*β*lacZ* RA reporter activity in wildtype (C,E) *and Cdx2*$^{+/-}$/*Cdx4*$^{-/-}$ mutants (D,F).**

3.4.2. A time-related change in the balance between trunk and posterior *Hox* genes instructs axis extension and caudal termination

Our data show that whereas trunk *Hox* gene products rescue the shortage in Cdx proteins, Hox proteins of paralogy group 13 have an opposite effect when expressed at similar levels and in the same spatio-temporal window. PG13 *Hox* genes expressed from the *Cdx2* promoter cause early axial truncations, in a way mimicking *Cdx* loss of function. Our data can be reconciled with a model according to which *Cdx* genes and trunk *Hox* genes would stimulate posterior elongation of tissues by sustaining posterior growth signaling required for the maintenance of progenitor activity. This would take place until the most posterior *Hox* gene products accumulate after E10.5, and dominantly compete with trunk Hox proteins, thus arresting axial extension. Sequential expression of the central versus posterior *Hox* genes would thus successively instruct a phase of tissue growth during the generation of the trunk, and a phase of slowing down (trunk-tail transition period indicated in Figure 3.8) leading to the arrest of tissue addition at the end of body axis extension. This mechanism, differentially involving the central and posterior *Hox* genes, would intimately couple posterior elongation of the embryonic body axis with patterning of the emerging tissues by the *Hox* combination expressed in the growth zone at that moment.

The biphasic action of *Cdx*/trunk *Hox* and posterior *Hox* genes would also differentially impact on the clearance of retinoic acid (RA), essential for posterior axial elongation (Abu-Abed *et al*., 2001; Sakai *et al*., 2001). RA is generated by *Raldh2* in the somites, and causes differentiation of nascent tissue arisen from the posterior progenitors (Olivera-Martinez and Storey, 2007 in the chick). RA clearance is crucial in the posterior growth zone and is carried out by the RA degrading enzyme *Cyp26a1*. Expression of *Cyp26a1* follows the dynamics of *Cdx2* expression, and is downregulated in the late phase of axial elongation (Figures 3.8 and 3.11). Transcription of *Cyp26a1* in posterior embryonic tissues of E8.5 $Cdx2^{+/-}/Cdx4^{-/-}$ mutant embryos was found to be lower than in wildtypes, and a similar situation was observed in embryos precociously expressing *Hoxc13*. The decrease in Cyp26a1 activity in these situations may contribute to a deficit of proliferation versus differentiation in the growth zone. It will be interesting to find out whether the effect of the Cdx or PG13 Hox proteins on *Cyp26a1* transcription is a direct effect.

3.4.3. A mode of action of *Cdx/Hox* genes via a timed positive feedback loop on Wnt signaling during trunk elongation

Remarkably, *Hox* genes can drive and orchestrate the complex process of posterior expansion of well-organized embryonic tissues during axial morphogenesis.

It is known that Wnt signaling regulates axial elongation in mice (Ikeya and Takada, 2001; Takada *et al*., 1994) and zebrafish (Shimizu *et al*., 2005). Early *Cdx* expression is controlled by Wnt (Ikeya and Takada, 2001; Gaunt *et al*., 2003; Pilon *et al*., 2006; Shimizu *et al*., 2005) and Fgf (Bel-Vialar *et al*., 2002; Pownall *et al*., 1996) in mice and amphibians. Chick *Cdx* genes have been shown to be under Fgf control, and to mediate this control on *Hox* genes (Bel-Vialar *et al*., 2002). It is possible that Wnt control of *Hox* genes (Forlani *et al*., 2003) is also *Cdx*-mediated. Given our findings that gain of function of *Lef1* in the posterior embryonic tissues rescues the *Cdx*

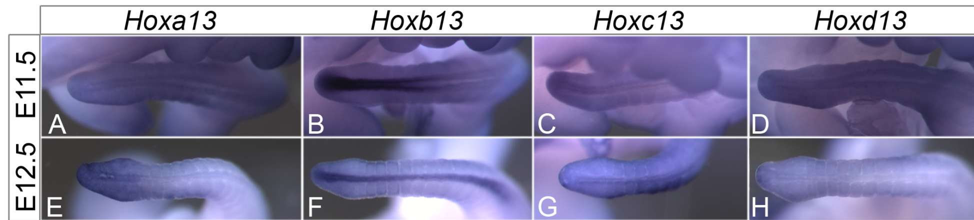

Figure 3.16. **Embryos were hybridized with probes for *Hoxa13* (A, E), *Hoxb13* (B, F), *Hoxc13* (C,G) and *Hoxd13* (D, H). Expression in axial tissues was not detected at E9.5 or E10.0 (see Figure 3.10.A-H) but was clear at E 11.5 and 12.5.**

truncation phenotype, we propose that, in turn, *Cdx* and central *Hox* genes exert a positive feedback loop on Wnt signaling during body axis elongation. Maintained Wnt signaling would sustain progenitor self-renewing and tissue elongation until "arresting *Hox*" genes intervene to slow down and stop this stimulation, after all axial structures have been laid down. The expression dynamics of *Wnt3a* indeed mimicks *Cdx*/central *Hox* gene expression and drops after posterior *Hox* genes are highly expressed (Figures 3.8, 3.16, and 3.11). From data base sequence search we know that there are several potential Cdx binding sites in the 5′ flanking sequences of both *Wnt3a* and *Lef1*, and future work will tell whether binding really takes place and mediates transcriptional control.

Wnt signaling maintenance during axial elongation by *Cdx*/central *Hox* genes is reminiscent of the recently elucidated relationship between *T Brachyury*, also known to be required for axial extension, and Wnt3a signaling in the zebrafish (Martin and Kimelman, 2008). Like *Cdx*, *T* is initially transcriptionally stimulated by *Wnt3a* (see also Yamaguchi *et al*., 1999), but *T* exerts a positive feed-back control on canonical Wnt signaling at later stages, that is absolutely required for maintening axial extension. It will be interesting to discover the relationship between *T* and *Cdx*/*Hox* genes that has remained elusive so far, since the expression of *Cdx* genes in early *T* mutants, and the expression of *T* in early *Cdx* mutants were found to be unaffected (S. Forlani and JD, unpublished).

In conclusion, *Hox* genes would control posterior axial elongation by differentially regulating maintenance of canonical Wnt signaling in the posterior growth zone. They exert this function in a globally collinear way, trunk *Hox* genes stimulating the elongation process via a positive regulation of Wnt signaling, whereas posterior *Hox* genes would compete and arrest this process. Given the evolutionary conservation of both *Hox* and *Cdx* genes, and the conserved involvement of *Cdx* genes in axial extension in species that extend their body axis by posterior addition of new tissues (Copf *et al*., 2004; Shimnyo *et al*., 2005), it is possible that the growth stimulation property of *Hox* genes in axis extension is ancient in origin.

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Chapter 4

Cdx deficiency and the Caudal Regression Syndrome

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Abstract

The Caudal Regression Syndrome encompasses a range of congenital defects of varying severity centred around sacral dysplasia and ano-rectal stenosis. It is likely that numerous pathological processes may result in the development of the condition involving both genetic and environmental factors such as maternal diabetes. Using gene inactivation by homologous recombination in a mouse model, we have defined a specific group of anomalies that fall within the umbrella of the Caudal Regression Syndrome and are the result of decreased expression of the *Cdx* group of *ParaHox* genes in the posterior region of the primitive streak and the tailbud of the developing embryo. The resultant deficit in the paraxial pre-somitic mesoderm and posterior endoderm results in sacro-coccygeal dysplasia and recto-urinary atresia. The consistency of the findings together with the absence of additional systemic malformations distinguishes this phenotype as a distinct group with a precise genetic aetiology.

4.1. Introduction

The *Drosophila* gene *caudal* (*cad*) (Mlodzik *et al*., 1985) has three mammalian homologues termed *CDX1*, *CDX2* and *CDX4* in the human and *Cdx1*, *Cdx2* and *Cdx4* in mice. *Cdx4*/*CDX4* is *X*-linked in mice and in humans. All three are expressed in embryos at the primitive streak stage of development. In the mouse, *Cdx1* transcripts appear at E7.2 in the posterior part of the streak, extending posteriorly to the base of the allantois; *Cdx2* has a more posterior but overlapping region of expression extending into the allantoic mesoderm while *Cdx4* expression is restricted to the most posterior part of the streak extending to the tip 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. All three genes are also expressed in the developing hindgut endoderm but only *Cdx1* and *Cdx2* expression persist into late gestation and postnatally (Beck, 2002). Comprehensive information concerning *CDX* expression in humans is not available but reported interspecific expression patterns throughout vertebrates makes it likely that they correspond with the murine observations.

Cdx1 and *Cdx2* are both concerned with antero-posterior patterning of the embryonic axis. *Cdx1*$^{-/-}$ mice exhibit anterior homeotic shifts involving the upper cervical vertebrae (Subramanian *et al*., 1995), while *Cdx2*$^{+/-}$ animals manifest similar homeotic effects situated more posteriorly in the lower cervical and upper thoracic regions (Chawengsaksophak *et al*., 1997). *Cdx2*$^{-/-}$ animals do not undergo uterine implantation and have therefore not been examined in this context (Strumpf *et al*., 2005). In *Drosophila cad* has been found to be the homeotic gene specifying 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 several insect and arthropod species that extend their body axis by posterior addition of tissues (Copf *et al*., 2004), a mode that is not used by long germ band insects like the fruit fly. The mammalian version of this effect is shown by the mice *Cdx2*$^{-/-}$ embryos that have been enabled to implant by fusion with tetraploid embryos (Chawengsaksophak *et*

al., 2004). These embryos survive to E10.5 but die because they lack the posterior extraembryonic mesoderm formed during gastrulation. The allantoic bud fails to grow properly so that the chorio-allantoic placenta does not develop. From the fifth somite onwards there is irregular segmentation and the embryo is severely truncated posteriorly with little development posterior to somite 17.

We have inactivated *Cdx4* by homologous recombination and reported (van Nes *et al.*, 2006) a minimal phenotype involving only a mild anterior transformation at the level of vertebra 15 with a very low penetrance and no other abnormality. However combined mutation producing $Cdx2^{+/-}/Cdx4^{-/0}$ male or $Cdx2^{+/-}/Cdx4^{-/-}$ female embryos causes high prenatal mortality due to loss of posterior mesoderm resulting in inadequate vascularisation of the placental labyrinth (van Nes *et al.*, 2006). These defects are only partially penetrant. We now report on the compound mutants that survive to birth. These show a constant and characteristic phenotype.

The caudal regression syndrome 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 reflected in sensory and motor deficit involving the lower limbs and abnormalities of cloacal derivatives. The latter include recto-anal atresia, recto-urinary or recto-vaginal fistulae and abnormalities of the bladder outflow tract. Interestingly, cases of caudal regression are often associated with maternal diabetes (Passarge and Lenz, 1966), which in turn is frequently connected with placental insufficiency (Desoye and Shafrir, 1996). There are also rare examples in which a familial element appears to be operative. Our observations highlight the role of *Cdx2* and *Cdx4* in the development of structures at the posterior end of the developing axis and in associated cloacal derivatives. We present an analysis of the defects of Cdx mouse mutants affecting posterior structures with a particular focus on the cloacal derivatives. Our observations reveal a phenotype in $Cdx2^{+/-}/Cdx4^{null}$ mutants that corresponds with a group of human congenital anomalies included in the broad spectrum termed the Caudal Regression Syndrome.

4.2. Materials and methods

4.2.1. Mice

Cdx2 heterozygous and *Cdx4* null mutant mice as well as the protocols to genotype them have been described previously (Chawengsaksophak *et al.*, 1997; van Nes *et al.*, 2006). $Cdx2^{+/-}/Cdx4^{-/0}$ and $Cdx2^{+/-}/Cdx4^{+/-}$ embryos and pups were generated by crossing $Cdx2^{+/-}$ and $Cdx4^{-/-}$ mice. $Cdx2^{+/-}/Cdx4^{-/-}$ embryos and pups were generated by crossing $Cdx2^{+/-}/Cdx4^{+/-}$ females with $Cdx4^{-/0}$ males. Animals were analyzed at embryonic stages E15.5, E18.5, at birth (P0), and two days after birth (P2). Mice were treated according to the "Law on animals in experiments", under the licences required in the Netherlands.

4.2.2. Cartilage and bone staining

Protocols for alizarin red and alcian blue staining for bone and cartilage, respectively, have been described previously (van den Akker *et al*., 2002).

4.2.3. Histology

Tissues were fixed with paraformaldehyde (4%) overnight at 4°C and embedded in paraffin. Hematoxylin and eosin staining of 10µm sections has been described previously (Chawengsaksophak *et al*., 2004).

4.2.4. Photographs

Mouse embryos, pups and skeletons were photographed on an Olympus SZX9 stereomicroscope using a 6.25/0.5X objective lens and a Leica DFC 420C camera.

4.2.5. 3D reconstruction

Haematoxylin and eosin stained sections were photographed on a Leica DM2500 microscope using a 2.5X/0.07 objective and a Leica DFC290 camera. Images were resized, cropped and changed to 8 bit grey scale. The spinal cord and bladder were coloured using ImageJ (http://rsb.info.nih.gov/ij/). The colouring was used to align sections for 3D reconstruction using the ImageJ plugin StackReg (Thevenaz *et al*., 1998). Aligned stacks were imported in Imaris (Bitplane, Switzerland) software to view the 3D reconstruction.

4.3. Results

Cdx2$^{+/-}$/*Cdx4*$^{-/0}$ male mice that survived to term were growth retarded with stumpy or absent tails. The anal opening was absent in all animals and there was no evidence of an anal pit (Figure 4.1). The abdomen was often distended and on transillumination this was seen to be due to an enlarged fluid filled bladder (Figure 4.2). There was no evidence of defaecation and post-natal survival was not possible; however, the animals were able to move and breathe normally and there was no cardiovascular distress. On serial sectioning the bladder was dilated and thin walled. The outflow tract was patent but appeared somewhat distorted. 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 specimens examined (Figure 4.3.A). The region at which the gut terminated was variable. In some specimens there was no evidence of stratified squamous epithelium characteristic of the proctodeum while in others the transition from the simple columnar epithelium of the rectum to a stratified squamous variety was clearly seen (Figure 4.3.B).

We also studied serial sections of *Cdx2*$^{+/-}$/*Cdx4*$^{-/0}$ embryos at E15.5. These exhibited similar features to those described for full term specimens (Figure 4.4) with the exception that the urinary outflow tract which at this stage of development normally opens to the exterior by a breakdown of the urogenital membrane (Figure

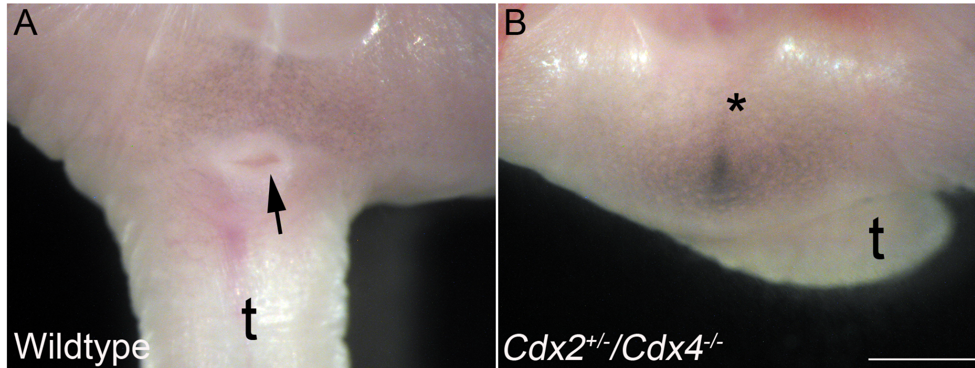

Figure 4.1. (A) Ventral view of wildtype newborn depicted in Figure 4.2.A.i., showing the anus (arrow in A). (B) Ventral view of *Cdx2*$^{+/-}$/*Cdx4*$^{-/-}$ mutant shown in Figure 4.2.A.iii, with absence of the anus (asterisk in B). Bar = 1mm; t = tail.

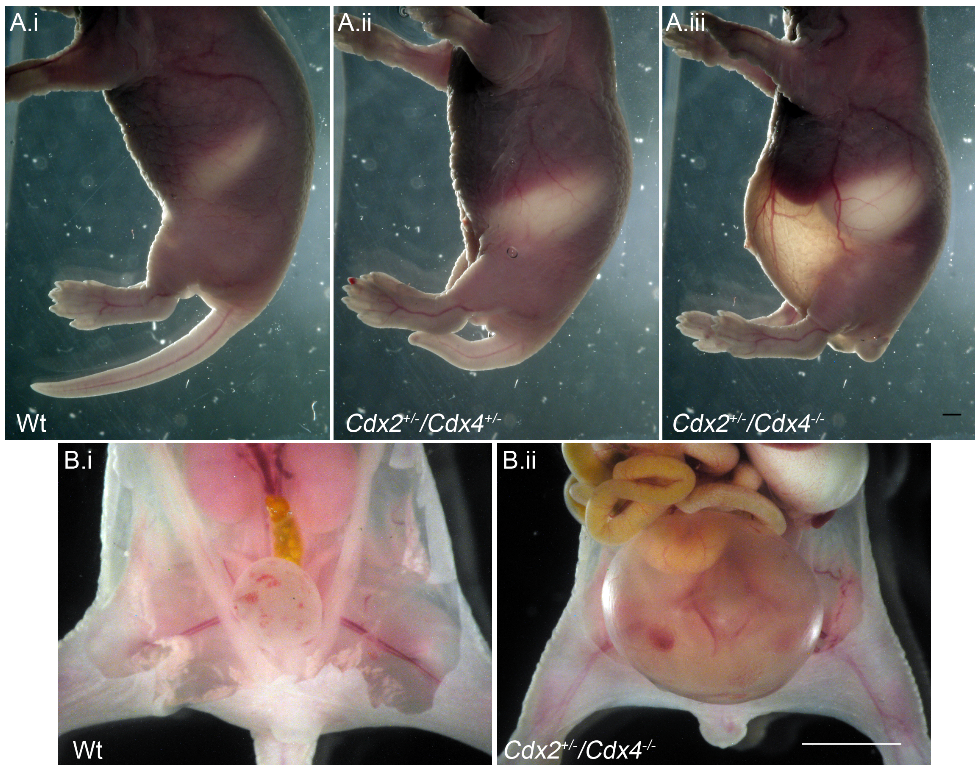

Figure 4.2. **(A) (i) Wildtype newborn mouse compared with (ii) *Cdx2*$^{+/-}$/*Cdx4*$^{+/-}$ and (iii) *Cdx2*$^{+/-}$/*Cdx4*$^{-/-}$ neonates. The degree of posterior abnormality and bladder distention is compatible with decrease in *Cdx* dosage. (B) (i) Bladder in wildtype neonate compared with (ii) distended bladder of *Cdx2*$^{+/-}$/*Cdx4*$^{-/0}$ neonate. Bar = 1 mm**

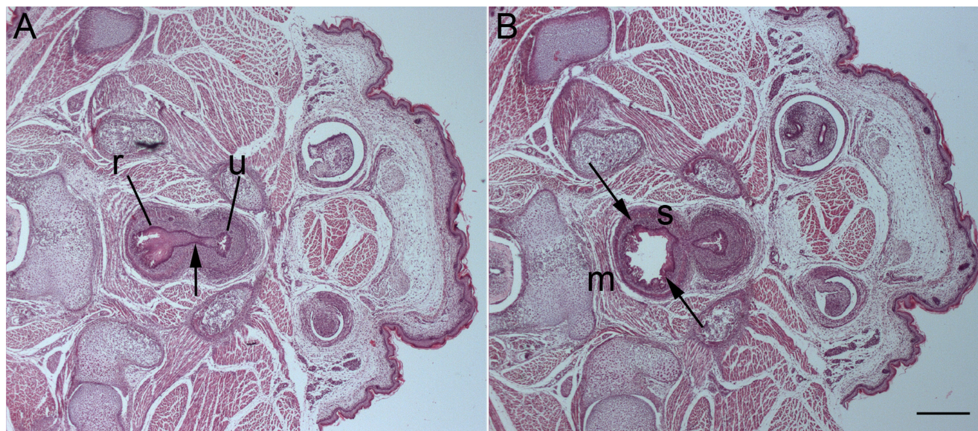

Figure 4.3. **(A) Transverse section through the pelvis of a neonatal *Cdx2*$^{+/-}$/*Cdx4*$^{-/0}$ male mouse showing a recto-urethral fistula (arrow). urethra; u. rectum; r. (B) Transverse section through the pelvis of the mouse illustrated in (A) but at a slightly higher level. The transition between the mucous (m) and squamous epithelium (s) of the blind ending rectum is clearly seen at arrows. Bar = 200µm**

4.4.A) now fails to do so (Figure 4.5 and supplementary video). This results in the dilated bladder seen in these and in full term animals. We conclude that in male (*Cdx2*$^{+/-}$/*Cdx4*$^{-/0}$) compound mutants some, possibly diminished, continuity of the urinary outflow tract is re-established with the development of the terminal (glandular) portion 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 to establish the median raphé on the lower surface of the penile shaft. In *Cdx2*$^{+/-}$/*Cdx4*$^{-/0}$ mutants, we postulate that the re-establishment of continuity is somewhat defective and insufficient to relieve the accumulation of urine in the bladder.

In order to confirm these findings we also examined serial sections of a *Cdx2*$^{+/-}$/*Cdx4*$^{-/-}$ female mouse embryo 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 this animal was enormously dilated and the urinary tract did not open to the exterior; however, as in all the other genetic combinations, 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.

Interestingly, examination of three *Cdx2*$^{+/-}$/*Cdx4*$^{+/-}$ E18 embryos showed anal atresia with bladder distension due to persistence of the urogenital membrane in one animal (not shown) while the other two were normal apart from the expected paracaecal homeosis consequent upon focal *Cdx2* haploinsufficiency (Beck *et al.*, 1999). This is explicable in terms of the random nature of *X* inactivation during normal development. If the pelvic region of a *Cdx2*$^{+/-}$/*Cdx4*$^{+/-}$ (female) mouse happens by chance to develop from clones bearing a preponderance of cells that express the func-

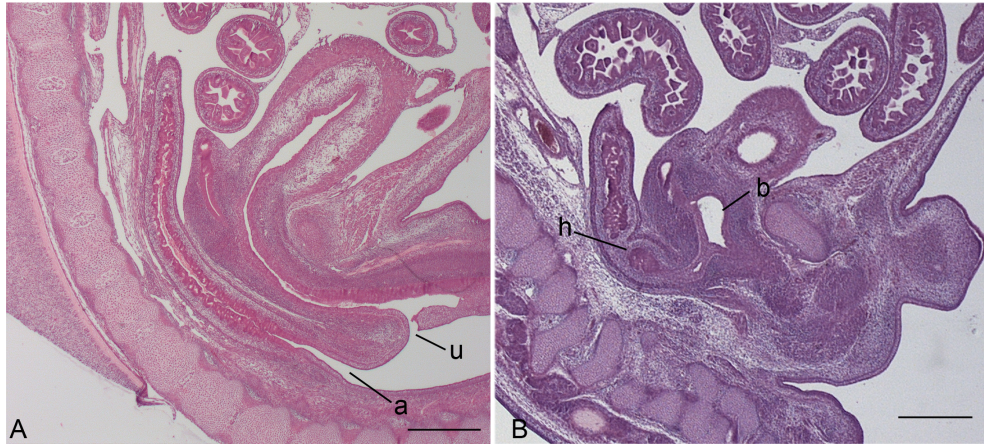

Figure 4.4. **(A) Saggital section through the pelvic region of an E 15.5 day wildtype male mouse. The anal orifice is clearly seen at (a). The urinary tract opening to the exterior which, at this stage of development, occurs as the result of a breakdown of the urogenital membrane is shown at (u). (B) Sagital section through the pelvic region of an E 15.5 day male *Cdx2*$^{+/-}$/*Cdx4*$^{-/0}$ mouse. The hindgut ends blindly at the level of the bladder neck and a recto-urinary fistula between the blind ending hindgut (h) and the bladder (b) is present. Bar = 200µm**

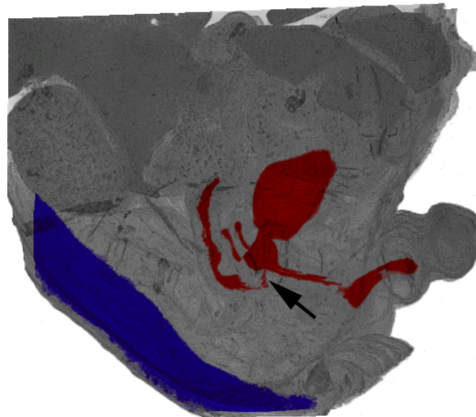

Figure 4.5. **Serial reconstruction of the hindgut and bladder from a 15.5 day male *Cdx2*$^{+/-}$/*Cdx4*$^{-/0}$ (red). The blind ending hindgut is associated with a recto-urinary fistula (at arrow). The urethra does not open to the exterior. Paired seminal vesicles are shown. The spinal cord is outlined in blue.**

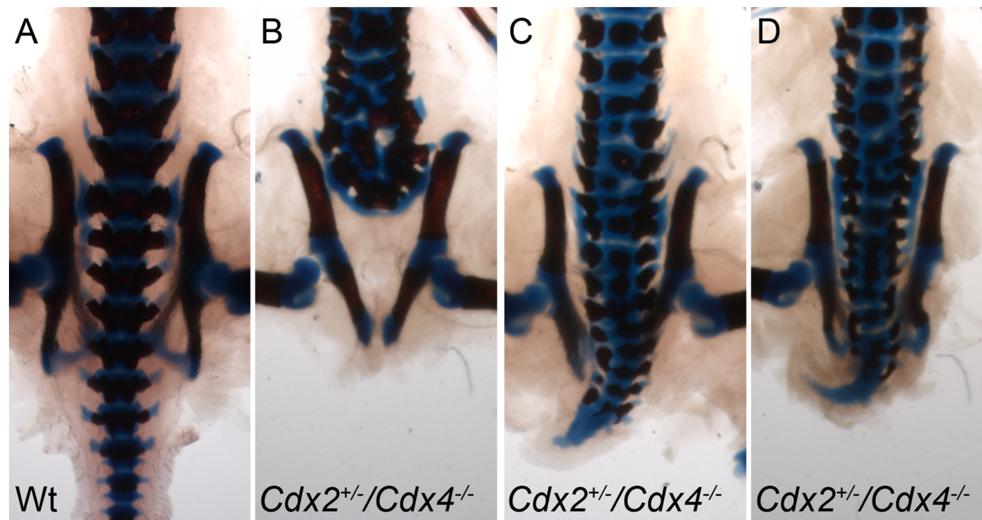

Figure 4.6. **Photographs of cleared and alizarin red/alcian blue stained wildtype (A) and three *Cdx2*$^{+/-}$/*Cdx4*$^{-/0}$ (B-D) mutants showing various degrees of lumbo-sacral and caudal anomalies.**

tional *Cdx4* allele then development will be normal while in animals that develop the appropriate regions from clones bearing mainly null mutant alleles the phenotype will approximate that of the *Cdx2*$^{+/-}$/*Cdx4*$^{-/-}$ female (Figure 4.2). Taken together with the phenotype described for *Cdx2*$^{-/-}$ embryos (see above and Chawengsaksophak *et al*., 2004), our results confirm the principle that normal posterior development depends upon *Cdx* dosage and that there is a degree of redundancy between *Cdx2* and *Cdx4* with respect to morphogenesis of the posterior spine and cloacal derivatives.

Alizarin red and alcian blue stained skeletons of full term male and female compound *Cdx2*$^{+/-}$/*Cdx4*^{null} mutants consistently showed extensive disorganisation of the lumbar and sacral spine (Figure 4.6). The coccygeal vertebrae were almost entirely absent, sacral vertebrae were grossly misshapen as were those of the lower lumbar region. In a minority of animals the upper lumbar vertebrae appeared grossly normal. The lower limb skeleton, on the other hand, including the hip bones was normal in all specimens.

The absence of caudalmost vertebrae in *Cdx2*$^{+/-}$/*Cdx4*^{null} mutants is reminiscent of the situation regarding the posterior presomitic mesoderm observed in tetraploid-rescued *Cdx2*$^{-/-}$ embryos in which generation of the caudal paraxial mesoderm slows down and arrests precociously causing posterior truncation (Chawengsaksophak *et al*., 2004). The *Cdx* mutant pathology indicated in the present findings involves a deficiency of both caudal mesoderm and endoderm resulting in vertebral defects as well as defects in cloacal derivatives affecting the gut and bladder outflow tracts.

4.4. Discussion

The defining feature of the caudal regression syndrome is sacral dysplasia. It includes a broad spectrum of abnormalities arising from defective development of the caudal mesoderm. These vary from minor sacro-coccygeal anomalies to syrinomyelia which latter comprises fusion of the lower extremities, sacral agenesis, anorectal stenosis, renal aplasia or dysplasia and malformed external genitalia. It is likely that numerous pathological processes can result in the development of the condition, ranging from primary defective development of the caudal paraxial mesoderm to interference with caudal blood supply during later development. An association with maternal diabetes or prediabetic states has long been recognised (Stewart and Stoll, 1979) and the involvement of multiple genetic factors has been suggested (Welch and Aterman, 1984). Secondary developmental defects include spina bifida, posterior meningocoele and club foot. The occasional co-expression of short femora, cleft lip and palate, congenital heart defects, trachea-oesophageal fistula, polydactyly and the Pierre-Robin syndrome have been reported but these are essentially unrelated in terms of developmental mechanisms.

All compound *Cdx2*$^{+/-}$/*Cdx4*^{null} mutants examined showed gross disturbance of the lumbo-sacral spine and cloacal derivatives. This contrasts with the absence of abnormalities of the sacro-lumbar region or of the hindgut/cloaca in *Cdx2*$^{+/-}$ animals (Chawengsaksophak *et al.*, 1997), and in *Cdx4* null mice. The abnormalities described here fall entirely within the spectrum of the caudal dysplasia syndrome but are restricted in variety. This suggests that a defined sub-section of this phenotype is genetic in origin, specifically caused by low posterior *Cdx* dosage in late gastrulation and tailbud stages and expressed as a deficiency in pre-somitic mesoderm, endoderm and tailbud elongation. Significantly, we did not observe renal or gonadal abnormalities implying that the intermediate mesoderm is unaffected.

Disturbance of a number of candidate genes are suggested as being involved in the genesis of various types of the Caudal Regression Syndrome. The homeobox gene *HLXB9* has been linked to the genesis of Currarino syndrome (Kohling *et al.*, 2001), which is characterised by partial sacral agenesis together with a presacral mass and anorectal malformations but Merello *et al.*, 2006 report that the gene is not involved in other variants of caudal regression including that described here. *CYP26A1* is an important enzyme involved in the metabolism of all trans retinoic acid (Lee *et al.*, 2007), which in turn is centrally involved in AP patterning during development. *Cyp26a1* knock out is embryolethal. The mutant mice have spina bifida, tail truncations, malformations of the lumbo-sacral regions and developmental defects in the hindgut, kidneys and hindbrain (Abu-Abed *et al.*, 2001; Sakai *et al.*, 2001). This phenotype is more extensive than that we describe in *Cdx2*$^{+/-}$/*Cdx4*^{null} deficiency. De Marco *et al.* (2006) could find no evidence for the implication of *CYP26A1* in the pathogenesis of caudal regression with the caveat that larger groups should be studied before reaching a definitive conclusion. *Gdf11*, is a member of the *TGF*β superfamily of genes (McPherron *et al.*, 1999). Null mutant animals survive to term and show some degree of posterior truncation and vertebral homeosis but their phenotype is different from that described here. They exhibit a range of palatal and renal abnormalities (Esquela *et al.*, 2003) and this may be related to the VACTERL-like phe-

notype associated with mutation in the proprotein convertase *Pcsk5* (Szumska *et al*., 2008) since *Gdf11* is cleaved and activated by *Pcsk5*. VACTERL involves oesophageal, renal, kidney and limb defects in addition to the vertebral and ano-rectal anomalies exhibited by our mice and is therefore unlikely to be relevant to an understanding of the aetiology of the pathology of the $Cdx2^{+/-}/Cdx4^{null}$ mice described here.

The vertebral patterning abnormalities we observed and the placental defects previously described by us in mutant animals (van Nes *et al*., 2006) are both the result of defective formation of posterior mesoderm. It could be argued that disturbed placental function in itself might contribute to the genesis of caudal regression in *Cdx* deficiency as it appears to do in diabetes associated caudal dysplasia. However, this seems unlikely since lumbar prevertebrae are formed by paraxial mesoderm generated at the 18 somite stage. This is about two days earlier than when the chorio-allantoic placenta is established and begins to function (at E10.5) (van Nes *et al*., 2006).

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Glossary

Agenesis: absence or incomplete development of an organ or body part.

Anal pit: the proctodeum of the embryo.

Anorectal malformations: imperforate anus, covered anus, ectopic anus.

Anorectal stenosis: congenital malformation where the anal or rectal opening is obstructed.

Atresia: the absence or closure of a normal body orifice or tubular passage such as the anus, intestine, or external ear canal.

Bladder: a solid, muscular, and distensible (or elastic) organ that sits on the pelvic floor in mammals. It is the organ that collects urine excreted by the kidneys prior to disposal by urination. Urine enters the bladder via the ureters and exits via the urethra.

Cleft lip: a congenital cleft or defect in the upper lip.

Cleft palate: a congenital anomaly of the oral cavity caused by the failure of fusion between the embryonic palatal shelves.

Cloacal: the common cavity into which the intestinal, genital, and urinary tracts open in vertebrates such as fish, reptiles, birds, and some primitive mammals.

Club foot (talipes): a congenital deformity in which the foot is twisted out of shape or position; it may be in dorsiflexion (t. calca´neus), in plantar flexion (t. equi´nus), abducted and everted (t. val´gus or flatfoot), abducted and inverted (t. va´rus), or various combinations (t. calcaneoval´gus, t. calcaneova´rus, t. equinoval´gus, or t. equinova´rus).

Coccygeal: referring to the coccyx, the small tail-like bone at the bottom of the spine, that is made up of 3-5 (average of 4) rudimentary vertebrae.

Coccyx: tail bone; the small bone caudal to the sacrum, formed by union of three to five rudimentary vertebrae, and forming the caudal extremity of the vertebral column.

Columnar epithelia: epithelial cells whose heights are at least four times their width. Columnar epithelia are divided into simple (or unilayered), and stratified (or multi-layered).

Defecation: the final act of digestion by which organisms eliminate solid, semisolid or liquid waste material (feces) from the digestive tract via the anus.

Dysplasia: an abnormality in maturation of cells within a tissue.

Esophageal: of or pertaining to the esophagus.

External genitalia: the reproductive organs external to the body, including pudendum, clitoris, and female urethra in the female, and scrotum, penis, and male urethra in the male.

Fistulae: an abnormal connection or passageway between two epithelium lined organs or vessels that normally do not connect.

Glandular: pertaining to the glans penis.

Homeosis: the transformation of one body part into another, arising from mutation in or misexpression of specific developmentally critical genes, i.e. *Hox* genes.

Hydronephrosis: distention and dilation of the renal pelvis, usually caused by obstruction of the free flow of urine from the kidney.

Hydroureter: distention of the ureter with urine or watery fluid, due to obstruction.

Maternal diabetes (gestational diabetes): a condition in which women without previously diagnosed diabetes exhibit high blood glucose levels during pregnancy.

Median raphé: perineal raphe extends from the anus, through the mid-line of the scrotum (scrotal raphe) and upwards through the posterior mid-line aspect of the penis (penile raphé).

Meninges: the system of membranes which envelops the central nervous system.

Mesentery: the double layer of peritoneum that connects a part of the small intestine to the posterior wall of the abdomen. Its meaning, however, is frequently extended to include double layers of peritoneum connecting various components of the abdominal cavity.

Palate abnormalities: cleft or velopharyngeal incompetence.

Pelvis: the lower (caudal) portion of the trunk, bounded anteriorly and laterally by the two hip bones and posteriorly by the sacrum and coccyx.

Pierre Robin Sequence (Pierre Robin Syndrome or Pierre Robin Malformation): a congenital condition of facial abnormalities in humans.

Polydactyly: a condition characterized by the presence of more than five digits on a hand or foot. Also called hyperdactyly.

Posterior meningocele: the outer faces of some vertebrae are open (unfused) and the meninges are damaged and pushed out through the opening, appearing as a sac or cyst which contains cerebrospinal fluid. The spinal cord and nerves are not involved and their function is normal.

Presacral mass: a mass lying in the presacral region.

Proctodeum: a depression of the ectoderm, behind the urorectal septum of the developing embryo, that forms the anus and anal canal when the cloacal membrane ruptures.

Renal aplasia: nondevelopment of a kidney or renal tissue.

Renal dysplasia: small, misshapen kidneys at birth. May be caused by intrauterine infection of the fetus by virus, but numerous inherited renal dysplasias occur in dogs. They occur in several breeds and are manifested by signs of chronic renal insufficiency, e.g. polyuria, polydypsia, poor growth and weight gain, pale mucous membranes, and renal secondary osteodystrophia fibrosa, from an early age.

Simple columnar epithelium: a columnar epithelium that is unilayered.

Spina bifida: a serious birth abnormality in which the spinal cord is malformed and lacks its usual protective skeletal and soft tissue coverings.

Stratified squamous epithelium: squamous (flattened) epithelial cells arranged in layers upon a basement membrane.

Syrinomyelia: a condition characterized by cavities or hollowed out areas in the spinal cord.

The Currarino syndrome (Currarino triad): an inherited congenital disorder where (1) the sacrum (the fused vertebrae forming the back of the pelvis) is not formed properly, (2) there is a mass in the presacral space in front of the sacrum, and (3) there are malformations of the anus or rectum. It can also cause an anterior meningocele or a presacral teratoma.

Trachea: windpipe; the cartilaginous and membranous tube descending from the larynx and branching into the left and right main bronchi.

Urethral folds: a pair of folds derived from the cloacal folds. In male embryos they close over the urethral plate and fuse to form the spongy urethra and ventral aspect of the penis, and in female embryos they fuse only anterior to the anus and form the labia minora.

Urogenital membrane: ventral part of the cloacal membrane separating the gut from the fetus's external environment.

Chapter 5

Cdx2 loss of function affects the primordial germ cell population

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Submitted

Abstract

Cdx expression is detected in the primitive streak where the progenitor cells of trunk and tail are located. *Cdx* genes have been shown to be involved in the morphogenesis of various axial tissue and organs such as allantois vasculature, paraxial and lateral mesoderm, neuroectoderm, hematopoietic stem cells, and intestinal epithelial endoderm. From this gene family in mouse, *Cdx2* is the most dominant gene in tissue generation. Loss of *Cdx2* in trophoblast, the extra embryonic ectodermal layer causes embryonic lethality at peri-implantation stage. We have generated a *Cdx2* conditional knock-out mouse strain to allow the natural generation of *Cdx2* null embryos and make it possible to study the role of this gene throughout development and adulthood. Upon loss of *Cdx2* function in all epiblast derived tissues, the embryos display a severe posterior truncation phenotype identical to the *Cdx2* null embryos generated from tetraploid rescue experiments. Our first analysis of these mutant embryos reveals that primordial germ cells counts are significantly lower than in the wildtype. In addition, a particularly high proportion of the germ cells are ectopically located in the yolk sac.

5.1. Introduction

The mouse genome has three orthologs of *Drosophila caudal*: *Cdx1* (Duprey *et al.*, 1988), *Cdx2* (James and Kazenwadel, 1991) and *Cdx4* (Gamer and Wright, 1993). The expression of *Cdx2* is first detected at E3.5 in the trophectoderm (Beck *et al.*, 1995) and is essential for proper trophectoderm differentiation (Strumpf *et al.*, 2005) to allow blastocyst implantation (Chawengsaksophak *et al.*, 1997). The *Cdx2*$^{+/-}$ knock-out mouse strain currently available carries a constitutive null *Cdx2* mutation, thus *Cdx2* homozygous mutant embryos obtained from this strain by natural breeding die at the peri-implantation stage. The *Cdx2* heterozygous mutant mice are viable and fertile although they exhibit intestinal lesions of a gastric-type epithelium around the midgut-hindgut junction and anterior homeotic shifts involving the cervical and upper thoracic vertebrae. In addition, they miss the last caudal vertebrae (Chawengsaksophak *et al.*, 1997).

In 2004, *Cdx2* null mutant embryos that developed beyond the implantation stage were made by aggregation of *Cdx2* null embryonic stem cells or morulae with wildtype tetraploid embryos (Chawengsaksophak *et al.*, 2004). This technique called the tetraploid aggregation overcomes implantation problems and trophectoderm deficiency in early embryos (Rossant and Spence, 1998). The *Cdx2* null mutant embryos produced by this method are severely truncated from the level of the forelimb bud in all germ layers, and die around E10.5 because they do not generate an allantois and no funcational chorio-allantoic placenta is made (Chawengsaksophak *et al.*, 2004).

At gastrulation (E7.5), *Cdx2* expression is detected in the chorion, ectoplacental cone, mesoderm of the allantoic bud, and primitive streak. Cell populations located in the primitive streak are progenitors of all the trunk and tail tissues as well as some extra embryonic structures such as allantois and yolk sac mesoderm (Lawson *et al.*, 1991). The three *Cdx* genes have been shown to be involved in posterior tissue

generation in all three germ layers (van den Akker *et al*., 2002). Partial loss of *Cdx* causes premature arrest of axial extension involving neuroectoderm, mesoderm and endoderm of the posterior trunk and tail (van den Akker *et al*., 2002; van Nes *et al*., 2006; Young *et al*., submitted/Chapter 3 and 4). In addition, combined mutations of *Cdx2* and *Cdx4* form an allantois but show impaired placental labyrinth formation (van Nes *et al*., 2006). *Cdx* genes were also shown to impair primitive and definitive hematopoiesis (Wang *et al*., 2008), precursors of which originate from the mesoderm that emerges from the primitive streak (Dzierzak and Speck, 2008).

Another cell progenitor population located in the *Cdx2* expression area at the base of the allantois is the primordial germ cell population (PGC). The germ cell lineage is the first linage to be segregated from the pluripotent epiblast adjacent to the extraembryonic ectoderm in early mouse embryos (Gardner and Rossant, 1979; Lawson and Hage, 1994). PGCs were first identified more than 50 years ago thanks to their high level of tissue nonspecific alkaline phosphatase (TNAP) (Chiquoine *et al*., 1954). At E7.2 of embryonic development, PGCs are found as a cluster of TNAP positive cells at the base of the allantois (Ozdzenski *et al*., 1967). The founding cluster consists of ~45 progenitor cells (Ginsburg *et al*., 1990; Lawson and Hage, 1994). Although TNAP is not necessary for germ cell survival (MacGregor *et al*., 1995) it allows the identification of PGCs from the time they become lineage restricted ~E7.2 until their entry into the genital ridges (future gonads) two days later.

Twenty-four hours after specification, the germ cell cluster formed at the base of allantois breaks down and PGCs start to migrate. Some PGCs end up in the allantois and/or other extraembryonic structures. Most of them move along the primitive streak into the definitive endoderm. As the endoderm starts to invaginate to form the hindgut, PGCs migrate through the hindgut portal and later along the hindgut, to arrive to its dorsal side eventually. PGCs at the lip of the hindgut pocket have rounded non-motile shapes suggesting that they are being carried by endoderm (passively incorporated). PGCs migrating along the hindgut tend to contact each other by thin cytoplasmic extensions thus form a network of migrating cells (Gomperts *et al*., 1994). During this time PGCs actively proliferate to reach ~6,000 cells by the time they enter the genital ridges at E10.5. Later on sex specification occurs. Proliferation of germ cells within the gonads continues till E13.5 when they reach approximate number of 20,000 cells. In males PGCs undergo mitotic arrest within seminiferous cords and in females germ cells enter meiosis.

We set out to investigate whether *Cdx2*, the *Cdx* gene with the most important role in tissue establishment from posterior progenitors, is involved in the laying down the germ line. Rather than generating *Cdx2* null embryos by tetraploid rescue, we generated a mutant line carrying a conditional *Cdx2* loss of function allele. We created *Cdx2* conditional knock-out mice strain utilizing *loxP/Cre* and *FRT/flp* technology (Nagy, 2000). In the *Cdx2* conditional allele, exon two that encodes the homeodomain, an important domain for *Cdx2* function, is flanked by *loxP* sites. *Cdx2* mice homozygous for the conditional allele are healthy and breed normally. *Cdx2* null homozygous mutant embryos that develop beyond the implantation stage were created by crossing the *Cdx2* conditional knock strain with a *Sox2Cre* transgenic mouse (Hayasi *et al*., 2002; Hayasi *et al*., 2003) that provides *Cre* recombinase activity in all epiblast derived tissues. The resulting *Cdx2* null homozygous mutant embryos

reached a maximal developmental stage of E10.5 and displayed severe posterior truncation, in a way similar to *Cdx2* null mutant embryos generated by tetraploid aggregation. We set out to investigate the influence of *Cdx2* on the PGC populations located in the base of the allantois where *Cdx2* is expressed. Our results indicate that the number and location of PGCs is altered in *Cdx2* null embryos.

5.2. Materials and methods

5.2.1. Gene targeting and mouse breeding

Mouse genomic DNA (supplied by S. Munson, Geneta, University of Leicester, Leicester) was used as a template for creating a targeting vector by standard techniques. A *pGK-neo*^{R} cassette flanked by *FRT* sites was inserted into intron two at the *NotI* and *XmaI* sites. *loxP* sites were introduced to flank exon two. The targeting vector was electroporated into ES cells (129/Ola). After G418 (200μg/mL) selection, positive clones were screened using a PCR strategy to detect left and right arm homologous recombination events, and verified by Southern-blot analysis with a *neo*^{R} probe. Chimeric mice were generated by blastocyst injection of two independently targeted *Cdx2*$^{+/FRT\text{-}loxP(ca)}$ ES cells into E3.5 C57BL/6 mouse blastocyst using standard procedures. Chimaeric males were mated with FVB females for generation of F1 animals allowing us to detect the targeted ES cell contribution to the germ line by coat color. Genotyping confirmed the presence of the *Cdx2*$^{+/FRT\text{-}loxP(ca)}$ allele in the targeted mice.

5.2.2. Genotyping

Primers OES30 (TCC TAG CCA GGA CAG GAG AA) and OES31 (GGG TTC CGG ATC CAC TAG TTC) that generate a 2.6kb PCR product were used to screen for correct left arm recombination, while OES34 (TCG CCT TCT TGA CGA GTT CT) and OES35 (AGG GAC AGG AAG TCC AGG TT) that generate a 2.4kb PCR product were used to screen for right arm recombination. The reaction conditions were 95°C for 30 seconds, 55°C for 1 minute and 72°C for 3 minutes for 35 cycles in a 20 μL mixture that contained 0.5μM of each primers, 0.2mM of each dNTP, 1mM $MgCl_2$ and 1x PCR Buffer. Isolated genomic DNA was digested with *HindIII* and processed according to standard Southern-blot procedure and hybridized with a *neo*^{R} probe to detect a 10.1kb band corresponding to the correct recombination event.

To distinguish the *Cdx2* wildtype, *FRT-loxP(ca)*, and *loxP(ca)* alleles after breeding with *CMV-Flp1* mice strain, 3 primers were used, namely *Cdx2*-ca-p1 (TGG GGC AAT CTT AAT GGG TA), *Cdx2*-ca-p2 (TGT AGC CTC GAC TTG GCT TT) and *Cdx2*-ca-p3 (ATA TTG GCT GCA GGT CGA AA). Combination of *Cdx2*-ca-p1 and *Cdx2*-ca-p3 produce a 708bp band that identifies the *FRT-loxP(ca)* allele, while the combination of *Cdx2*-ca-p1 and *Cdx2*-ca-p2 can detect the either wildtype allele (140 bp) or the conditional allele (363bp). Upon Cre recombinase action, the *Cdx2* mutant allele can be detected with *Cdx2*-ca-p4 (ACG CGT TCC AAG TAG AAG GA) and *Cdx2*-ca-p5 (CGC TCT TTC TCT GTC CAA GTG) that produce a 264bp band. The

PCR reaction conditions to detect all these *Cdx2* alleles was 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds for 35 cycles in 10μL of reaction mixture contain 0.5μM of each primers, 0.2mM of each dNTP, 2mM $MgCl_2$ and 1x PCR Buffer.

Primer *Cre* rec 2F (CCG GGC TGC CAC GAC CAA) and *Cre* rec 2R (GGC GCG GCA ACA CCA TTT TT) were used to detect the *Cre* recombinase gene in 10μL reaction mixture containing 0.5μM of each primers, 0.2mM of each dNTP, 2mM $MgCl_2$ and 1x PCR Buffer. The reaction condition was 95°C for 30 seconds, 58°C for 30 seconds, and 72°C for 30 seconds for 35 cycles.

Cdx2$^{+/-}$ mutant mice strain were genotyped by PCR as described previously (Chawengsaksophak *et al.*, 1997).

In all PCR reactions, the Polymerase used was Promega GoTaq M830B with 5x Flexi Green GoTaq Buffer M891A.

5.2.3. Whole mount detecting and counting of PGC

Embryos with intact yolk sac were dissected from the deciduas in PBS. Embryos were then flattened to make PGC counting easier after tissue non-specific alkaline phosphatase (TNAP) staining. Embryos were immediately fixed in 4% paraformaldehyde (PFA) in PBS for 1.5 hours and then washed 3X 10 minutes in PBS. The fixed embryos were incubated in 70% ethanol/PBS for at least 1 hour followed by 3X 5 minutes washing in water before staining. The TNAP staining solution was prepared freshly and was composed of 0.5% sodium 5′5′diethyl barbiturate (veronal), 0.06% $MgCl_2$, 0.1 mg/mL α-naphthyl phosphate, and 0.5 mg/mL Fast Red TR. The staining reaction was monitored from time to time and stopped by rinsing the embryos in water as soon as PGCs were detected (about 8-20 minutes). The stained embryos were stored in 70% glycerol. The head piece of each embryo was removed for genotyping before flat mounting the rest of the embryo on a 18x18 mm coverslip. PGCs were identified as single cells with a stained ring and an intracellularly intense single dot stained under a 20X objective of a compound microscope. All steps were done under gentle shaking at 4°C except staining, staging and flat mounting which were done at room temperature. The protocol for whole mount detecting and counting of PGCs was adopted from Lawson *et al.* (1999).

5.2.4. Statistics on PGC counting

The data distribution of each group was analyzed with the Kolmogorov Smirnov test. The Z value $\leq$ 0.05 was used as criterium indicating normal data distribution. Since the data distribution was not normal, the Mann-Whitney test was used to compare the difference between genotypes. $p \leq 0.05$ was considered statistically significant.

5.3. Results

5.3.1. Generation of the *Cdx2* conditional knock-out strain

Combination of gene targeting and site-specific recombination has been demonstrated to be a powerful method to create conditional knock out mice and overcome the lethality of null mutant strains. In this method, the targeting construct is made by introducing *loxP* sites on both sides of an exon with important function or interest. In the same construct, a selection marker flanked by *FRT* sites is introduced into an intron. Since insertion of a marker into an intron has been shown to often disrupt the normal function of the gene, the marker can be removed upon action of a *FRT* recombinase (*flippase*) allowing the allele to function normally in the presence of *loxP* flanked insert. The mutants can be obtained by crossing the mouse with a strain of mice expressing *Cre* recombinase in a tissue specific or time restricted way. *Cre* will remove the important exon, thus compromising the gene function (Nagy, 2000).

Cdx2 is a homeobox gene; the protein therefore contains a homeodomain, allowing it to act as a transcription factor. The murine *Cdx2* gene locus consists of three exons, of which exon two encodes the homeodomain (Figure 5.1.A). Deletion of exon two will render *Cdx2* non-functional i.e. "knocked out". A targeting construct to generate a conditional *Cdx2* allele (designated $Cdx2^{FRT\text{-}loxP(ca)}$) was therefore designed so that exon two was flanked by *loxP* sites, and a $pGK\text{-}neo^R$ cassette flanked by *FRT* sites as a selection marker was inserted into intron two and positioned 5′ to the second *loxP* site (Figure 5.1.B). Neomycin resistant colonies were screened after electroporating the construct into ES cells and applying a G418 (gentamicin B1 analog) selection. Neomycin resistant colonies that have undergone homologous recombination (Figure 5.1.C) were identified by PCR using primer sets OES 30-31 and OES 34-35 (Figure 5.2.A-B) to detect left and right arms recombination respectively. Subsequently, Southern blot analysis was performed using the neo^R probe that detects a 10.1 kb fragment, verifying the correctness of the homologous recombination event (Figure 5.2.C). From 1800 neomycin resistant colonies screened in Leicester (600 colonies) and Utrecht (1200 colonies), only 4 positive clones were found to have undergone a homologous recombination event showing a low targeting efficiency in the *Cdx2* locus. The only clone found in Leicester was injected into blastocysts and generated several chimeras, that failed to give any germ line transmission. In addition to having the diagnostics neo^R positive 10.1kb band corresponding to the homologous recombination event, the Leicester clone also possessed a smaller band (Figure 5.2.C) representing random integration of the construct into the genome. This random integration might have caused lethality during embryogenesis, possibly explaining why no germ line transmission was obtained from this ES clone. Two out of three targeted ES clones isolated in Utrecht were injected into blastocysts and generated chimeras that successfully gave germ line transmission.

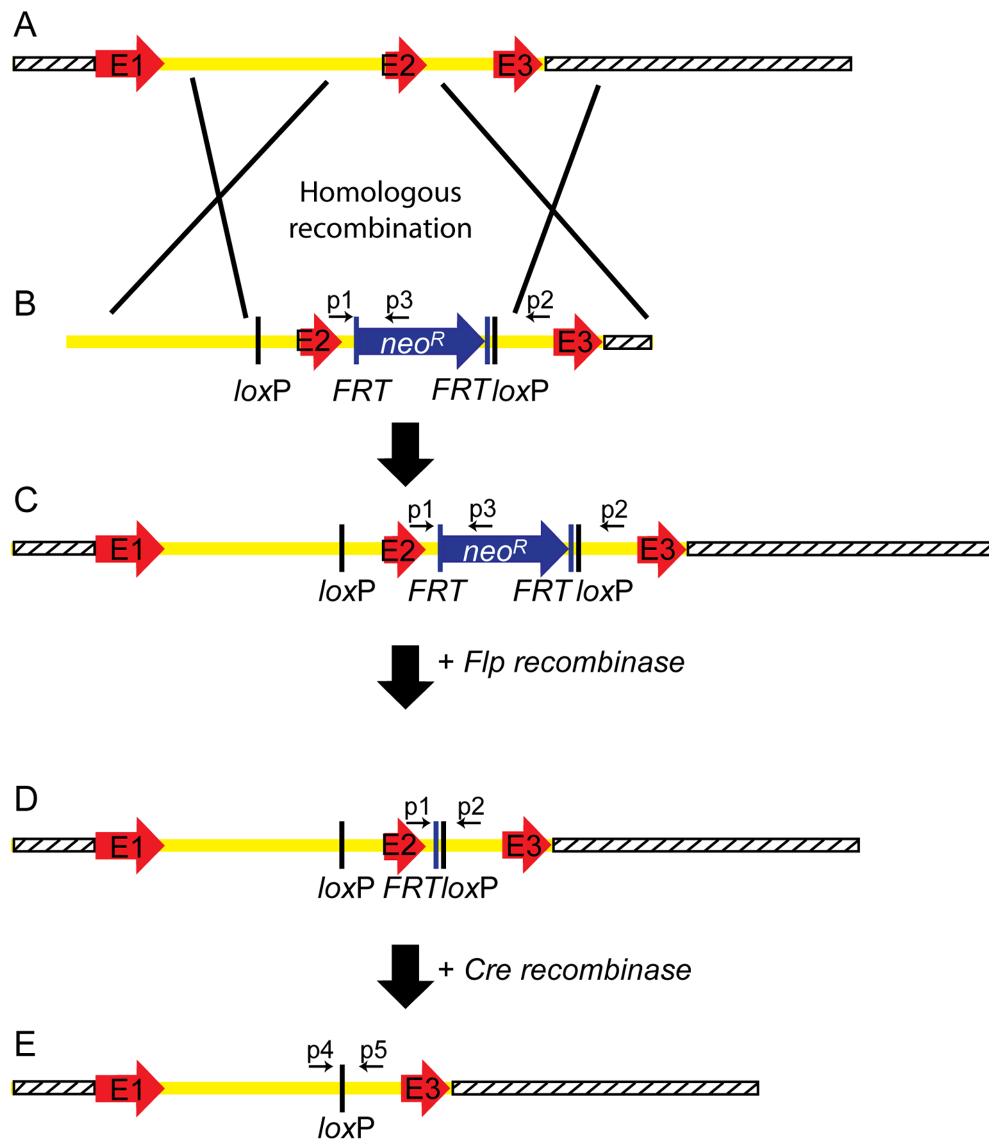

Figure 5.1. **Generation of a *Cdx2* conditional allele. (A) The endogenous *Cdx2* locus consists of three exons. (B) The targeting construct contained a *neo*^{R} cassette flanked by the *FRT* sites inserted into intron two and two *loxP* sites located in intron one and intron two after the *neo*^{R} cassette. The left and right arms of this construct are homologous to the DNA of intron one, and to sequences surrounding exon three including, the exon three itself. (C) *Cdx2* locus organization after the homologous recombination event between the targeting construct and the *Cdx2* endogenous locus. (D) Situation after the action of *flippase1* that exises the *neo*^{R} cassette, leaving the *Cdx2* locus with two *loxP* sites flanking exon two. (E) A *Cdx2* null allele is created by removal of exon two after the recombination activity of *cre*. Exons, red arrow; introns, yellow lines; intragenic regions, shaded line; *loxP* sites, black vertical lines; *FRT* sites, blue vertical lines.**

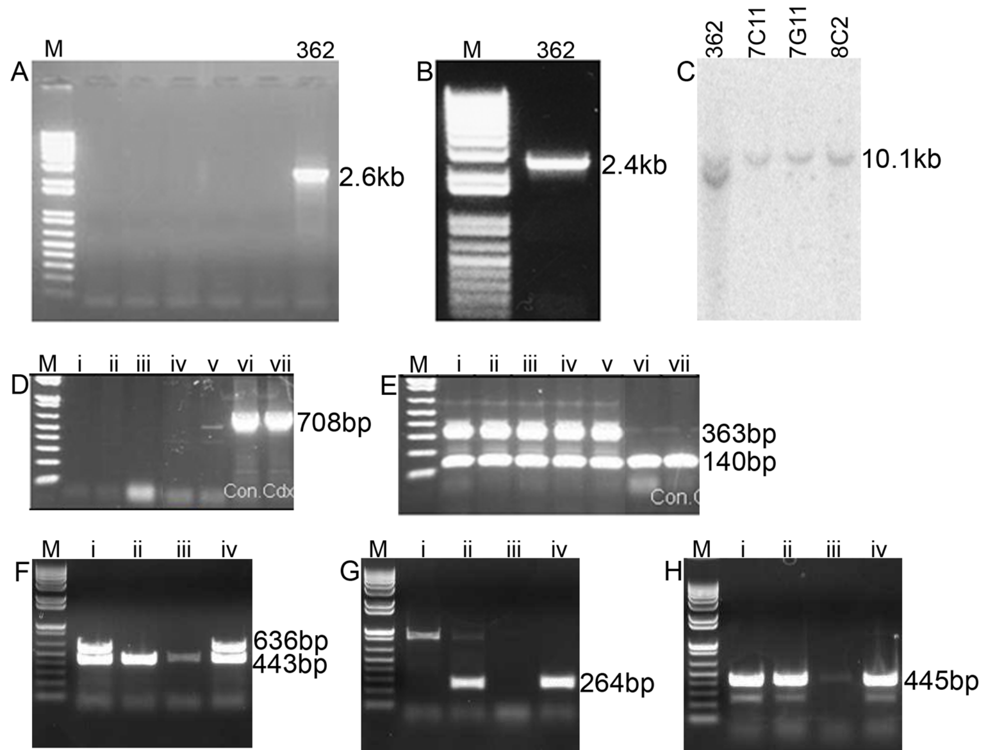

Figure 5.2. **PCR assays to identify the *Cdx2* conditional allele. (A-B) PCR assay used to detect left and right arm recombination producing 2.6 and 2.4 kb bands respectively, from clone 362 generated in Leicester, the same bands are seen for clones 7C11, 7G11 and 8C2 isolated in Utrecht (not shown). (C) Southern blot analysis of all the positive clones using *neo^R* as a probe to detect the 10.1kb band of the correct homologous recombination event between the targeting construct and *Cdx2* endogenous locus. (D-E) PCR analysis of the mice after the removal of the *pGK-neo^R* cassette. The 708bp band detecting the presence of *pGK-neo^R* cassette are only seen in mice vi and vii and absent in mice i-v that possess *loxP(ca)* (363bp) and wildtype (140bp) alleles. (F-H) Genotyping of embryos generated from the crosses between *$Cdx2^{+/loxP(ca)}$* female mice with *$Cdx2^{+/-}$/Sox2Cre* male. *$Cdx2^{+/-}$* alleles (Chawengsaksophak *et al.*, 1997) are distinguished by the presence of 636 and 443bp bands respectively (F). A *Cdx2* null allele resulted from the removal of exon two after *Cre* recombination from the conditional allele, detected by the presence of a 264bp band (G). The *Sox2Cre* transgene is detected by the presence of a 445bp band (H). Thus, only embryo iv is the *Cdx2* null mutant. M is 1kb plus DNA ladder.**

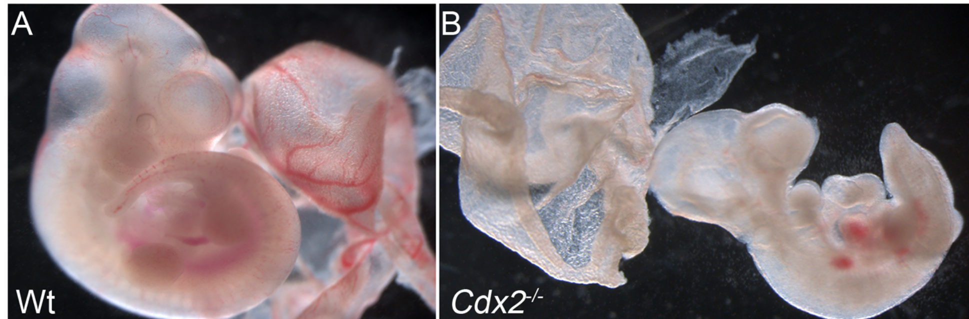

Figure 5.3. **Wildtype (left) and *Cdx2* null (right) embryos at E10.5.**

5.3.2. Presence of a *neo*^{R} cassette in the *Cdx2* intron causes inactivation of the gene

To generate a *Cdx2* null embryo supported by wildtype tropholasts, the breeding strategy employed in this work was to cross the *Cdx2* conditional mice with *Cdx2*$^{+/-}$ (Chawengsaksophak *et al*., 1997) mice carrying a *Sox2Cre* transgene (Hayashi *et al*., 2002). *Sox2Cre* transgenic mice display efficient *Cre* mediated recombination in epiblast derived tissue. Because maternal *Cre* activity in the oocyte of the transgenic females has been reported (Hayashi *et al*., 2003), the *Sox2Cre* transgene was delivered paternally. Crossing *Cdx2*$^{+/FRT\text{-}loxP(ca)}$ females with *Cdx2*$^{+/-}$ (Chawengsaksophak *et al*., 1997) males carrying the *Sox2Cre* transgene failed to produce any *Cdx2* null embryos after the implantation stage. This indicated that the presence of a *pGK-neo*^{R} cassette in the intron probably disrupts the normal function of the gene, which in agreement with previous experiments with other genes (Nagy, 2000; van Nes *et al*., 2006). The *pGK-neo*^{R} cassette was removed from the *Cdx2*$^{FRT\text{-}loxP(ca)}$ allele resulting in the generation of the *Cdx2*$^{loxP(ca)}$ allele (Figure 5.1.D). Intercrosses between *Cdx2*$^{+/loxP(ca)}$ mice produced *Cdx2*$^{loxP(ca)/loxP(ca)}$ mice that live and breed normally. This confirmed that the *Cdx2*$^{loxP(ca)}$ conditional allele functions normally as a wildtype allele.

5.3.3. Characterization of *Cdx2* null embryos

Crossing *Cdx2*$^{+/loxP(ca)}$ female mice with *Cdx2*$^{+/-}$/*Sox2Cre* male generated *Cdx2* null embryos that survived beyond the implantation stage. These *Cdx2* null embryos isolated at E10.5 (Figure 5.3) were very similar to the *Cdx2* null embryos generated from tetraploid rescue experiments (Chawengsaksophak *et al*., 2004) confirming the successful conditional knock-out procedure. The *Cdx2* null mutant embryos were truncated posteriorly beyond the forelimb bud. The maximum number of somites counted was 17. Blood circulation in the yolk sac was not observed possibly due to blood leakage out of the yolk sac vessels, often observed in cases of placental failure. The posterior neural tube was open. Some of the mutant embryos still had a heartbeat and exhibited hemorrhage in addition to a dilated pericardium.

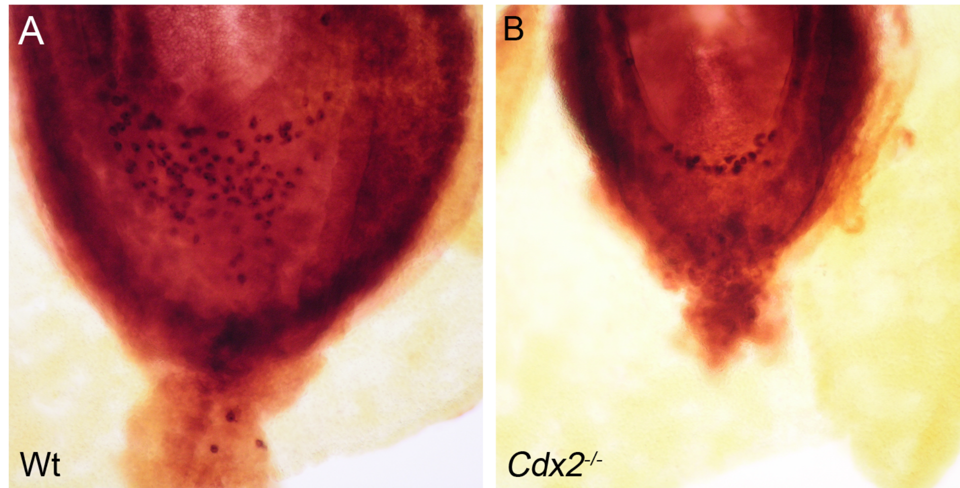

Figure 5.4. **Tissue non specific alkaline phosphatase staining reveals the PGC population in wildtype (A) and *Cdx2*$^{-/-}$ (B) embryos. With this staining, each individual PGC was identified with a single dark spot and a ring shaped staining. The number of PGCs in *Cdx2*$^{-/-}$ embryo was reduced significantly compared to that of the wildtype embryo.**

5.3.4. Reduced PGCs population in *Cdx2* homozygous mutant embryos

Around E7.5 *Cdx2* expression is detected in the primitive streak and allantoic bud (Deschamps and van Nes, 2005). These expression regions include the posterior proximal epiblast where clustered PGC founder cells are located (Ginsburg *et al*., 1990; Lawson and Hage, 1994). To evaluate whether *Cdx2* plays a role in PGC development, we compared PGC numbers in wildtype and *Cdx2* homozygous mutant embryos at early somites stages (around E8.5). At this stage, PGCs are in their migration phase along with the developing hindgut endoderm to reach their final destination in the genital ridge. During this phase as earlier, individual PGCs can be identified easily due to their high endogenous levels of TNAP. Intraembryonically, the number of PGCs in *Cdx2* homozygous mutant embryos (n=15) was significantly reduced ($p<0.01$) compare to that of wildtype embryos (n=40) (Figure 5.4 and Table 5.1). During the course of embryonic development, the intraembryonic PGC number in *Cdx2* homozygous mutant embryos showed a tendency to decrease (Figure 5.5).

5.3.5. Ectopic location of PGCs in the yolk sac in *Cdx2* null embryo

In wildtype embryos a small number of ectopic PGCs is usually seen in the yolk sac, a double layer of extraembryonic mesoderm and endoderm. In the *Cdx2* homozygous mutant yolk sacs, the PGC number was significantly increased ($p<0.01$) compared to the wildtype situation. This is in marked contrast with the PGC number in the embryo proper, that is lower in mutants than in wildtypes. During normal development, some PGCs also migrate into the allantois, and this was also observed

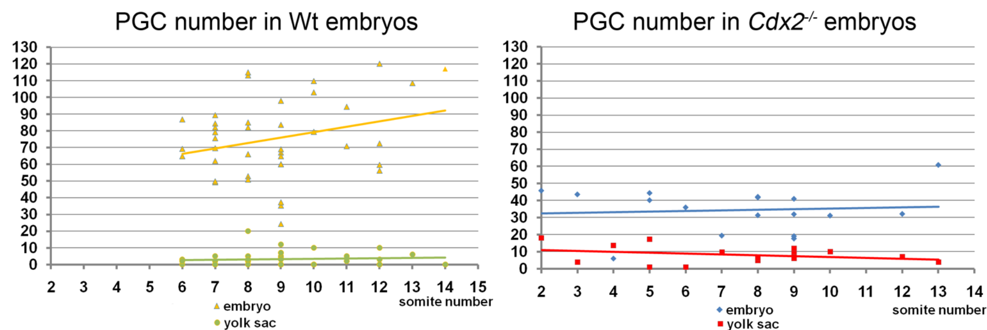

Figure 5.5. **Plot of PGCs numbers vs somite stage in wildtype (left) and *Cdx2*$^{-/-}$ (right) embryos. PGC number are indicated along the vertical axis, somite stages along the horizontal axis.**

in the wildtype control in this study. However, allantois development was impaired in the *Cdx2* homozygous mutant embryos, possibly explaining the absence of PGCs in the allantois of these mutants.

5.4. Discussion

Cdx2 behaves as the most dominant *Cdx* genes in functions related to the generation of embryonic tissues from posterior progenitors. Like the other two *Cdx* genes, *Cdx2* is expressed in the primitive streak during the generation of trunk and tail tissues, and it controls the morphogenesis of various axial tissue and organs such as the allantoic vasculature, hematopoietic stem cells, paraxial and lateral mesoderm, neuroectoderm, and intestinal epithelial endoderm. (Chawengsaksophak *et al.*, 1997; van den Akker *et al.*, 2002; van Nes *et al.*, 2006; Wang *et al.*, 2008; Young *et al.*, submitted/Chapter 3 and 4). One of the progenitor populations in which *Cdx2* might play a role, that had not studied before is the primordial germ cell population. PGCs originate from proximal posterior epiblast in closed contact with extraembryonic ectoderm.

We took advantage of the easy generation of early *Cdx2* null embryos from the conditional *Cdx2* mutant we generated to study the role of this gene during initial and later establishment of the germ line. At E7.2 after PGCs are specified, they are found as a cluster of about 45 cells at the base of the allantois (Ginsburg *et al.*, 1990; Lawson and Hage, 1994). A day later, this cluster breaks down and individual PGCs start their migration along the developing hindgut endoderm toward their final destination in the genital ridge at E10.5. During this migration phase, the PGC number increases. Our observations in *Cdx2* homozygous mutant embryos at this migration stage revealed that the PGC number was decreased significantly compared to the situation in wildtype embryos. Several possibilities could account for this observation. The *Cdx2* mutation might compromise the initial number of PGCs in the cluster at the base of the allantois. That idea is supported by the fact that only 2 out of 15

Descriptive statistics: number of PGCs

	N	Min.	Max.	Mean	St. Error	St. Deviation
Wildtype embryo	40	24.2	120.2	75.43	3.69	23.36
Wildtype yolk sac	40	0.0	20.0	3.23	0.66	4.20
Wildtype embryo+yolk sac	40	31.2	120.2	78.66	3.70	23.40
Cdx2$^{-/-}$ embryo	17	6.1	60.8	34.42	3.19	13.15
Cdx2$^{-/-}$ yolk sac	17	1.0	18.0	8.13	1.21	4.97
Cdx2$^{-/-}$ embryo+yolk sac	17	19.7	64.8	42.55	3.22	13.26

Kolmogorov Smirnov test

	Z-value
Wildtype embryo	0.50
Wildtype yolk sac	1.40
Wildtype embryo+yolk sac	0.51
Cdx2$^{-/-}$ embryo	0.69
Cdx2$^{-/-}$ yolk sac	0.56
Cdx2$^{-/-}$ embryo+yolk sac	0.53

Mann-Whitney test

	p-value
Wildtype embryo vs *Cdx2*$^{-/-}$ embryo	0.000
Wildtype yolk sac vs *Cdx2*$^{-/-}$ yolk sac	0.000
Wildtype embryo+yolk sac vs *Cdx2*$^{-/-}$ embryo+yolk sac	0.000

Table 5.1. **Statistical analysis of PGC number.**

Cdx2 null embryos contained a wildtype-like number of PGCs (~45) just after the cluster dissociated (Figure 5.5). At this early stage, the *Cdx2* mutation does not affect posterior tissue morphogenesis yet. Mutations in several *Bmp*s (*Bmp2*, *Bmp4*, and *Bmp8b*) have been shown to lead to a reduced PGC number (Lawson *et al*., 1999; Ying *et al*., 2000; Ying *et al*., 2001). In some cases, we observed undissociated clumps of TNAP positive cells suggesting that the cluster did not break down fully and PGCs were not able to migrate away from it. A very similar situation is observed in *Blimp1* mutant embryos (Ohinata *et al*., 2005). *Bmp* is involved in the specification of PGCs as a signal from the extraembryonic ectoderm and visceral endoderm to the epiblast. In the epiblast, Smad proteins (*Smad4/5/8*) respond to this signal and lead to PGC specification by inducing *Fragilis* expression in precursor cells and later *Blimp1*. *Blimp1* is the first lineage restricted gene (Lange *et al*., 2008; Ohinata *et al*., 2005; Saitou *et al*., 2002). It will be interesting to see if loss of *Cdx2* in epiblast changes any of these components.

Since we observed a decrease in PGC number, it is possible that the migrating PGCs of *Cdx2* null mutant embryos become less proliferative and/or more apoptotic. *Nanos3* expression in the migrating PGCs protect them from apoptosis (Suzuki *et al*., 2008). In *Nanos3* null PGCs, an increased number of apoptotic cells was detected without any change in the proliferation rate. *Kit* and *Kit* ligand/*steel* prevent apoptosis and stimulate proliferation (Godin *et al*., 1991; Runyan *et al*., 2006). Mutations in *Kit* and *Kit* ligand/*steel* caused a dramatic reduction of the PGC number (Dolci *et al*., 1991). Loss of *Dnd1* (*dead end1* ortholog or *ter*) caused a progressive loss of PGCs from E8.5 on due to apoptosis, but without any effect on PGC migration to the genital ridge (Noguchi and Noguchi, 1985; Sakurai *et al*., 1994). Future work will test whether expression of these genes is impaired in *Cdx2* mutant embryos, possibly leading to a decrease in the PGC number. It also remain to be investigated whether the effect of the *Cdx2* mutation on posterior tissue generation and organization plays a role in the reduction of the PGC population at stages later than early somites.

A second striking feature charactering the *Cdx2* mutant PGC population is the difference in PGC distribution between tissues in mutant versus wildtype. There is a lot of uncertainty regarding the control of germ cell migration. After specification PGCs become transcriptionally silent. Genome wide reprogramming is taking place which includes histone modification, such as the erasure of H3K9me2 and upregulation of H3K27me3. The *C-kit/Steel Factor* (*SF*) signal transduction pathway is required for normal migration of PGCs. Disruption of either receptor and/or ligand causes abnormalities in migration and proliferation accompanied by formation of cell clumps in the hindgut, and apoptosis (Buehr and McLaren, 1993). Some PGCs also fail to leave the base of the allantois. Another gene product involved in PGC migration might be *E-cadherin*, a cell adhesion molecule. Hindgut epithelium and PGCs express *E-cadherin*, and this expression is necessary for strong adhesive properties of epithelial cells, but not for PGCs (Bendel-Stenzel *et al*., 2000). This may allow germ cells to move freely along the epithelium and to remain within the hindgut. The movement of PGCs in the hindgut seems not to be organized in any way. Although PGCs are motile they do not migrate anteriorly in a directed manner. Initial random movements become more directed as germ cells move to the dorsal side of the hindgut (~E9.5) to finally colonize the genital ridges. Migration along the

genital ridge is a directed process and depends on another receptor-ligand system: *CXCR/SDF1* (Ara *et al.*, 2003; Molyneaux *et al.*, 2003). Mutant mice for either receptor or ligand have genital ridges with only a few germ cells with unaltered division rate and survival. Progressive loss of PGCs starting from the beginning of migration is also observed in *Ter* mice carrying a mutation in *Dnd1* gene as well as in *Nanos3* knockout mice. In both cases PGC survival is severely altered resulting in apoptosis.

In contrast to the significantly reduced PGC number in the *Cdx2* homozygous mutant embryo compared to the wildtype, the PGC number in the mutant yolk sac was significantly higher. The proportion of ectopic versus total PGC number is thus much higher in the mutant than in the wildtype. PGCs migration along the hindgut might depend on cues present in this tissue, like *E-cadherin* (Benzel-Stenzel *et al.*, 2000). *Cdx2* mutation caused endodermal defects (Beck *et al.*, 2003; Young *et al.*, submitted/Chapter 4). It is possible that the migration cues expressed on the hindgut epithelium of *Cdx2* mutant embryos are compromised causing more germ cells to take a path towards the yolk sac in contrast to what they do in the normal situation. The yolk sac comprises two cell layers, its endodermal layer is continuous with the hindgut making this finding intriguing. A detailed comparative study of endodermals defect in the hindgut and yolk sac in the *Cdx2* mutant might give some insights about this aberrant PGC migration in the mutants.

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Part III

Discussion

Chapter 6

Discussion

The work described in this thesis concerns the developmental function of *Cdx* and *Hox* genes. Cdx genes belong to the *ParaHox* gene family, a sister group of the *Hox* genes. Like the *Hox* genes, *Cdx* genes have a function in the specification of antero-posterior identity of axial and paraxial embryonic tissues. In addition, *Cdx* genes play a role in tissue generation during axial extension. This work shows that *Cdx* genes share this role with the *Hox* genes, possibly by regulating the later. *Cdx* genes are expressed in the primitive streak where all the trunk and tail tissue progenitors are located (Cambray and Wilson, 2007; Deschamps and van Nes, 2005; Lawson *et al*., 1991). The formation of a functional chorio-allantoic placenta (van Nes et al., 2006), and hematopoietic stem cells (Wang *et al*., 2008) were also shown to require the *Cdx* genes. The generation of all three germ layers at the posterior end of growing embryos is depending on *Cdx* genes (van den Akker et al., 2002; van Nes 2005; and this thesis).

The experimental results reported in chapters 3, 4, and 5 have allowed us to better document the role of *Cdx* genes in the generation and patterning of posterior mesoderm and endoderm, to discover a differential function of the *Hox* genes in axial extension and to partly elucidate the genetic mechanism underlying *Cdx*/*Hox* mediated posterior tissue growth during extension of the body axis. In addition, this work reveals the involvement of *Cdx* genes in the establishment of the germ line, and provides the research community with a conditional mutant allele of *Cdx2* that might allow investigations of *Cdx* function in the adult.

The *Cdx* compound mutant embryos studied in chapters 3 and 4, *Cdx2*$^{+/-}$ /*Cdx4*$^{-/-}$ embryos, exhibit a spectrum of phenotypes including posterior axial truncation and chorio-allantois placental failure that caused death around E10.5 in most cases. Survivors were born but did not live longer than a day or two due to arrested development of the hindgut and urethra (van Nes et al., 2006). Overexpression of middle *Hox* genes (*Hoxa5* and *Hoxb8*) in *Cdx2*$^{+/-}$ /*Cdx4*$^{-/-}$ embryos rescues the posterior axial extension process, and restores the function of the chorio-allantois placenta. On the contrary overexpression of *Hox* paralogous 13 elicits a posterior axial truncation phenotype similar to the one observed in *Cdx2*$^{+/-}$ /*Cdx4*$^{-/-}$ embryos. This demonstrates a differential role of *Hox* genes belonging to different paralogous group in the axial elongation process, with the middle groups favoring tissue growth while the posterior group favors termination of the growth process. The work in this thesis also, for the first time reveals insights into the mechanism whereby the transcription factors *Cdx* and *Hox* regulate embryonic body axis elongation (Chapter 3). Posterior axial extension in *Cdx2*$^{+/-}$ /*Cdx4*$^{-/-}$ was rescued by the overexpression of an activated *Lef1* construct, constitutively turning on the canonical Wnt downstream targets. This indicates that *Cdx* gene products maintain Wnt signaling in the embryonic posterior growth zone by a positive feedback loop on the Wnt pathway.

Interestingly, a positive feedback loop exists between *Brachyury* (*T*), a transcription factor known since long to be required for embryonic axial elongation (Dobrovolskaia-Zavadskaia, 1927) and canonical Wnt signals (Martin and Kimelman, 2008, Yamaguchi *et al*., 1999). However the relationship between *Brachyury* and *Cdx* is still elusive. In *Cdx2* null embryos, *T* expression seems unaffected at least at early stages (Chawengsaksophak et al., 2004), and unpublished data of S. Forlani and J. Deschamps. In *T*^{WIS} mutant mice, *Cdx4* expression is not dependen on *T* (Conlon

et al., 1995). Whether complete *Cdx* loss of function will affect *T* expression remains to be seen. Future investigations to understand the relationship between *Cdx* and *Brachyury* at their initiation stage as well as during the maintenance phase might help to decipher the genetic cascade underlying posterior body axis elongation in vertebrates.

A second mechanism of action of *Cdx* transcription factor in sustaining axial elongation in the embryonic trunk affected in our *Cdx* mutants is the clearance of retinoic acid (RA) diffusing from the somites. Mutants in the gene encoding the RA degrading enzyme *Cyp26a1* exhibit a posterior axial truncation resembling that in *Cdx2*$^{+/-}$/*Cdx4*$^{-/-}$ mutants (Abu-Abed *et al.*, 2001; Sakai *et al.*, 2001). The decrease in expression of *Cyp26a1* in our *Cdx* mutant embryos probably contribute to their premature arrest of axial extension.

In mouse embryos, the most severe axial truncation reported so far arrests development from a level caudal to the forelimb (*Wnt3a*-Takada *et al.*, 1994; *Brachyury*-Dobrovolskaia-Zavadskaia, 1927; *Cdx2*-Chawengsaksophak *et al.*, 2004). It is still unclear why the anterior trunk remains unaffected by any mutation so far. Interestingly, loss of *Cdx* function in the short germ band insect, *Tribolium* arrest axial extension at the level just caudal to the head (Copf *et al.*, 2004). Since a *Cdx* mutant allele combination with the most severe phenotype still leaves a *Cdx2* allele intact, it will be interesting to use the *Cdx2* conditional allele generated in this work to produce a total *Cdx* loss of function and investigate whether it affects the anterior trunk during axial elongation.

Cdx2 is required at several steps of embryogenesis. Complete loss of *Cdx2* at the zygotic stage caused embryonic lethality at implantation due to defective extraembryonic trophoblasts (Chawengsaksophak *et al.*, 1997; Strumpf *et al.*, 2005). If active *Cdx2* is supplied in the extraembryonic tissues, either by aggregation of a *Cdx2* deficent morula with a tetraploid wildtype morula (Chawengsaksophak *et al.*, 2004), or by conditionally inactivating *Cdx2* in the epiblast exclusively (Chapter 5), the embryos die around E10.5 (in most cases) of placental failure. Those that survive fail to generate their posterior tissues in the three germ layers. The *Cdx2* conditional knock-out (*Cdx2*^{loxP}) mice that we generated are healthy and breed normaly. Complete loss of *Cdx2* in embryonic tissues was obtained by crossing the conditional knock out strain with the epiblast *Cre* deleter mouse strain (*Sox2Cre*-Hayasi *et al.*, 2002). *Cdx2* null embryos obtained in this way die around E10.5 as they fail to establish a functional placental labyrinth, a structure with contribution from the epiblast. They also exhibit a very severe posterior truncation from a level caudal to the forelimb. Their phenotype is therefore similar to that of *Cdx2* null embryos produced by tetraploid aggregation, but they can be produced more easily by natural breeding. To address questions regarding the role of *Cdx2* in later developmental events, the conditional *Cdx2* allele can be inactivated by a *Cre* deleter strain driven by a promoter active at a later stage or in a specific subset of tissues. This was recently done for the intestinal endoderm by Gao *et al.* (2009). These authors inactivated *Cdx2* in the early gut endoderm using a *Foxa3Cre* deleter strain. Their work demonstrates an additional and later requirement of *Cdx2* in the specification of posterior gut endoderm identity. At E12.5, markers of anterior (oesophagus and stomach) epithelium were expressed in the mutant posterior intestine. Subsequently, activation of the foregut

transctiptional program in the mutant posterior gut occurred (between E14.5 and 18.5), indicating acquisition by this posterior endoderm of an anteriorized identity. The *Cdx2* null phenotype recapitulates the occurrence of squamous metaplastic areas that did not express *Cdx2* around the caecum in *Cdx2* heterozygous mutant mice by the laboratory of Felix Beck (Beck *et al.*, 1999). These findings provide molecular evidence that *Cdx2* promotes posterior intestinal cell fate and antagonizes the foregut differentiation program. Strikingly, these changes in intestinal identity were not accompanied by a change in the enteric *Hox* expression domains, showing that they occur independently of the acquisition of *Hox*-mediated positional information.

In conclusion, the data involve *Cdx2* in the early function of specifying trophectoderm around E3.5. This function is unique to *Cdx2*. It is neither shared by the other two *Cdx* genes, nor does it involve the *Hox* genes. Around E9.5 to 10.5, *Cdx2* is required to generate a functional chorio-allantoic placental labyrinth. *Cdx4* participated in this function as well, and so do *Hox* genes since they can recue the placental failure of *Cdx* mutants. In an overlapping time window of development (between E8.0 and E11.5), *Cdx2*, and the other *Cdx* genes are essential for elongation of the embryonic body axis, and this function involves the *Hox* genes, since they can take over the axial growth arrested in *Cdx* mutants (Chapter 3). So, posterior tissue generation and acquisition of *Hox*-mediated positional identity occur concomitantly during expansion of the axial and paraxial tissues in the three germ layers. Around E14.5-18.5, *Cdx2* performs an even later function in the regionalization of the intestinal epithelium promoting posterior intestinal cell fate and preventing the foregut differentiation programme in the mid-and hindgut (Gao *et al.*, 2009). This function, that occurs after posterior axial elongation is terminated, when the gut has already been laid down and has fully expanded, may involve *Cdx1*, but it is *Hox*-independent.

In this thesis we have used our new *Cdx2* conditional knock-out mutant strain to study the involvement of *Cdx* genes in the establishment of the germ line. Primordial germ cells (PGCs) are laid down from the epiblast during gastrulation at a position at the base of the allantois in the posterior primitive streak reagion (Ginsburg *et al.*, 1990; Lawson and Hage, 1994). This area expresses *Cdx* genes and *Cdx2* in particular at the time of PGC allocation. Work described in chapter 5 of this thesis shows that the absence of *Cdx2* affects the establishment of the germ line. *Cdx2* homozygous null embryos have a significantly lower number of PGCs at their migration stage and later. In addition, a significantly higher proportion of PGCs in the mutant was found in the yolk sac, an extra-embryonic structures. Although more work is needed to understand the mechanism of PGCs population defects in *Cdx2* mutants, we conclude that loss of *Cdx2* function compromises the generation of a correct number of PGCs founders from posterior proximal epiblast at E7.2 (Ginsburg *et al.*, 1990; Lawson and Hage, 1994). PGCs founders form a cluster of about 45 cells in the wildtype embryos (Lawson and Hage, 1994). A day later the cluster fragment and PGCs start their migration along the developing hindgut endoderm. Cues for migration, survival and proliferation during migration must be present in the hindgut. *Nanos3* (Suzuki *et al.*, 2008; Tsuda *et al.*, 2003), *Kit* and its ligand/*Steel* (Dolci *et al.*, 1991), and *Dnd1* (Noguchi and Noguchi, 1985; Sakurai *et al.*, 1994) are essential for the survival of the germ cells during migration. Since mutation in *Cdx2* caused endodermal defect, these cues might be disrupted and alter PGCs migration and/or proliferation all

through this process. This might cause both the lower number of embryonic PGCs and the higher number of PGCs ending up in the yolk sac. Future studies of endodermal defects of *Cdx* mutant hindgut and yolk sac might shed light into the cues required for PGCs migration as well as survival and proliferation during migration.

Summary

The mouse genome harbors three *Cdx* genes, orthologs of the invertebrate caudal gene. *Cd*x and *Hox* gene share common ancestors, the *ProtoHox* genes. An additional link between *Cdx* and *Hox* gene is that Cdx proteins regulate at least a number of *Hox* genes.

In chapter 1, I survey the expression and function of *Cdx* genes in vertebrates and some invertebrates. A comparative analysis of the early and later expression of *Cdx* and *Hox* genes in the mouse is then presented in chapter 2.

Chapter 3 reports on a detailed analysis of *Cdx* mutant defects in the generation of posterior axial and paraxial tissues, and reveals that overexpression of trunk *Hox* genes can rescue these defects whereas the posterior most *Hox* genes induce an arrest of posterior tissue elongation. This chapter also presents a novel insight into the mechanism whereby *Cdx* and trunk *Hox* genes stimulate whereas posterior most Hox genes arrest axial extension: the former genes maintain active canonical Wnt signaling in posterior embryonic tissues whereas the latter negatively interfere with this function, possibly by a post transcriptional phenomenon called *Posterior Prevalence*, also at work in *Drosophila*. A second mechanism contributing to the modulation of posterior embryonic growth is the *Cdx*/trunk *Hox*-dependence of *Cyp26a1*, the gene encoding an enzyme causing degradation of retinoic acid, a morphogen that inhibits stem cell maintenance.

Chapter 4 describes the specific defects of *Cdx* mutants in the intestinal endoderm and caudal mesoderm, also rescued by trunk *Hox* genes.

Chapter 5 reports on the use of the conditional *Cdx2* mutant that I made, to shed light on the role of *Cdx* genes in the establishment of the mouse germ line. The *Cdx2* conditional mutant should allow future research on the role of *Cdx* genes in developmental processes beyond the stage when *Cdx* mutants die, such as gut endoderm differentiation and homeostasis.

In chapter 6, I discuss the implications of my results and the priorities for future research along the paths opened by my work.

Samenvatting

Drie *Cdx*-genen, ortholoog aan het caudal-gen van ongewervelde dieren, worden in het genoom van de muis gevonden. *Cdx*- *Hox*- genen hebben dezelfde gezamenlijke voorouders, de *ProtoHox* genen. Nog een verband tussen *Cdx* and *Hox* genen is dat minstens een aantal *Hox*-genen door *Cdx* eiwitten wordt gereguleerd.

In Hoofdstuk 1 geef ik een overzicht van functie en expressie van *Cdx*-genen van vertebraten en evertebraten. Vervolgens geef ik in Hoofdstuk 2 een vergelijkende analyse van *Cdx*- en *Hox*- expressie in de muis.

Hoofdstuk 3 is het verslag van een gedetailleerde analyse van defecten in *Cdx*-mutanten bij het vormen van de caudale weefsels. Het blijkt dat bij overexpressie van *Hox*-genen de zogenaamde trunk *Hox*- genen, met een expressie-grens in de romp van het embryo, deze defecten kunnen compenseren, in tegenstelling tot de *Hox*-genen met de meest posterieure expressiegrens, die een blokkade van de asverlenging veroorzaken. Dit hoofdstuk verschaft ook nieuw inzicht in het mechanisme van stimulering van asverlenging door *Cdx* en trunk-*Hox* genen tegenover het blokkeren daarvan door de posterieure *Hox*-genen. De eerste zorgen voor het op peil houden van actieve Wnt signalering in posterieure embryonale weefsels, terwijl de laatstgenoemde genen hiermee juist interfereren, mogelijk via '*posterior prevalence*' een post- transcriptioneel verschijnsel bekend van werk in *Drosophila*. Een tweede mechanisme dat van invloed is op regulatie van posterieure embryonale groei is *Cdx*/trunk *Hox*-afhankelijke expressie van *Cyp26a1*. Dit gen codeert voor een enzym dat verantwoordelijk is voor afbraak van retinoc acid (retinolzuur), een morfogen dat de instandhouding van stamcellen remt.

Hoofdstuk 4 beschrijft specifieke defecten in het endoderm van de darm en het posterieure mesoderm van *Cdx*-mutanten. Ook deze defecten worden gecompenseerd door overexpressie van trunk *Hox*-genen.

Hoofdstuk 5 doet verslag van het gebruik van een door mij gemaakte conditionele *Cdx2*-mutant waarmee een rol van *Cdx*-genen in het vastleggen van de kiembaan van de muis wordt bevestigd. De conditionele *Cdx2* mutant maakt toekomstig onderzoek mogelijk naar de rol van *Cdx*-genen in ontwikkelingsprocessen als darm-endodermdifferentiatie en homeostase op ontwikkelingsstadia voorbij het voor de reguliere *Cdx*-mutant letale stadium.

Hoofdstuk 6 is een algemene discussie waarin ik een overzicht geef, niet alleen van de implicaties van mijn waarnemingen, maar ook van de prioriteiten bij toekomstig onderzoek dat voortvloeit uit mijn werk.

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Curriculum vitae

Teddy Young was born on the 21st of August 1979 in Medan-Indonesia. In May 1996, he finished his pre-university education at the SMA Katolik Budi Murni 1, in Medan. He then studied Chemical Engineering at the Universitas Katolik Parahyangan in Bandung where he earned B.Sc. in August 2000. Immediately after his graduation, he worked in this university as teaching assistant for Purification and Separation subject and later on he taught Introduction to Biochemistry till October 2002. In November 2002 he went to Dresden, a beautiful city situated in a valley on the River Elbe in the eastern Germany, to start a master degree study in Molecular Bioengineering at the Technische Universität Dresden. To finish this master study, he did a final project at the Max Planck Institute of Molecular Cell Biology and Genetics, Dresden under the supervision of Dr. Andrew C. Oates. Nine months after the start of this final project, he graduated in January 2005. In May 2005 he started his Ph.D. project in the research group of Dr. Jacqueline Deschamps at the Hubrecht Institute in Utrecht that is described in this book. In the future he plans to continue science in Singapore.