Cdx genes and the maintenance of tissue progenitors in the mouse

Monika Białecka

"Research is to see what everybody else has seen, and to think what nobody else has thought"

"A discovery is said to be an accident meeting a prepared mind"

Albert Szent-Gyorgyi

Utrecht 2012

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Cdx genes and the maintenance of tissue progenitors in the mouse

Cdx-genen en voorlopers van embryonale en volwassen weefsels in de muis
(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof.dr. G.J. van der Zwaan, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op donderdag 19 januari 2012 des middags te 12.45 uur

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SUMMARY

The three Cdx genes play important functions in the embryonic and adult life of a mouse. *Cdx2* has an early onset of expression in the trophectoderm of the blastocyst stage embryos (E3.5) where it is crucial for implantation in the uterine tissue. All three genes, *Cdx1*, *Cdx2* and X-linked *Cdx4*, are expressed during gastrulation and axial elongation and are involved in axial growth. Mutations in Cdx genes cause axial truncation with a varying severity depending on the allele combination. **Chapter 1** contains an overview of the embryonic development of the mouse embryo and the role of Cdx genes in this process.

In **chapter 2** we challenged one of the hypotheses regarding the impact of Cdx mutations on axial elongation. Posterior elongation of the mouse trunk and posterior body had been shown to depend on tissue generation from progenitor cells located in the posterior of the embryo that we called the posterior growth zone. In particular a small cell subpopulation called the long term progenitors with stem cell properties plays a crucial role in axial growth. We asked whether we could identify cell-autonomous defects in long term axial progenitor of Cdx mutants by grafting them into a wild type growth zone environment. No difference between the contribution of descendants from wild type and mutant grafted progenitors was detected, indicating that rescue of the Cdx mutant graft occurred by the wild type recipient growth zone. Our data suggested that Cdx genes function to maintain a signaling-dependent niche for the posterior axial progenitors.

The Cdx genes, and *Cdx2* in particular, start to be expressed in the primitive streak and the base of the allantois at mid/late-primitive streak stages, where and when the PGC population emerges, within the posterior growth zone. In **chapter 3** we report that the expression of *Cdx2* around the newly specified PGCs is necessary for proper maintenance of this population at early stages of embryonic development. In $Cdx2^{null}$ embryos the number of PGCs was significantly lower than in wild type littermates. We found that *Wnt3a* loss of function reduces the PGC population to the same extent as *Cdx2* inactivation. Moreover, the decrease in PGC number in $Cdx2^{null}$ posterior embryonic tissues in *in vitro* cultures was corrected by adding *Wnt3a* to the medium. The phenotype of $Cdx2^{null}$ embryos also resembles that of the epiblast-specific $Bmp4^{null}$ mutants. Bmp4 added to the cultures of posterior explants of $Cdx2^{null}$ embryos, recues the PGC population. Since *Cdx2* is not expressed in the PGCs themselves, we proposed that *Cdx2* expression in posterior embryonic tissues ensures a proper niche for the germ cell progenitors by stimulating canonical Wnt and Bmp signaling.

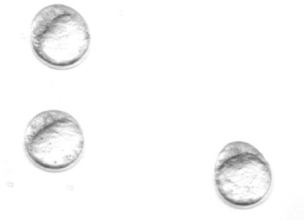
The axial defects of Cdx mutants are corrected by posterior gain of function of the Wnt effector *Lef1*. Precocious expression of Hox paralogous 13 genes induces vertebral axis truncation by antagonizing Cdx function. In **chapter 4** we report that the phenotypic similarity regarding axial growth also applies to patterning of the caudal neural tube and of uro-rectal tracts. Inactivation of *Cdx2* after placentation leads to posterior defects including incomplete uro-rectal septation. Compound mutants carrying one active *Cdx2* allele in the $Cdx4^{null}$ background ($Cdx2^{+/-}Cdx4^{null}$), transgenic embryos precociously expressing Hox13 genes, and a novel Wnt3a hypomorph mutant all manifest a phenotype with similar uro-rectal defects. These mutants also present neural dysmorphology including ectopic neural structures sometimes leading to neural tube splitting at caudal axial levels. These findings involve the Cdx genes, canonical Wnt signalling, and the temporal control of posterior Hox gene expression in posterior morphogenesis in the different embryonic germ layers. Interestingly, our data show that the embryonic defects observed at relatively late stages are caused by a much earlier disruption of signaling in the growth zone.

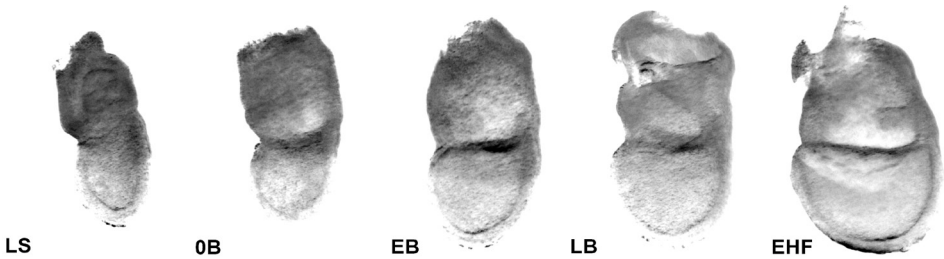
The expression of *Cdx1* and *Cdx2* is maintained in the embryonic endoderm after it is down-regulated in the tailbud by E12.5. In the adult *Cdx1* and *Cdx2* are expressed in the endoderm of the intestine and are involved in establishing the identity of the intestinal epithelium. The strongest *Cdx2* expression is observed at the A-P level of the cecum area. In **chapter 5** we describe the effect of *Cdx2* inactivation in the adult intestinal stem cells using a *Cdx2* floxed allele and $Lgr5CreER^{T2}$. *Cdx2* negative crypts produced subsurface cystic vesicles that express gastric

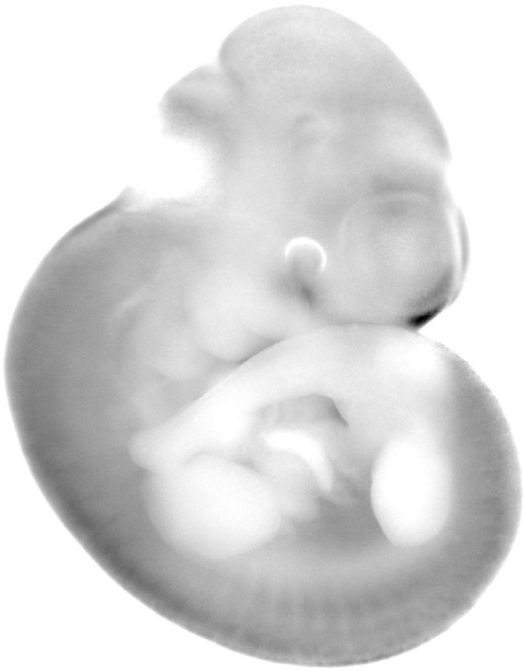
markers and did not contribute to populate the crypt surface any more. Inactivation of *Cdx2* in intestinal stem cells in culture abolishes their ability to form long term growing intestinal organoids in intestine specific conditions in matrigel. These Lgr5^{+ve} *Cdx2*^{null} intestinal stem cells were rescued once cultured in conditions appropriate for pyloric tissues, and generated organoids that could be passaged over a long period of time. These organoids expressed gastric genes instead of intestinal markers, contained some Lgr5 expressing cells and failed to express the gastric endoderm-specific transcription factor *Sox2*. The absence of Cdx2 therefore partly transforms the intestinal into gastric epithelium.

The implications of the work described in this book are discussed in **chapter 6**.

Chapter 1

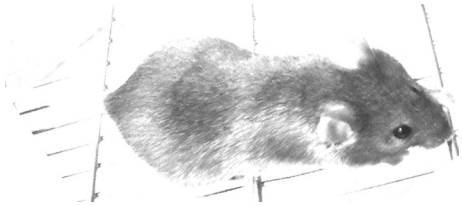






Introduction

Development of the mouse



1. GASTRULATION

The mouse embryo develops in the maternal uterine environment and although not easily accessible, it proved to be a very useful model to study developmental processes and underlying pathological conditions. During gestation, which in mouse takes around 19 days, the fertilized egg divides and later forms a blastocyst that subsequently implants in the uterine wall and undergoes complex morphological processes, leading to the development of the fetus (Figure 1). Gastrulation, a process by which the three germ layers are formed starts around the 6th day of development (E6.0). At this point the embryo proper is of a cup-shaped epiblast, enveloped by a single layer of visceral endoderm (VE, Figure 1). The extraembryonic ectoderm (ExE) is crucial for the initiation of gastrulation as well as for the allocation of some lineage-restricted progenitors. Gastrulation commences with formation of the primitive streak (PS), at the posterior side of the embryo. Primitive streak formation is the first visible sign of the antero-posterior polarity of the embryo. Epiblast cells ingress through the primitive streak to form mesoderm and definitive endoderm (Figure 1C-E). The third germ layer, the ectoderm, is formed from the non-ingressing epiblast cells.

1.1. GENETIC CONTROL OF GASTRULATION

Before the antero-posterior (A-P) axis becomes evident a proximo-distal axis is established by differential gene expression. *Nodal*, initially uniformly expressed in the epiblast (1) activates the expression of its downstream effector, *Smad2* in the overlying visceral endoderm at the tip of the embryo, which initiates formation of the distal visceral endoderm, DVE (2). *Nodal* is required for the maintenance of *Bmp4* in the ExE. Bmp4 in the ExE induces the expression of *Wnt3* and *Nodal* in the epiblast in a proximo-distal gradient. The DVE initiates a cascade of signaling resulting in the expression of *Dkk1* and *Lefty1, Wnt* and *Nodal* antagonists, respectively (3, 4). *Wnt3* is expressed in both posterior visceral endoderm (PVE) and the epiblast. The expression of *Wnt3* in the PVE precedes epiblast expression suggesting a hierarchical relationship between those two sites (5). *Wnt3* signal from the PVE is crucial for initiation of *TBrachyury* expression in the posterior epiblast, thus mesoderm formation (Rivera-Perez, personal communication, EMBO meeting in Leuven, 2011). The DVE migrates proximally to become anterior visceral endoderm (AVE). By continuous expression of *Nodal* and *Wnt* antagonists the AVE shifts the gene expression from proximo-distal to the posterior of the embryo. This breaks the radial symmetry of the embryo and establishes the antero-posterior polarity. Loss of *Nodal* signaling leads to the absence of the anterior primitive streak and in consequence to the loss of axial and paraxial mesoderm as well as the definitive endoderm (6-8).

Besides the dynamic gene expression, cell rearrangements involving cell movement and cell shape changes take place in the gastrulating embryo. Epithelial cells of the epiblast that ingress through the PS aquire mesenchymal characteristics in a process of epithelial-to-mesenchymal transition (EMT). Active Wnt and Fgf signaling is necessary for EMT to occur. Embryos with impaired Fgf signaling (*Fgf8^{null}* and *Fgfr1^{null}* mutants) initiate EMT, but cells at the PS are not capable of migrating away or sustaining the mesenchymal state, thus remain in the vicinity of the PS (9, 10). Additionally EMT requires downregulation of E-cadherin in the adherens junctions (11).

Gastrulation is one of the most crucial events in embryonic life. Embryos, that fail to undergo proper gastrulation, die in utero. By day 7.5 of embryonic development, three germ layers have been formed. Posterior progenitor populations progressively generate the extraembryonic mesoderm and embryonic tissues of the trunk and tail.

2. AXIAL ELONGATION AND THE PROGENITOR POPULATIONS

Embryonic axis elongation occurs in three ways: cell proliferation, convergent extension, and posterior addition of tissues. Convergence and extension include change of the cells shape, cell rearrangements, and directed movement. This results in morphological changes of the developing embryo. The antero-posterior axis extends and becomes narrower by movement of laterally located cells towards the embryonic midline. Convergent

extension is regulated by the non-canonical Wnt pathway, also called planar cell polarity (PCP) pathway (12).

Trunk and tail tissues of the mouse embryo are generated in the process of posterior addition of tissues from a pool of progenitors located at the caudal end of the embryo. This process is similar to body extension in some insects that extend their body from the posterior growth zone. By analogy this region in the mouse was called the posterior growth zone (81). In the process of generating new tissues, progenitor populations allocated during gastrulation lay down descendants and generate organs and structures posteriorly to the head. All primordia of trunk and tail tissues are formed by E13.5. Until then, the embryo forms around 60-65 somites and these mesodermal blocks will form the axial skeleton, tendons and musculature. Somites flank the neural tube, the prospective of spinal cord, and the notochord, a midline mesodermal tissue that patterns the neural tube and the somites.

2.1. PROGENITOR POPULATIONS IN THE PRIMITIVE STREAK

2.1.1. The fate map of cells flanking the primitive streak in the pre-somitic stage embryo (E7.5)

From E6.25 the primitive streak elongates and extends towards the distal tip of the embryo. During this period distinct mesodermal progenitor populations are formed according to time and position of the cells ingressing from the epiblast (13). Mouse embryos can be cultured as whole mounts, over certain time periods *in vitro* in medium supplemented with mouse or rat serum. This allows to label cells by incorporation of a fluorescent dye (like DiI-1,1'-dioctadecyl-3,3,3',3'-tetramethyl indocarbocyanine perchlorate in sucrose solution) or to label single cells by electroporation with the horse radish peroxidase (HRP) to follow their contribution to the axis after in vitro culture (usually 24-48hrs). This technique allowed the establishment of a fate map of the embryo at different early developmental stages (15).

The posterior-most mesoderm contributes descendants to the extraembryonic tissues: mesoderm of the allantois, yolk sac, the chorion and blood islands of the yolk sac. Intermediately and more anterior levels of the primitive streak contribute to the cardiac, lateral plate mesoderm and paraxial mesoderm. The anterior tip of the primitive steak (the node) gives rise to the axial mesoderm, the notochord and definitive endoderm (DE). With the progress of gastrulation DE progressively intercalates into the VE (14).

2.1.2. The fate map of cells flanking the primitive streak in the early somite stage embryo (E8.5)

Lineage tracing experiments in E8.5 embryo (15-17), revealed that in contrast to the primitive streak of the gastrulating embryo at E7.5, the posterior streak of the E8.5 embryos does not contribute cells to the extraembryonic lineages anymore. The mesoderm cells located in the posterior third of the streak contribute descendants to the lateral mesoderm. The middle and anterior streak continue to give rise to paraxial (somitic) mesoderm and axial mesoderm. The somitic mesoderm which comes from the anterior streak is found medially in the somite, whereas mesoderm that originated from more posterior streak levels contributes to the dorso-lateral side of the somite (15). The node contributes cells to the axial mesoderm (notochord). Cells emerging from the node also contribute to the definitive endoderm and floor plate of the neural tube. Neither node nor the anterior streak gives rise to lateral plate mesoderm (15).

2.2. EVIDENCE FOR THE EXISTENCE OF AN AXIAL STEM CELL POPULATION

2.2.1. Tissue transplantations

The primitive streak of early embryos persists in the tail bud at later stages (16, 17). The descendants of a small population of cells in the anterior steak and the caudal node, labeled at E8.5, is found later on in the chordoneural hinge (CNH) area in the tail bud of E10.5 embryos. This population residing at the junction of the node and the streak in E8.5 embryos has been named the node streak-border, NSB. With the use of tissue transplantations, a GFP labeled NSB area was grafted into the NSB of non-GFP recipient embryo (18). It was shown that the NSB contributes descendants to two germ layers: neuroectoderm (ventral neural tube) and paraxial mesoderm (somites) (17, 18), as well as to the CNH area. The CNH contains descendants of the node and anterior streak

(17). Serial transplantations of the CNH area from E10.5 embryos into the NSB area of E8.5 embryos has shown that progenitors located in the tail bud of the donor have the potency to contribute to rostral through caudal somites, neural tube and notochord, in the recipient embryo, suggesting the presence of a resident stem cell like population (17). These observations provide evidence for the presence of a stem cell population in the node-streak border and CNH area.

2.2.2. Retrospective clonal analysis.

Retrospective clonal analysis uses a *LaacZ/LacZ* system to label a cell population with β-galactosidase, as a result of a single cell event that subsequently has to be traced back. *LaacZ* is a modified version of the E.coli *LacZ* gene containing an inactivating sequence duplication inserted into the transgene. A random intragenic recombination event re-establishes the open reading frame for the reporter gene, thus reverting it to a functional *LacZ* and labeling the cells' progeny (19). β-Gal positive clones of cells in the embryo are traced back to original single cell in which, the recombination has occurred. Studies using this system under tissue specific promoter have been used to investigate the cellular genealogy of the embryonic muscle, myocardium, CNS, and melanocytes (20-25). Retrospective clonal analysis with the use of the *Rosa26* promoter revealed the existence of neuro-mesodermal (N-M) progenitors with bipotent properties that contribute descendants to the neuroectoderm and mesoderm during the whole axial elongation (19). These progenitors presumably locate to the NSB and later on in the CNH. It is indeed likely that the population of cells in the NSB and later in the CNH comprises the long-term stem cell population for the axis. Further support of that hypothesis comes from experiments in which a CNH graft from the E12.5 embryo was inserted orthotopically into the E10.5 recipient. Descendants of the E12.5 graft also contribute to mesoderm and neuroectoderm suggesting that the CHN retains multipotency over long periods (17).

2.3. SHORT-TERM PROGENITORS IN THE PRIMITIVE STREAK

2.3.1. Paraxial mesoderm

While some progenitors for the paraxial mesoderm belong to the axial stem cell-like population described above, the dorso-lateral part of the somites derives from progenitors in the primitive streak that do not self-renew. Paraxial mesodermal progenitors leaving the streak end up flanking the notochord and become pre-somitic mesoderm (PSM), which forms the somites. The PSM contains enough mesoderm to form several somites (depending on the developmental stage) and is continuously supplied by the posterior growth zone. The somites are formed periodically (2hrs per somite) in a rostro-caudal sequence and later differentiate into vertebrae and intervertebral disks of the vertebral column, skeletal muscles, tendons and much of the dermis of the skin. A molecular oscillator termed the segmentation clock governs the periodicity of somite formation. Somites and their derivatives acquire the morphological identity corresponding to their position along the antero-posterior axis, which is mainly imposed by Hox genes. The first 5 somites are incorporated into the basi-occipital bone at the base of the skull and the following somites form the vertebral column. Forelimbs develop at the level of somites 7-12 and hindlimbs at the level of somite 24-30. The cervical part of the vertebral column contains 7 vertebrae (C1-C7); the thoracic part 13 (T1-T13) of which the first 7 form the rib cage and the rest contains free floating ribs. The lumbar part of the vertebral column consists of 5 vertebrae (L1-L5) and the sacral is formed from 4, fused vertebra. The caudal vertebral column contains variable number of vertebrae, 31-35 in mouse, forming the tail. The process of somitogenesis is controlled by the Wnt, Fgf and Noth pathways and is described in (26).

2.3.2. Lateral plate mesoderm

The lateral plate mesoderm emerging from the streak area gives rise to the mesodermal lining of endoderm derived tissues and organs (gut tube and associated organs and the respiratory tract) the mesodermal lining of the body wall, as well as to the hart, circulatory and uro-genital systems.

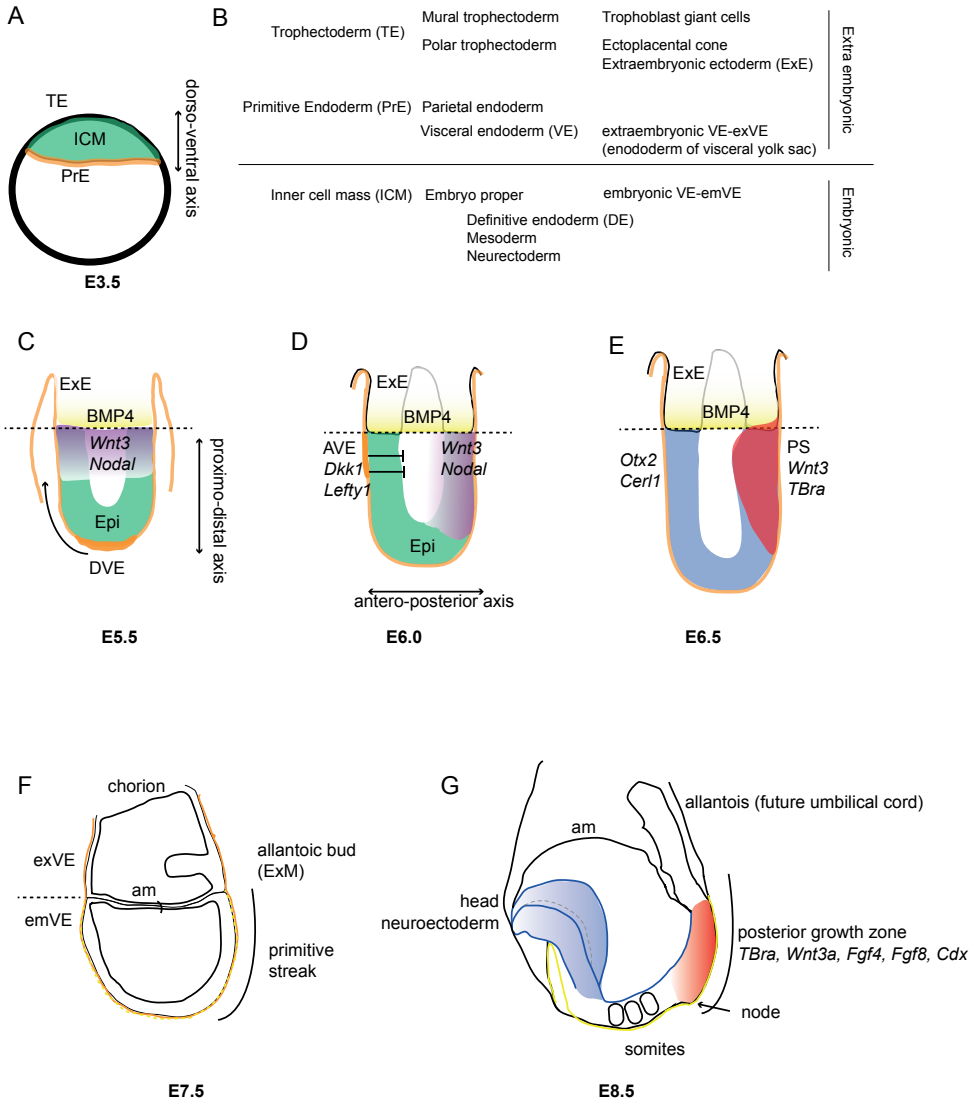


Figure 1 Gastrulation and axial elongation in the mouse

Three days after the fertilization, the mouse embryo forms a blastocyst (**A**). Pluripotent cells of the inner cell mass (ICM) are surrounded by trophectoderm cells (TE). Adjacent to the ICM, a layer of *Gata4* positive cells, which give rise to the primitive endoderm (PrE) and determine the first axis of the embryo, the dorso-ventral axis. **B**-Scheme representing cell lineage relationships in the early mouse embryo. After the implantation in the uterine wall, the embryo acquires a cylindrical shape (**C**). The proximo-distal axis is set by the distal visceral endoderm (DVE) by E5.5. *Wnt3* and *Nodal* are expressed in the epiblast in a proximo-distal gradient (**C**). The DVE migrates to the future anterior side of the embryo and by expressing the antagonists of Wnt and Nodal pathways (**D**) restricts the formation of the primitive streak, marked by *Wnt3* and *Nodal* expression, to the future posterior of the embryo (**E**). **F**-Schematic representation of the E7.5 embryo. Note that the VE shown in orange is being displaced by the definitive endoderm shown in yellow. **G**- Schematic representation of the E8.5 embryo. Neural folds forming the head neurectoderm are shown in blue. The VE is displaced by definitive endoderm and forms the anterior gut tube. The posterior growth zone, shown in red, supplies descendants for the trunk and the tail. TE-trophectoderm, ICM-inner cell mass, PrE-primitive enododerm, VE-visceral endoderm, ExE-extraembryonic ectoderm, DE-definitive endoderm, AVE anterior visceral endoderm, DVE-distal visceral endoderm, Epi-epiblast, PS-primitive streak, am-amnion, ExM-extraembryonic mesoderm.

2.3.3. Neurectoderm

The AVE overlying the anterior epiblast of the E6.5 embryo, patterns the head neurectoderm, which develops into the brain later on. The anterior neural tube is shaped from the ectoderm layer by convergence and extension, under control of the PCP pathway in the process called the primary neurulation. The posterior neural tube is formed in the process of the secondary neurulation, from a pool of neural progenitors located in the tailbud. These neural progenitors, in the dorsal tailbud undergo condensation and epithelialization to form the secondary neural tube, which is continuous with the neural tube formed by the primary neurulation. Secondary neurulation generates the lowest part of the spinal cord posteriorly from the sacral region. The primary neural tube closes at 3 points. Closure 1 is initiated at the hindbrain/cervical boundary and proceeds in both directions caudal and rostral. Closure 2 is initiated at the forebrain/midbrain boundary and closure 3 at the extreme rostral end of the forebrain. Mutations in components of the PCP pathway lead to neural tube defects, which result in an open neural tube at different positions of the axis.

2.3.4. The germ line

The germ cell lineage is the first lineage to be segregated from the pluripotent epiblast adjacent to the extraembryonic ectoderm in the early mouse embryo (27, 28). Primordial germ cells (PGCs) were first identified over 50 years ago due to their high level of tissue nonspecific alkaline phosphatase (TNAP) (29). At an early stage of embryonic development (E8.0) PGCs are found as a cluster of approximately 45 TNAP positive cells at the base of the allantois (28, 30, 31). Although TNAP is not necessary for germ cell survival (32) it allows to identify PGCs from the time they become lineage restricted around E7.2 until their entry to the genital ridges (future gonads) two days later.

Twenty-four hours after specification the germ cell cluster formed at the base of the allantois brakes down and PGCs begin to migrate. Some PGCs are found at ectopic positions in the allantois, amnion or yolk sac, however most of them move into the definitive endoderm. As the endoderm starts invaginating to form the hindgut, PGCs migrate through the hindgut portal and later on remain confined within the hindgut epithelium (Figure 2). The PGCs at the lip of the hindgut pocket have rounded non-motile shape suggesting that they are being carried along by endoderm (passively incorporated). During the migration period along the hindgut, PGCs tend to contact each other by thin cytoplasmic extensions thus form a network of migrating cells (33). Simultaneously PGCs actively proliferate to reach ~6000 cells by the time they enter the genital ridges at E10.5. Later on PGC sex specification occurs based on the sex of the somatic gonadal tissue occurs. Proliferation of germ cells within gonads continues till E13.5 when they reach the approximate number of 35 000 cells. In males PGCs undergo mitotic arrest within seminiferous cords whilst in females germ cells enter meiosis.

PGC specification

PGCs emerge from the proximal epiblast upon Bmp4/Bmp8b signaling as a population of cells expressing *Fragilis/Ifitm3* (34). Among these cells PGC precursors can be distinguished as early as E6.25 as a small subpopulation expressing the transcriptional regulator *Blimp1*. *Blimp1* is responsible for repressing the somatic program while establishing the germ cell character (34) in precursor cells. "True" PGCs start expressing *Stella*, the first germ cell restricted marker. As the epiblast loses its pluripotent characteristics, the PGCs are the only population retaining expression of pluripotent markers such as *Oct3/4*, *Nanog* and *Sox2*. Fragilis initially marking precursor cells is still expressed until PGCs enter the genital ridges. In *Blimp1*^{null} embryos a PGC cluster is formed but cells do not migrate away from it or proliferate properly. Also repression of Hox genes is aberrant in *Blimp1*^{null} *Stella* expressing PGCs, all suggesting a crucial role of *Blimp1* in germ cell development (34). *Fragilis* and *Stella* are both dispensable for germ cell development and/or survival since mutants in these genes do not display any defects in germ line development (35, 36).

PGC migration

After specification, PGCs become transcriptionally inactive/silent. Genome wide reprogramming takes place,

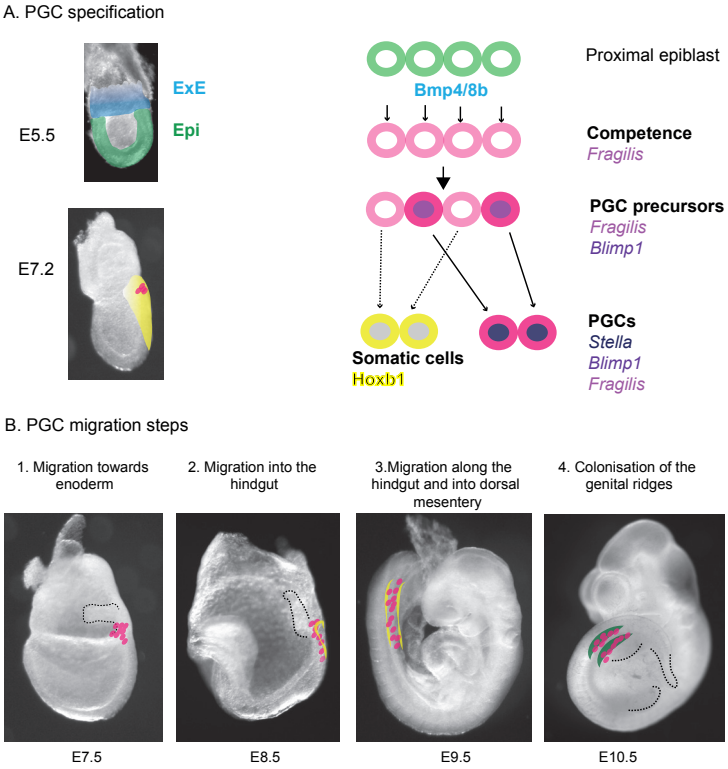


Figure 2 The germ line development in the mouse
A-Germ cell specification (based on (82). Development of early postimplantation embryo from E5.0 to E7.5, depicting the formation of PGCs. The proximal epiblast cells respond to signals from the extraembryonic tissues, which induce expression of *fragilis* in the epiblast, and of *Blimp1* in the PGC precursor cells at one end of the short axis before gastrulation. After gastrulation, the PGC precursors locate to the posterior proximal region, where they undergo specification to form the founder population of Stella-positive PGCs. **B**- PGCs migration in mice (based on (83). PGCs specified in the proximal epiblast, migrate from the primitive streak to the endoderm (future hindgut) at embryonic day 7.5 (E7.5; step 1) and migrate along the endoderm (step 2). At E9.5, PGCs migrate bilaterally towards the dorsal body wall (step 3). At E10.5, PGCs reach the genital ridges to form the embryonic gonad (step 4).

which includes histone modification such as the erasure of H3K9me2 and up regulation of H3K27me3. The c-kit/Steel Factor (SF) signal transduction pathway is required for normal migration of the PGCs. Disruption of either the receptor and/or the ligand, causes abnormalities in PGC migration and proliferation accompanied by formation of PGC clumps in the hindgut and apoptosis (37). Additionally some PGCs also fail to leave the base of the allantois. 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. Initially 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 dependent on another receptor-ligand system: CXCR/SDF1 (38). Mutant mice for either CXCR or SDF1 have genital ridges containing a few germ cells, with unaltered division rate and survival. Progressive loss of PGCs from the beginning of their migration is also observed in Ter mice carrying a mutation in the *Dead-end1* gene (39) as well as in *Nanos-3* knockout mice (40). In both cases PGC survival is severely compromized and results in apoptosis.

3. GENETIC CONTROL OF AXIAL ELONGATION

The canonical Wnt pathway and TBrachyury

Several signaling pathways control axial elongation. *TBrachyury*, which is initiated by *Wnt3*, is expressed in the primitive streak, nascent mesoderm and later on in the tail bud for the entire period of axis elongation (41). It is also expressed in the node and notochord and at the base of the allantois (42). *TBrachyury*^{null} embryos present

severe axial truncation and form only 7-8 somite pairs. Mutant embryos have very short allantois, which fails to grow and fuse with the chorion, thus prevents development beyond E10.5. *TBrachyury* is necessary for normal morphogenetic movements during earlier stages of gastrulation. Experiments with chimeric embryos containing *TBrachyury*^{null} mouse ES cells (mES) cells shown that *TBrachyury*^{null} cells accumulate in the primitive streak and the node and are not able to move away. Additionally cell accumulation is also observed at the base of the allantois (43, 44). Consistent with that finding, *TBrachyury* is required for the elongation and vascularization of the allantois (45). The neural tube of *TBrachyury* mutants is abnormally kinked with ectopic structures of a neural character, located ventrally to the endogenous neural tube (46). *TBrachyury* is co-expressed with *Wnt3a* in the primitive streak and was shown to be a direct target of the canonical Wnt pathway (46). *Wnt3a*^{null} embryos present a similar phenotype to that of *TBrachyury*^{null} embryos with severe truncation of the axis and ectopic neural structures (47). However, on the contrary to *TBrachyury*^{null} , *Wnt3a*^{null} mutants do not present defects in allantois development and can develop to term. *Wnt3a* is the only canonical Wnt expressed in the posterior during axial elongation. It is activated in the primitive streak area later than *TBrachyury* (47) and its expression overlaps with *Wnt3* expression in primitive streak stage embryos. In *Wnt3a*^{null}mutants the appearance of ectopic neural tubes correlates with the shortage of paraxial mesoderm. This suggests that in the absence of *Wnt3a* signaling, paraxial mesoderm cannot sustain its character and adopts the "default", neural fate (48). This is supported by the absence of *TBrachyury* expression in the posterior of *Wnt3a*^{null} mutants (46). *Tcf1* and *Lef1* are downstream effectors of the canonical Wnt pathway and are expressed in the posterior mesoderm of the embryo during axial elongation. Double *Tcf1*^{null}/*Lef1*^{null} mutants display very similar phenotype to that of *Wnt3a*^{null} mutants but additionally have defect in placental formation due to impaired allantoic growth (49). *Lef1* is dispensable for the initiation of *TBrachyury* expression, however it is necessary to maintain *TBrachyury* expression in the posterior (50). The canonical Wnt pathway in cooperation with *TBrachyury* governs mesoderm formation and mesoderm vs. neurectoderm fate choice.

The Fgf pathway and epithelial-to-mesenchymal transition

The Fgf pathway is also implicated in axial elongation. Fgfr1 (Fgf receptor 1) is expressed throughout the epiblast prior to gastrulation, and its expression becomes preferentially located around the posterior streak as gastrulation proceeds. *Fgfr1*^{null} mutants die at gastrulation. In these mutants, cells traversing the primitive streak accumulate posteriorly severely impairing formation of the paraxial mesoderm (51, 52). Embryos, which carry a mutation in the Fgfr1 ligand, Fgf8 present a similar phenotype to *Fgfr1*^{null} mutants. In *Fgfr8*^{null} embryos mesoderm is not formed since ingresing cells fail to migrate away from the primitive streak (9). Cells, which accumulate beneath the primitive streak, express abnormally high level of E-cadherin, normally down-regulated as cells undergo EMT (10). Similarly to the situation in the *Wnt3a*^{null} mutants *TBrachyury* expression is down-regulated in *Fgfr1*^{null} embryos in a similar domain of the primitive streak (10, 46). *TBrachyury* and the Fgf and Wnt pathway cooperate together to ensure correct signaling in the posterior growth zone, thereby creating a suitable microenvironment for axial progenitors. Additionally, the transcription factors encoding Cdx genes, belonging to the para-Hox gene family, are essential for posterior development of the mouse embryo and for its A-P patterning.

4. CDX GENES IN AXIAL ELONGATION AND PATTERING

The Cdx genes are ortologues of *Drosphila Caudal* and are involved in the determination of the posterior part of the body. The mouse genome contains three Cdx genes: *Cdx1, Cdx2* and the X-linked *Cdx4. Cdx2* is expressed the earliest of all Cdx genes, since it is transcribed in the trophectoderm (TE) of the blastocyst (53) and is crucial for embryo implantation in the uterine wall (54). The *Cdx2*^{null} mutation is therefore perimplantation lethal. At the postimplantation stages *Cdx2* expression is detected in the TE derivative, the ExE. In the embryo proper, *Cdx2* expression is initiated around the late streak stage in ectoderm and mesoderm of the primitive streak and at the base of the allantois. Later on *Cdx2* is expressed in the posterior growth zone in the tailbud. *Cdx1* and *Cdx4* are both expressed similarly to *Cdx2*, in the primitive streak area and the tailbud. *Cdx4* is expressed in the allantois while *Cdx1* is not. At somite stages, all three genes have different rostral boundaries of expression with the most

anterior being for *Cdx1* and the less anterior for *Cdx4. Cdx4* expression is down-regulated around E10, while *Cdx1* and *Cdx2* remain expressed until E12.5 in the tail bud. Both *Cdx1* and *Cdx2* remain expressed in the gut endoderm until with a rostral boundary at the duodenum.

Cdx genes belong to the para-Hox genes family and they cause vertebral homeotic transformations, when mutated, in a similar way to Hox genes. $Cdx1^{null}$ and $Cdx4^{null}$ single or double mutants are viable and fertile and have no defect in axial elongation. They present homeotic transformations of a varying severity (55-57). $Cdx1^{null}$ mutants show vertebral transformations from the level of the first cervical vertebra (C1; the atlas) to the level of the first lumbar vertebra (L1) and the $Cdx4^{null}$ mutation slightly increases the severity and penetrance of $Cdx1^{null}$ phenotype. *Cdx2* heterozygote animals present anterior transformation of vertebrae from the level of C6 to the level of T8 with incomplete penetrance (54, 56). Additionally a mild axial truncation and a kinky tail are observed in these animals. Besides axial defects, *Cdx2* heterozygotes develop multiple intestinal adenomatous polyps, particularly in the proximal colon where *Cdx2* expression is normally the strongest (54) also described in more detail in chapter 5).

$Cdx1^{null}Cdx2^{+/-}$ animals present more severe axial truncation. These double mutant mice exhibit more alterations and their anatomical boundaries are shifted more posteriorly (56). Most of $Cdx2^{+/-}Cdx4^{null}$ mutants die at E10.5 due to placental deficiency. Their shortened allantois prevents fusion with the chorion and in cases when fusion occurs, branching and elongation of embryonic vessels in the placenta is defective (57). Due to variation in the severity of the mutant phenotype, around 10% of $Cdx2^{+/-}Cdx4^{null}$ survive until birth. Newborns have a very short or absent tail, with malformations in the lower vertebral column, dilated bladder and blindly ending gut which all lead to neonatal death (58, 59). These defects, reminiscent of the caudal regression syndrome are described in chapter 4. $Cdx2^{+/-}Cdx4^{null}$ mutants thus have severely compromised axial development. A shortening of the pre-somitic mesoderm is the first manifestation of the phenotype, visible at the 7/9-somite stage. The most posterior somites are smaller and sometimes fused.

$Cdx2^{null}$ mutants have been first obtained by aggregation of tetraploid wild type morula with $Cdx2^{null}$ ES cells (60). Since the generation of a *Cdx2conditional* allele (84), the use of the *Sox2Cre* recombinase (83) enables us to ablate *Cdx2* in the epiblast from the beginning of its expression. Generation of the conditional allele has been described elsewhere (84). $Cdx2^{null}$ embryos develop about 17 somite pairs and are severely truncated posterior to the forelimb. Mutants can be recognized at E7.5 (late bud stage) by the severely underdeveloped allantois. The aberrant phenotype regarding axial elongation can be observed from the 5/6-somite stage as a shortening of the PSM. $Cdx2^{null}$ embryos do not extend the hindgut endoderm properly and at E9.5 they are developmentally retarded and die around E10 due to placental failure.

5. ENDODERM AND ITS DERIVATIVES

5.1. DEVELOPMENT OF THE EMBRYONIC ENDODERM

The primitive endoderm of the mouse embryo (PrE, Figure 1) is a single cell layer derived from Gata4 expressing cells of the inner cell mass. In the pre-streak stage PrE covers the entire epiblast and later on, it also covers the mesoderm of the yolk sac (61). Recently it was found that PrE could contribute to the embryonic gut (14, 62). The VE surrounding the embryo is displaced by the definitive endoderm (DE) recruited from the epiblast through the primitive streak (Figure 1,3). The DE forms the gut tube and associated organs (pancreas, liver, respiratory tract). Fate mapping analyses (62-64) revealed that distal endoderm of the MS stage embryo together with cells overlying the primitive streak are fated to become the foregut and the posterior endoderm (Figure 3). The allocated endoderm precursors rapidly expand from anterior and posterior sites of the embryo to intercalate within the visceral endoderm to generate the middle part of the gut endoderm (65). The definitive endoderm uniformly expresses genes like *Foxa2* and *Sox17*, which have been shown to be crucial for proper endoderm expansion (66). Morphogenesis of the embryonic gut tube commences with the formation of anterior and posterior intestinal pockets at early somitic stages (Figure 3). Within one day of further embryonic development, anterior and posterior pockets close and elongate to form the primitive gut tube by the time the embryo

has formed around 20-25 somites. At this developmental stage the antero-posterior tissue identity is already established. Foregut and midgut give rise to the liver, the pancreas, the duodenum and part of the intestine while the hindgut gives rise to the large intestine. Posterior endoderm development is dependent on several transcription factors among which are the Cdx and Hox genes (58, 67). *Cdx1* and *Cdx2* are expressed in the posterior endoderm throughout embryonic development, and adulthood. In adult mice *Cdx2* is expressed at different levels in the intestinal endoderm with the highest expression at the level of the posterior small intestine/ cecum, whereas *Cdx1* is expressed the strongest in the distal gut.

5.2. STEM CELLS IN THE ADULT GASTRO-INTESTINAL TRACT

Every tissue and organ faces cell loss within the adult lifetime. In order to compensate the cell/tissue loss and keep the homeostasis in the organism, new cells have to replace the old ones. A small pool of organ specific stem cells supports this renewal. These cells self renew and have the capacity to produce multipotent descendants capable of differentiating into all cell types of the tissue they reside in.

Unlike embryonic stem cells (ESCs) and induced pluripotent cells (iPS), which can be identified by the expression of certain markers (e.g. *Oct4, Sox2, Nanog*), adult stem cells do not seem to have a transcriptional signature characteristic for every organ. Homeostasis in these organs is controlled by regulatory mechanisms in the microenvironment in which stem cells reside (the niche). Although stem cells in different adult organs express different sets of genes, one gene, *Lgr5*, is expressed in all types of stem cells in the gastro-intestinal tract, GI (68). The Lgr5 (leucine-rich repeat-containing G-protein coupled receptor 5), also known as Gpr49, is a Wnt responsive gene encoding an orphan receptor. The function of Lgr5 and its binding partner(s) as well as their downstream transcriptional program are still unknown; however Lgr5 has been proven to be a marker of many stem cell types in the adult organism (68-70).

Small intestine

The epithelium of the small intestine is organized into villi and crypts. Each crypt supplies cells for several villi and one villus receives cells from multiple crypts. The bottom of the crypt contains approximately 14 columnar base cells (CBCs,71), which have been shown to be the intestinal stem cells, expressing *Lgr5* (68) and supplying all the cells for the intestinal epithelium. CBC cells are in intimate contact and intermingled with terminally differentiated and long-lived Paneth cells. Lgr positive ($Lgr5^{+ve}$) stem cells are actively cycling as shown by the expression of Ki67, histone H3 phosphorylation and BrdU incorporation. The main driving force behind the crypt biology is the Wnt signaling. Paneth cells have been shown to be a source of *Wnt3*, essential for the survival of $Lgr5^{+ve}$ stem cells (72). Mutant mice with a reduced number of Paneth cells (e.g. *Gfi1-, Sox9-* deficient animals) also have a greatly reduced number of stem cells. Gene expression profiling performed on CBC cells and Paneth cells (72), revealed that stem cells have high *Notch1* and *Fzd7* expression, while Paneth cells are expressing *Egf, Notch* ligand *Dll4* and components of the Wnt pathway (*Wnt3* and *Wnt11*). Thus, Paneth cells are sustaining the niche for their support by providing essential signals for stem cells support. Above the crypt bottom the progeny of $Lgr5^{+ve}$ stem cells forms a compartment of transit amplifying cells (TA) containing rapidly proliferating cells which move upward to the crypt/villus border (73), to eventually differentiate and give rise to mature and functional epithelial cells of the small intestine. Cell production from the crypt bottom is balanced by the apoptosis at the top of the villi. Differentiated cells on the villi surface move towards the tip of the villus and eventually are shed in to the intestinal lumen. This process takes approximately 5 days in the mouse intestine. In the colon, $Lgr5^{+ve}$ cells are also found at the bottom of the colon crypts and similarly to $Lgr5^{+ve}$ cells in the small intestine, they have been demonstrated to be the stem cells of the large intestine.

Stomach

The stomach is a glandular organ consisting of two parts the corpus and the pylorus. The stomach shares a number of features with the small intestine, such as an endodermal origin and constantly renewing endodermal epithelium supplied by a small population of resident stem cells. The stomach epithelium is organized into

multiple gastric units comprised of flask shaped tubular glands, equivalent of the small intestinal crypt, which feed into a pit that opens out to the stomach surface. Lineage tracing studies confirmed the existence of two populations of stem cells in the pyloric part of the stomach (74). The quiescent, "label retaining",Vilin^{+ve} cells reside in the isthmus part of the pyloric gland and have a multilineage potential after stimulation with γ -interferon, a proinflamatory cytokine (74). This dormant population of stem cells is activated only in the event of an injury and does not contribute cells to the gastric epithelium under normal, homeostatic conditions. The active stem cell population, consisting of Lgr5^{+ve} cells resides in the bottom of gastric glands. Lgr5^{+ve} cells are actively cycling, as shown by BrdU labeling and Ki67 staining, and have been shown to have a multi lineage potential and supply cells for the stomach epithelium under normal, homeostatic conditions (69). The progeny of the Lgr5^{+ve} cells populate the whole gastric unit with a turnover rate at 7-10days.

Lineage tracing experiments with the use of a mouse strain carrying *Lgr5-EGFP-CreER^{T2}* transgene crossed with strain carrying the *Cre*-activatable *Rosa26-LacZ* reporter revealed that Lgr5^{+ve} cells give rise to all cell types of the small intestine, colon (68) and pyloric part of the stomach (69). These studies confirmed that Lgr5 is a marker of actively cycling stem cells in the adult GI tract.

Intestinal crypt cultures

To study the long-term biology of the intestinal stem cells and the relationship between intestinal crypt and villi, a crypt culture system, which mimics the intestinal crypts, has been developed in the Clevers lab (75). Crypts from the small intestine can be isolated and put into a laminin rich matrigel-based culture system. Isolated intestinal crypts are able to grow and expand (75). In the medium containing R-spondin 1 (Wnt agonist), EGF, normally associated with the crypt growth (76), and Noggin (Bmp antagonist), which is necessary for the expansion of the crypt number in vivo (77). Organoid structures, which are formed, contain buds with stem cells and clearly recognizable Paneth cells at the bottom. The organoids that develop in vitro mimic the in vivo organization of the crypt and villus. The buds are equivalent to the crypts and contain stem cells and Paneth cells intermingled with each other. The upper part of the bud structure contains proliferative cells reminiscent of transit amplifying cells and the epithelium forming the lining of the lumen contains differentiated cells normally found on the villi surface. Apoptotic cells are shed to the inside of the organoid, which corresponds to shedding cells from the tip of the villus, causing the necessity of mechanical disruption after a week of culture. The gene expression profile of the organoids is highly similar to that of the intestinal crypts. Lineage-tracing experiments confirmed that Lgr5^{+ve} stem cells populate the whole organoid as in the in vivo situation (72). Furthermore, single Lgr5^{+ve} stem cells isolated from the intestinal crypt can reconstitute organoids, which are indistinguishable from the ones formed from isolated crypts with CBC cells and Paneth cells at the bottom of the bud, fully polarized enterocytes, goblet cells and enteroendocrine cells. The process of organoid formation from single cells has a very low efficiency (6%, (72). This efficiency can be increased up to 76%, by re-association of Lgr5^{+ve} cells with Paneth cells which underlines the importance of creating a suitable microenvironment for stem cell survival (72).

Small intestinal organoids, independently of their origin (single cell or the whole crypt) can be maintained in vivo over a long period of time without loss of morphology, continuously forming new buds with stem cells and Paneth cells.

Pyloric cultures

Similarly to small intestinal crypts, pyloric glands with Lgr5^{+ve} stem cells form stomach organoids in matrigel-based culture in medium supplemented with Wnt3a, (as gastric units are strictly dependent on Wnt signals) and Fgf10, (which is essential for the budding process) (69). The morphology of gastric oragnoids is similar to that of small intestinal organoids (spherical, with multiple buds containing Lgr5^{+ve} stem cells) with the presence of all differentiated cell types characteristic for pyloric glands (pit mucus cells, parietal cells, gland mucus cells and endocrine cells). Additionally single Lgr5^{+ve} cells are able to form organoids with the same characteristics as organoids made from pyloric glands.

The identity of the endoderm is acquired in the embryonic life. Cdx genes are among the factors that establish the identity of the posterior endoderm. Endodermal tissues caudal to the stomach retain Cdx

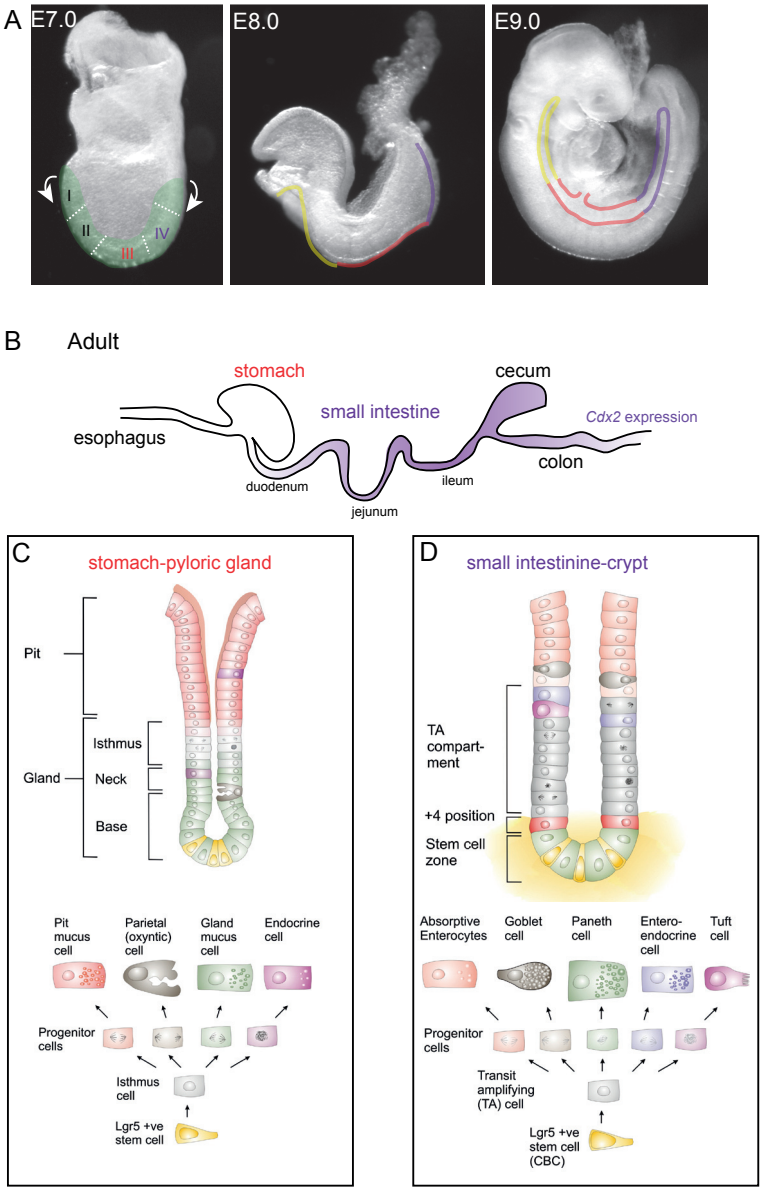


Figure 3 Development of the endoderm
A-Fate map of the endoderm (85). The endoderm of the E7.5 embryo was fate mapped to the forming E8.0 gut tube. The original fate map, of which this is a schematic representation, was generated by injection of single endoderm cells with a tracer (HRP), followed by 24 to 48 h culture (64). Roman numerals I–IV represent regions of E7.5 endoderm that generate different regions of the E8.5 gut. The anterior-most endoderm (region I) of the E7.5 embryo contributes to the ventral foregut adjacent to the developing heart and derives organs such as the liver and ventral pancreas (yellow lines in E8.0 an E9.0 embryos). Regions II and III give rise to dorsal foregut and midgut (red lines in E8.0 an E9.0 embryos) that are adjacent to the notochord and somites. These regions ultimately contribute to stomach, pancreas, duodenum, and part of the intestine. Region IV forms the hindgut (purple lines in E8.0 an E9.0 embryos), which contributes to the large intestine and colon. The foregut tube forms as region I folds over region II (left arrow) and migrates in a posterior direction, whereas the hindgut tube forms when region IV folds over and migrates in an anterior direction (right arrow). B-Schematic overview of the gastro-intestinal tract with *Cdx2* expression depicted in purple. C-Schematic representation of a pyloric gland in the stomach with depicted differentiated cell types present in the gland. D- Schematic representation of a small intestinal crypt and villus with depicted differentiated cell types present in the intestinal epithelium.

expression, while derivatives of more anterior endoderm, namely the stomach, are marked by *Sox2* expression. This histodifferentiation into stomach or intestinal morphology is acquired by day 14 of embryonic development. Cdx genes belong to the para-Hox gene family and share some characteristics with the Hox genes, like the ability to cause vertebral homeotic transformations when mutated, as shown for several Cdx mutants. Homeotic transformations within the endoderm, resulting in metaplasia have been found in the GI tract of Cdx mutants. *Cdx2*$^{+/-}$ mice present lesions in the intestine with expression of *Sox2* and epithelial morphology characteristic for the stomach (54). Conditional inactivation of *Cdx2* at different embryonic stages results in the conversion of the intestinal epithelium into foregut/esophageal (78) or stomach epithelium (79, 80). In chapter 5 we made an attempt to shed more light on this process by inactivating *Cdx2* in vitro exclusively in the adult stem cells of the small intestine using the organoid culture system described above.

6. CDX GENES, MASTER REGULATORS OF DIFFERENT PROGENITOR POOLS IN EMBRYONIC DEVELOPMENT AND THE ADULT INTESTINE- THE SCOPE OF THIS THESIS.

All the progenitor populations within the primitive streak area and the posterior growth zone are affected in Cdx mutants and some of these defects are described in this thesis. Cdx genes are expressed in all three germ layers thus have the potential to act as major regulators of the embryonic morphogenesis. Defects seen in Cdx mutants are complex and encompass a variety of organs and cell populations at different developmental stages. Before this work, a possible hypothesis regarding the Cdx mechanism of action was that Cdx mutations could impair progenitors in a cell autonomous way by disabling cell migration of their descendants, or change their properties like altering the differentiation choice. A second hypothesis proposed that Cdx genes are critical for maintaining the suitable microenvironment (the niche) for the progenitor populations, possibly via different signaling pathways. Affecting the niche would disturb the balance between descendants of cell populations and result in a variety of defects at different levels and developmental time. In this thesis we provide the evidence that Cdx genes act on different progenitor populations by ensuring the permissive microenvironment for their maintenance.

In **chapter 2** we provide the evidence that axial progenitors in posteriorly truncated Cdx mutants are not cell autonomously inactivated but are rescued when transplanted into a wild type host embryo. We demonstrate, using a grafting technique and subsequent embryo culture, that the node-streak border harboring axial stem cell-like cells explanted from a mutant *Cdx2*$^{+/-}$*Cdx4*^{null} GFP donor embryo and inserted homotopically and isochronically into a wild type recipient normally contributes cells along the axis to the mesoderm and neurectoderm. We also found that mutant cells contribute descendants to the CNH area proving that, mutant axial progenitor are highly dependent on their microenvironment and are able to behave as wild types if localized in a healthy niche.

Progenitors for the extraembryonic mesoderm (allantois) are located in the most posterior part of the primitive streak. In cases known so far, mutants (e.g. *Bmp4*^{null}, *Smad5*^{null}) that have allantoic defects also present defects in the PGC population. Progenitors for both tissues are transiently located in the most posterior mesoderm, in the *Cdx2* expression domain. In **chapter 3** we show that the PGC population in *Cdx2*^{null} mutants is impaired and thus we provide the evidence that also progenitors that do not contribute to the embryonic axis are also strictly dependent on Cdx mediated signaling.

In **chapter 4** we present evidence that the short-term progenitors for the lateral plate mesoderm, are affected in Cdx mutants. We observed defects in tissues, the progenitors of which were exposed to Cdx expression at early phase of embryonic development and do not express Cdx genes anymore. These defects are reminiscent of those in the human caudal regression syndrome. We also show that mutants in the canonical Wnt pathway also present a similar altered phenotype. We show that concerted expression of Cdx and Hox genes together with the Wnt pathway orchestrate the development of the uro-rectal tract.

Cdx genes play an important role in the maintenance of homeostasis of the adult intestine. *Cdx1* and *Cdx2* are both expressed in the adult intestine posteriorly of the stomach. In embryonic life *Cdx2* marks the identity of the posterior GI tract since it is expressed in the intestine but not in the stomach, whereas *Sox2* is

expressed in the stomach but not the intestine. In **chapter 5** we describe the effect of a stem cell specific *Cdx2* knock out in the intestine and show in an in vitro culture system that *Cdx2* is a master regulator of the intestinal stem cell. When *Cdx2* is inactivated in small intestinal epithelium it causes its transformation into the epithelium with stomach resembling phenotype.

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Chapter 2

Cdx mutant progenitors are rescued by the wild type niche

Impaired contribution of Cdx mutant axial progenitors to posterior growth is rescued by surrounding cells after grafting to a wild type environment

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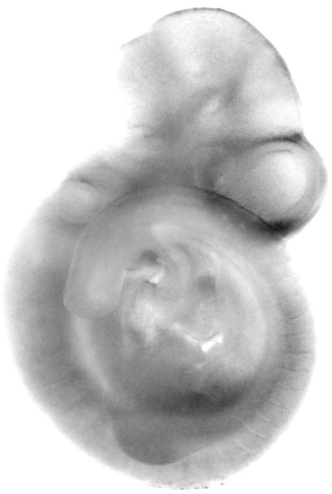

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ABSTRACT

Cdx transcription factors are required for axial extension. Cdx genes are expressed in the posterior growth zone, a region that supplies new cells for axial elongation. *Cdx2*$^{+/-}$*Cdx4*^{null} (*Cdx2/4*) mutant embryos show abnormalities in axis elongation from E8.5, culminating in axial truncation at E10.5. These data raised the possibility that the long term axial progenitors of Cdx mutants are intrinsically impaired in their ability to contribute to posterior growth. We investigated whether we could identify cell-autonomous defects of the axial progenitor cells by grafting mutant cells into a wild type growth zone environment. We compared the contribution of GFP-labeled mutant and wild type progenitors grafted to unlabelled wild type recipients subsequently cultured over the period during which *Cdx2/4* defects emerge. Descendants of grafted cells were scored for their contribution to differentiated tissues in the elongating axis, and to the posterior growth zone. No difference between the contribution of descendants from wild type and mutant grafted progenitors was detected, indicating that rescue of the Cdx mutant progenitors by the wild type recipient growth zone is provided non-cell autonomously. Recently we showed that premature axial termination of Cdx mutants can be partly rescued by stimulating canonical Wnt signaling in the posterior growth zone. Taken together with the data shown here, this suggests that Cdx genes function to maintain a signaling-dependent niche for the posterior axial progenitors.

INTRODUCTION

Progenitors for trunk and tail tissues of the mouse embryo have been localized in the primitive streak and adjacent territories and in the node region at pre-somitic and early somite stages (1-6). Some of these progenitors, located at the border between the node and the anterior primitive streak (NSB region) at early somite stages possess self-renewing properties and behave as long term axial progenitors for somitic mesoderm and neural tube (5).

Elongation of the vertebrate anterior-posterior axis depends on the progressive addition of new tissues from a "growth zone" at the posterior end of the embryo. This region, known as the primitive streak in mouse embryos up to E9.5, and thereafter the tail bud, was shown by grafting experiments to comprise the border region between the node and anterior primitive streak, called the node-streak border (NSB), and the epiblast adjacent to the anterior 4/5th of the streak (5-7). These regions contribute neurectoderm and mesoderm descendants to the elongating axis, while maintaining self renewing progenitors in the chordoneural hinge (CNH, (8) within the tail bud (5, 6). Therefore these progenitors are called "long term axial progenitors" (7). Recently, clonal analysis showed the existence of bi-potent neuro-mesodermal axial progenitors in the posterior part of the embryo throughout axial elongation ((9). These axial progenitors self renew and deliver descendants to the extending neuraxis and mesoderm for an extensive period of time (9), and are therefore probably equivalent to the long-term axial progenitors defined by grafting experiments.

Embryonic axial elongation by somite addition and neural tube extension normally ceases at E13.5 (after formation of about 65 somites) in mice. Mutants affecting the Wnt and Fgf signaling pathways (i.e. *Wnt3a, Tcf1/Lef1, Tcf1/Tcf4, FgfR1*) (10-14) undergo a premature arrest of posterior axial growth. Genes encoding the Cdx transcription factors were also shown to be required for the embryonic axis to extend to completion. Cdx mutations lead to posterior axial truncations affecting all germ layers (15-17). *Cdx2* plays a prevalent role in body axis elongation since loss of a single allele leads to a slight posterior truncation, whereas a *Cdx2*^{null} mutation results in arrest of axial extension beyond the level of the forelimb bud (15, 18, 19). Loss of *Cdx1* and *Cdx4* does not compromise axial elongation even in double homozygous null mutants. Combining mutant alleles of *Cdx1* or *Cdx4* with heterozygosity for *Cdx2* increases the severity of the truncations, indicating that all three Cdx genes contribute to axial growth. The *Cdx2*$^{+/-}$*Cdx4*^{null} allelic combination (called from now on *Cdx2/4*) generates an axial truncation of intermediate severity that we studied extensively (16, 17). Recently, the action of Cdx transcription factors in stimulating axial growth at trunk levels was shown to be exerted at least in part through the stimulation of canonical Wnt signaling, since posterior gain of expression of Lef1 could partially rescue the truncation

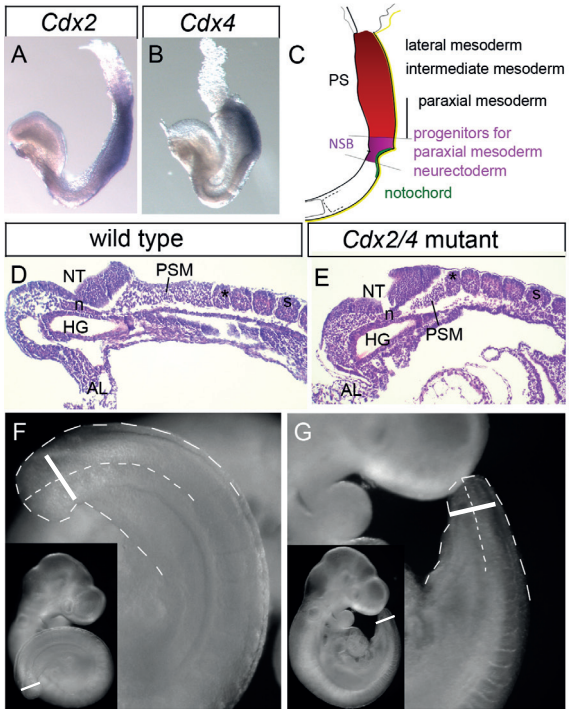

Figure 1. Cdx expression and axial progenitors in the mouse embryo, and phenotype of *Cdx2/4* mutants. A, B, Expression of *Cdx2* (**A**) and *Cdx4* (**B**) in early somite embryos shown after in situ hybridization. Both genes are expressed in the posterior of the embryo, in the primitive streak and the node area. **C**, Schematic representation of the progenitor populations in the primitive streak area of an E8.0 embryo, contributing descendants to different types of mesoderm. The progenitor population within the node-streak border (marked in purple) contributes descendants to two germ layers: neuroectoderm and mesoderm. **D, E**, Longitudinal section of the posterior part of a 19-somite wt (**D**) and a *Cdx2/4* mutant (**E**) showing the reduction in the presomitic mesoderm (PSM) length in the mutant. **F, G**, phenotype of a 32-somite wild type (**F**), and *Cdx2/4* mutant (**G**) embryo at E10.0. Note that the *Cdx2/4* mutant is posteriorly truncated. The tail bud is shorter and narrower than in wild type embryo. The distance between the last formed somite and the caudal tip is marked with a dashed white line. PS, primitive streak; NSB, node-streak border; NT, Neural tube; PSM, presomitic mesoderm; HG, hindgut; AL, allantois. The bar in F and G indicates the level of the CNH.

phenotype of *Cdx2/4* mutants (17). This genetic manipulation did not lead to a complete restoration of posterior morphogenesis. Therefore, we set out to investigate directly whether there was a cell-autonomous deficiency in *Cdx2/4* mutant axial progenitors to produce posterior axial descendants by comparing the contribution of *Cdx2/4*mutant progenitors with wild-type ones on grafting to wild-type recipient embryos and subsequent culture. We find no evidence for defects in either contribution to differentiated tissues or maintenance of progenitors in the CNH, and conclude that the effect of the Cdx mutations on posterior axial growth can be compensated by signaling from surrounding wild type tissue in the embryonic growth zone.

RESULTS

Contribution of the posterior axial progenitors of *Cdx2/4* mutants to trunk and tail extension of wild type embryos

Cdx2 and *Cdx4* are both expressed in the embryonic region corresponding to the posterior growth zone, which is the primitive streak and adjacent territories, and the node region (Figure 1A, B; (17). In *Cdx2/4* mutants axial extension slowed down and ceased prematurely. A reduction in the length of pre-somitic mesoderm (PSM) becomes visible at the 7 to 9 somite stage (Figure1D-G), and the axis is truncated at a level just posterior to the position of the hindlimb buds (17). We isolated the border region between node and streak (NSB) from GFP positive *Cdx2/4* mutant embryos at early somite stages, before the first manifestation of the truncation phenotype, and grafted this area into the equivalent position of stage matched wild type embryos (Figure S1). Successfully grafted embryos were cultured in vitro for 48 hours. Littermate Cdx4 heterozygote embryos were used as controls since embryonic axis extension is not compromised in mice with this genotype (16). We determined the distribution of GFP-labeled descendants of the newly introduced axial progenitors after 2 days of culture of the recipient embryo.

Recipient embryos developed normally during the 48 hours of in vitro culture. In our experiments, embryos that received mutant grafts (n=17) formed on average an additional 29.5 somites, and embryos that received control grafts (n=14) 31 somites (Figure S2). This minor difference is statistically non significant (p=0.215). We did not observe any malformations of the posterior trunk or tail bud in embryos grafted with *Cdx2/4* mutant

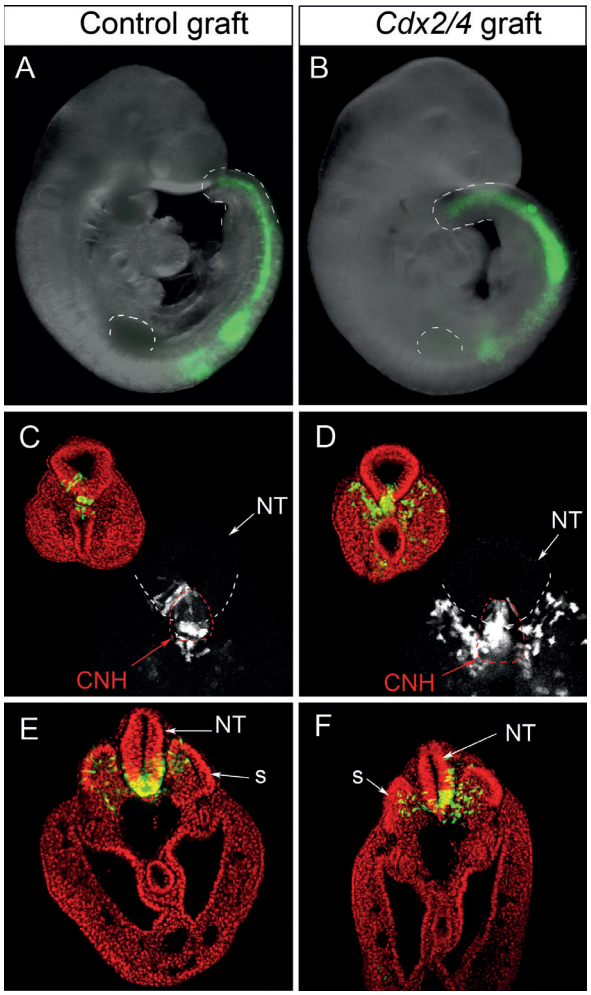

Figure 2. Distribution of GFP labeled descendants from control and *Cdx2/4* mutant grafts in wild type recipients. A-F, Contribution of grafted NSB areas from control (**A, C, E**) and *Cdx2/4* mutant (**B, D, F**) in wild type recipients. Panels **A** and **B** present whole mount view of grafted embryo (with control and *Cdx2/4* mutant graft, respectively). Transplanted GFP cells contribute to trunk and tail tissues caudally to the level of the forelimb. **C, D,** representative posterior sections showing contribution of grafted GFP progenitors originating from control and *Cdx2/4* mutants, at the level of the CNH in the recipient. Inserts show close ups of the labeled areas. **E, F**, sections at the level of the most anteriorly labeled somite, showing the contribution of grafted GFP progenitors from control and mutant grafts, respectively, to the somites and neural tube. FLB- fore limb bud; NT- neural tube; CNH- chordoneural hinge; S, somite.

NSB after the 2-day culture period. Overall, the contribution of grafts was in agreement with results obtained previously (5, 6). In both types of embryos after culture, GFP grafts were well incorporated and their descendants were found in posterior embryonic tissues and all along the antero-posterior axis with a rostral limit at the forelimb level (Figure 2 A-F), as expected from the developmental stage at their insertion time. Occasionally, clumps of tissue remained associated with the graft site. This unincorporated tissue was not considered in the quantification of the graft contribution. We also observed occasional anterior neural tube irregularities in embryos that received both mutant and control grafts, probably resulting from a local disruption of the morphology of the tissue upon grafting (Figure S3). GFP positive cells at these irregularities in the neural tube represent descendants from the grafts at the insertion position, and they were thus included in the analysis. We scored the contribution of grafted cells to different tissues in the embryonic trunk along the A-P axis after culture (Table 1). A first observation from scoring the GFP positive axial descendants from the *Cdx2/4* and control grafts was that the average total number of descendants between embryos within each group was very similar (Figure 3). These data show that the grafting protocol is reproducible. In addition, the average numbers of descendants from mutant versus control grafts was also very similar, showing that *Cdx2/4* mutation does not compromise overall proliferative ability in this context (Figure 3). Likewise, examination of the contribution to individual tissues showed that both *Cdx2/4* mutant and control grafts contributed similarly to mesoderm (tail bud mesenchyme, pre-somitic mesoderm and somites, and notochord) and neurectoderm (neural tube) (Figure 2 C-F and Figure 3). These data indicate that the *Cdx2/4* mutant axial progenitor cells can contribute normally to posterior growth at axial levels

Control graft	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12	Average
CNH	15	25	48	18	26	39	53	39	26	26	12	24	29.3
Neurectoderm	74	19	53	91	212	60	4	115	138	202	304	144	118.0
Mesoderm	383	271	661	46	618	83	253	169	73	102	280	396	277.9
Endoderm	1	0	5	8	0	19	0	12	22	0	0	0	5.6
Total	**473**	**315**	**767**	**163**	**856**	**201**	**310**	**335**	**259**	**330**	**596**	**564**	**430.8**

Mutant graft	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10	M11	M12	M13	M14	Average
CNH	20	26	45	13	59	21	17	56	27	19	53	27	34	12	30.6
Neurectoderm	62	69	148	77	209	226	94	47	254	67	136	242	56	16	121.6
Mesoderm	373	206	129	242	488	265	154	327	405	30	367	358	228	162	266.7
Endoderm	0	8	0	0	0	0	4	15	2	0	23	32	22	1	7.6
Total	**455**	**309**	**322**	**332**	**756**	**512**	**269**	**445**	**688**	**116**	**579**	**659**	**340**	**191**	**426.6**

Table 1 Contribution of descendants of GFP cells in the posterior embryonic tissues of wild type recipients. In each embryo the total number of cells found in the neurectoderm, mesoderm and in the CHN was scored. Only embryos with GFP cells in the CNH area, neurectoderm and mesoderm have been considered in this analysis

and in the temporal window where/when *Cdx2/4* mutants in vivo are deficient, if grafted into a surrounding wild type environment.

The self renewing properties of the long term posterior axial progenitors are not affected by Cdx mutations

To determine whether *Cdx2/4* mutant grafts show any depletion in long-term progenitors in the CNH, the contribution of mutant and control grafts in the CNH was examined. The contribution of mutant and control grafts to the CNH was similar, both qualitatively (Figure 2 C, D) and quantitatively (Figure 3A). Thus the *Cdx2/4* mutant and control grafts maintain a similar self renewing population of progenitors in the posterior growth zone at E10.5. *Cdx2/4* mutations therefore do not impair the ability of posterior axial progenitors to self renew in the CNH in a wild type environment.

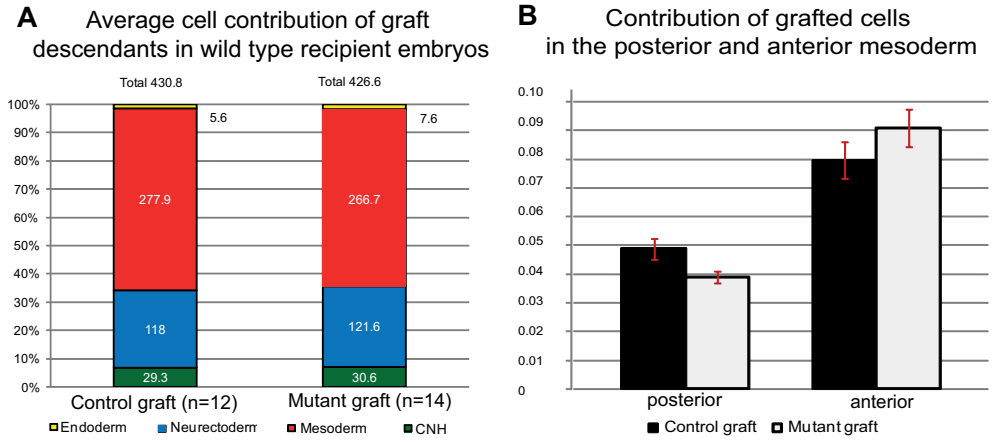

Figure 3. Contribution of the control and mutant grafts to each germ layer of the recipients and to the chordoneural hinge (CNH). A, The contribution of graft descendants to the three germ layers is expressed as a percentage of the total number of graft descendants. The average number of GFP cells contributing to the different tissues (see Table 1) is very similar in embryos grafted with control and *Cdx2/4* mutant NSB. The contributions in the CNH are also similar in both cases. We also normalized the contribution of each graft in mesoderm and neurectoderm for the total contribution of that graft. Again, there was no significant difference between control and mutant grafted embryos (p value for mesoderm =0.997, for neurectoderm =0.820). **B**, Quantification of the contribution of the mutant and control grafts to posterior and anterior mesoderm. The numbers of graft descendants in posterior versus anterior mesoderm were scored for mutant and control grafts. See text for the axial levels selected. The numbers have been normalized for the total contribution of the graft. Error bars are Standard deviations; p values are 0.651 (posterior mesoderm) and 0.786 (anterior mesoderm).

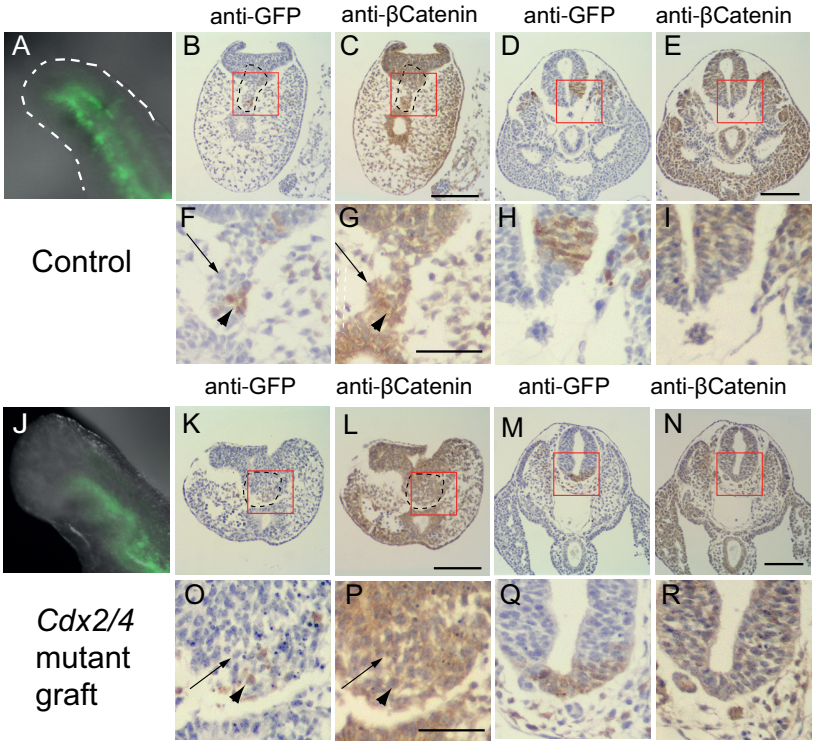

Figure 4 Localization of activated β-catenin in the CNH region of embryos grafted with wt and *Cdx2/4* mutant NSB. A–R, Analysis of GFP and β-catenin protein localization in embryos grafted with control (**A–I**) and *Cdx2/4* mutant (**J–R**) NSB after 48 hours of culture. Consecutive sections are stained with anti-GFP antibody to visualize graft descendants, and anti-β- catenin antibody to reveal the activation of the Wnt pathway in grafted cells in comparison to the wild type CNH environment. In the CNH of both grafted embryos (**B, C, F,** and **G** for control graft and **K, L, O**, and **P** for *Cdx2/4* graft) GFP positive cells show similar levels of nuclear β-catenin (arrowheads) as surrounding recipient wt CNH cells (arrows). In more anterior sections (**D, E, H**, and **I** for control graft and **M, N, Q**, and **R** for mutant graft), GFP positive cells in the neural tube do not differ either from neighboring wt cells in localization of β-catenin, which is not nuclear in these anterior tissues. White dashed line in panel A indicates the tail bud outlines. Red squares in panels **B–E** and **K–N** show the areas magnified in panels below (**F–I** and **O–R**, respectively). Dashed black lines on panels **B** and **C, K** and **L** depict the CNH area. Scale bars: **B–E** and **K–N,** 200 µm; **F–I** and **O–R**, 100 µm.

Cdx2/4 mutant progenitors contribute normally to posterior mesoderm in wild types embryos whereas nascent mesoderm stops being generated in *Cdx2/4* mutants in vivo

The NSB grafting experiments directly challenge the properties of the Cdx mutant axial progenitors transplanted into a proficient growth zone. The mutant and control grafts contributed similar numbers of descendants in mesoderm and in neurectoderm (Figure 3A). Altogether these observations show that tissue contribution in general, and the ability of cells to remain as progenitors was apparently unaffected by *Cdx2/4* mutations. A clear manifestation of the axial extension defect of *Cdx2/4* mutants is the decrease in PSM length after the 7-somite stage (Figure 1 D, E) to almost nothing at E10.5 (17). We reasoned that if the Cdx mutant progenitors were even slightly impaired in generating posterior tissues, this would be reflected by a decrease in the relative contribution of mutant progenitors at caudal levels (laid down late and impaired in Cdx mutants in vivo), versus more anterior, forelimb levels (laid down earlier and not affected in Cdx mutants in vivo). To evaluate whether the *Cdx2/4* mutant axial progenitors are increasingly impaired in their contribution to PSM with time, we compared the mesoderm contribution of *Cdx2/4* mutant grafts at posterior versus anterior axial levels (Figure 3B).

We scored the number of GFP labeled mesoderm descendants from the grafts at a posterior level just anterior to the chordo-neural hinge after culture. This level is located well behind the hindlimb buds at this stage (see indication in Figure 1F, G), and corresponds to the caudal part of the axis, which is affected in the Cdx mutant embryos. We also scored the number of GFP descendants close to the most anterior axial level to which the

control graft		C1	C2	C3	C4	C5	C7	C8	C9	C10	C11	C12	Average
	P	0.023	0.140	0.038	0.037	0.026	0.065	0.048	0.015	0.036	0.015	0.073	**0.047**
	A	0.032	0.063	0.144	0.196	0.054	0.135	0.009	0.015	0.124	0.030	0.038	**0.077**

mutant grafft		M1	M4	M5	M6	M7	M8	M9	M10	M11	M12	M13	M14	Average
	P	0.075	0.042	0.030	0.012	0.015	0.065	0.020	0.052	0.028	0.018	0.024	0.068	**0.037**
	A	0.022	0.127	0.132	0.023	0.264	0.092	0.038	0.052	0.040	0.049	0.121	0.089	**0.087**

Table 2 Normalized contribution of graft descendants to the posterior of the recipient embryo. The number of GFP cells was scored in the pre-somitic mesoderm in the section anterior to that containing the CNH and normalized for the total graft contribution size. One embryo grafted with the control graft and two embryos grafted with Cdx2/4 mutant graft were excluded from the analysis since they did not contain GFP cells in the section immediately flanking that with the CNH. P- posterior; A-anterior; CNH-chordoneural hinge.

graft contributed, thus behind the forelimb buds, which is a level that is not affected by the Cdx mutations. We compared the cell counts at these two axial levels for mutant and wild type grafts. The relative contribution of the graft at posterior compared with anterior levels was the same for wild type and mutant grafts. We found that *Cdx2/4* progenitors contributed descendants to the posterior mesoderm to the same extent as control progenitors (Figure 3B and Table 2).

Taken together our data indicate that the defects in contribution to mesoderm and neurectoderm of the *Cdx2/4* axial progenitors are fully rescued after transplantation in a wild type environment.

In search for a mechanism of axial growth stimulation by Cdx genes

Since we had shown before that posterior gain of Lef1 expression improved axial extension of Cdx mutant embryos (17), we set out to test whether Cdx mutant progenitors in the CNH region express lower Wnt levels than the corresponding wild type (wt) recipient cells, and whether activated β-Catenin, a read out of Wnt response, accumulates in the nuclei of the mutant graft that is rescued by the wild type surrounding.

We analysed mutant and control grafts at the end of the culture period, two days after insertion in the wild type recipient growth zone for the presence of nuclear β-Catenin. We observed that the GFP-labelled grafted mutant cells exhibit the same nuclear β-Catenin staining as the surrounding wild type recipient CNH cells, as do the cells of a control graft (Figure 4). The distribution of *Wnt3a* transcripts was also assayed in the growth zone of *Cdx2/4* mutant and wild type at a corresponding stage (Figure 4). As we expected from previous work (17), the effect of the *Cdx2/4* mutation on the expression of *Wnt3a*, the only canonical Wnt gene expressed in the growth zone around E 10.5, is a restriction of the expression domain rather than a decrease in the expression level in the CNH (Figure 4A-F). Even if we presume that the restriction in *Wnt3a* distribution must reflect some decrease in Wnt3a synthesis, this possible small decrement was not detected by the in situ hybridization procedure. Therefore, the fact that the presence of nuclear β-Catenin in the mutant graft is similar to that in the control graft may mean that a subtle shortage of Wnt signaling in the mutant progenitors is compensated by the cell response to the surrounding wt signal of the recipient. Alternatively, it may mean that the rescue of Cdx mutant progenitors during the axial extension phase studied (from E8.0 to E10.5, when the Cdx mutant axis is already truncated) is involving signaling pathways other than canonical Wnt signaling alone.

DISCUSSION

Cdx transcription factors are required for axial elongation(15, 16, 18, 20, 21). Our recent study showed that the premature axial termination of Cdx mutants, can be at least partly rescued by supplying canonical Wnt signals in the posterior growth zone (20). The partial character of the rescue might have resulted from an incapacity of the transgene to exactly compensate for the deficient Wnt signaling by mutant Cdx. Alternatively, it might be due to the existence of a direct impairment by the Cdx mutations of the long term axial progenitors themselves, in which case the defects of these progenitors would not be cured by a proficient niche in the growth zone. The present study aimed at investigating whether we could identify defects of the axial progenitor cells that would not be rescued by being grafted to a wild type growth zone environment. Two main properties of posterior

axial progenitors were the same in *Cdx2/4* mutants compared to wild types upon transplantation to a wild type surrounding. On the one hand, the maintenance of similar numbers of GFP labeled cells from both Cdx mutant and control grafts in the CNH at the end of the culture period indicates that the grafted progenitors kept on self renewing in the wild type environment. The self renewing property, essential for the generation of nascent tissues during axial extension is therefore compensated by the wild type context. On the other hand, the similar contribution of mutant and control grafts to axial tissues, and in particular to the posterior mesoderm known to be strongly reduced in the *Cdx2/4* mutants in vivo, indicates that there is no intrinsic impairment of the capacity of mutant axial progenitors to supply differentiated descendants to elongating axial structures.

Our previous work showed that the skeletal truncations in Cdx mutants were partially rescued by a posterior gain of function of the canonical Wnt signaling. The present analysis establishes that the failure of posterior axial progenitors to sustain axial growth in E10.5 Cdx mutants can be compensated by surrounding wild type signaling. This analysis, and *Wnt3a* expression and nuclear β-Catenin localization in and around mutant and control grafts, are compatible with the involvement of additional signaling pathways in maintaining posterior growth of the axis.

Acknowledgments

We thank M. Sutija for initial experiments, Wim de Graaff for help with the laboratory facilities, and the Hubrecht Institute animal facility staff for their care to the mice. This work was supported by a grant from the Dutch NWO ALW and by the Dutch Government Bsik programme "Stem Cells into Development and disease" (to JD).

MATERIALS AND METHODS

Mice

All animals used in this work are of mixed C57Bl6/JXCBA background. Donor embryos at E8.0 were recovered from *Cdx2*$^{+/-}$GFP x *Cdx4*^{null} crosses. The GFP transgene was described earlier (23). We used *Cdx2*$^{+/-}$*Cdx4*^{null}GFP embryos as donors of *Cdx2/4* mutant graft and *Cdx2*$^{+/+}$*Cdx4*$^{+/-}$GFP (Cdx4 heterozygotes) embryos as controls since they exhibit no mutant phenotype regarding axis extension. Recipient embryos at E8.0 were recovered from (C57BL/6J x CBA) F1 matings.

Graft dissection and preparation of recipient embryos

Embryos were dissected in M2 medium (Sigma- Aldrich) at room temperature. Embryos that had 1-7 pairs of somites and a clearly visible node and primitive streak were used as graft donors. A piece of tissue containing the NSB zone was microdissected using fine glass needles as described previously (5). The size of the graft was approximately 200 μm wide and 500 μm long (Figure S1), and contained on average 186 cells. The remaining embryonic tissue was used for genotyping. Recipient wild type embryos were stage matched with graft donor embryos to within 3 somite pairs.

Tissue transplantation and embryo culture

Homotopic, isochronic grafts were performed using a hand-drawn capillary pipette as described before (5). Briefly, grafts were inserted into the NSB of recipient embryo without removing the host NSB area. After grafting, embryos were left in medium for 15-30min to recover. This ensured that the graft would not fall out when the embryo was transferred to the culture tube. During the recovery period, proper insertion of the graft at the midline and at the correct antero-posterior (A-P) position was confirmed by fluorescence microscopic examination. Grafted embryos were placed individually in 25 ml containers (Nunc) and incubated as rolling cultures, as described by (22). The culture medium consisted of 50% or 75% rat serum (respectively for the first and the second day of culture) in DMEM (Sigma Aldrich or Invitrogen) supplemented with nonessential amino acids, sodium pyruvate and glutamine (Invitrogen). At the end of the culture period (48hrs in total) embryos were fixed in 4% paraformaldehyde overnight at 4°C, and photographed. Somites were counted using a LeicaMZ16FA microscope with a DFC480camera.

Embryo analysis

Embyos were embedded in 4% low melting point agarose and sectioned (200 μm sections) using a Vibratome. Sections were stained with DAPI (Invitrogen) to visualize nuclei and mounted on slides in Vectashield mounting medium (Dako). Sections were analyzed with a Confocal Laser Scanning Microscope using Leica Application Suite software. GFP labeled cells were manually counted on Z-stacks of the optical scans within the vibratome sections, using the Adobe Photoshop counting tool. Statistical analysis was done using the Mann-Whitney test.

Histology, Immunohistochemistry, and In Situ Hybridization.

All embryos were fixed in 4%PFA overnight at 4°C, embedded in paraffin and sectioned according to standard procedures. For the antibody staining adjacent sections (6 μm) were placed on separate slides to enable two different stainings at the time. Antibodies used were: mouse anti-GFP (1:200) Santa Cruz and mouse anti-β-Catenin (1:200) BD Sciences. Peroxidase conjugated secondary antibodies used were Mouse EnVision+ (DAKO). Signal was developed with 3,3' diaminobenzidine (DAB, Sigma) and sections were counterstained with Hematoxylin. In situ hybridization was performed as described previously (16).

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SUPPLEMENTARY MATERIAL

Supplementay material contains three figures

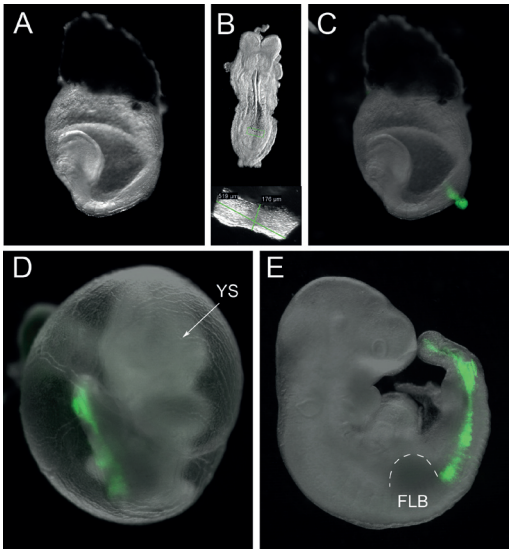


Figure S1. Overview of the grafting procedure. A, recipient embryo with intact yolk sac and ectoplacental cone. B, localization of the donor NSB area and excised graft. C, recipient embryo with graft placed in the host NSB area. D, E view of the embryo after 48 hrs of culture with and without the yolk sac respectively.

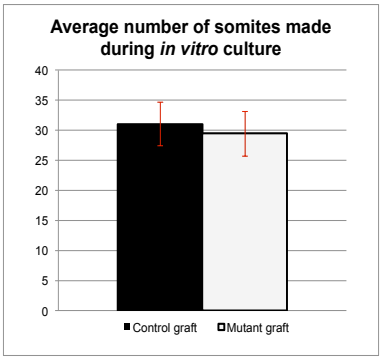


Figure S2. Number of somites added during the culture period in embryos that received mutant and control grafts. p value is 0.336.

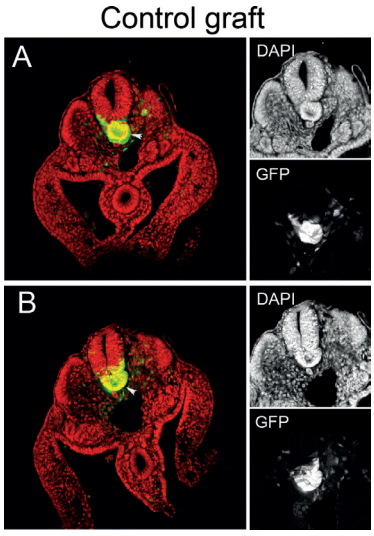
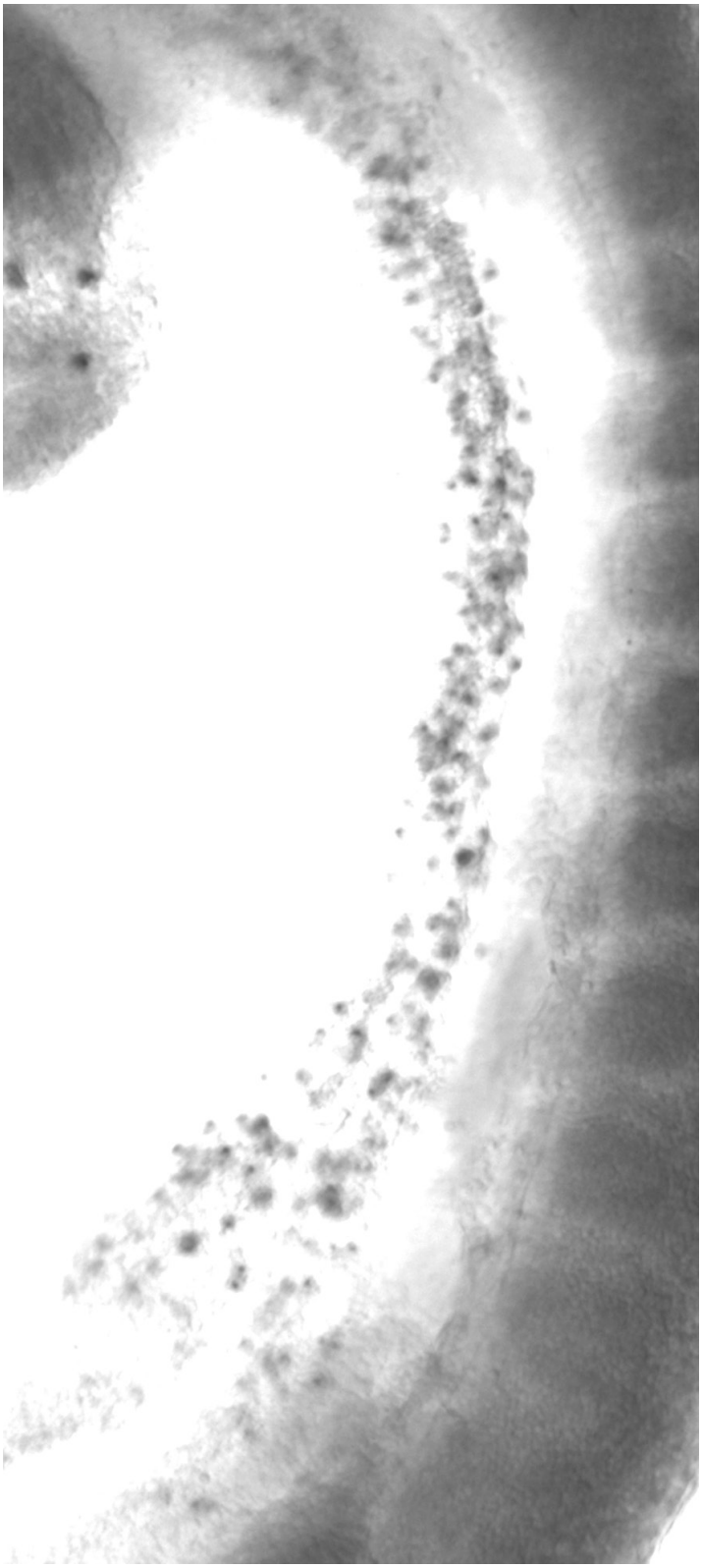


Figure S3. Local irregularities in the anterior neural tube, resulting from the graft insertion. In some cases, the graft descendants forming such irregularities at the graft insertion site did generate tubular structures very locally in the most anterior section (A, B). These cells were counted as neurectoderm contribution.

Chapter 3



Cdx2 acts via Wnt and Bmp signaling to maintain the early PGC population in mouse embryos

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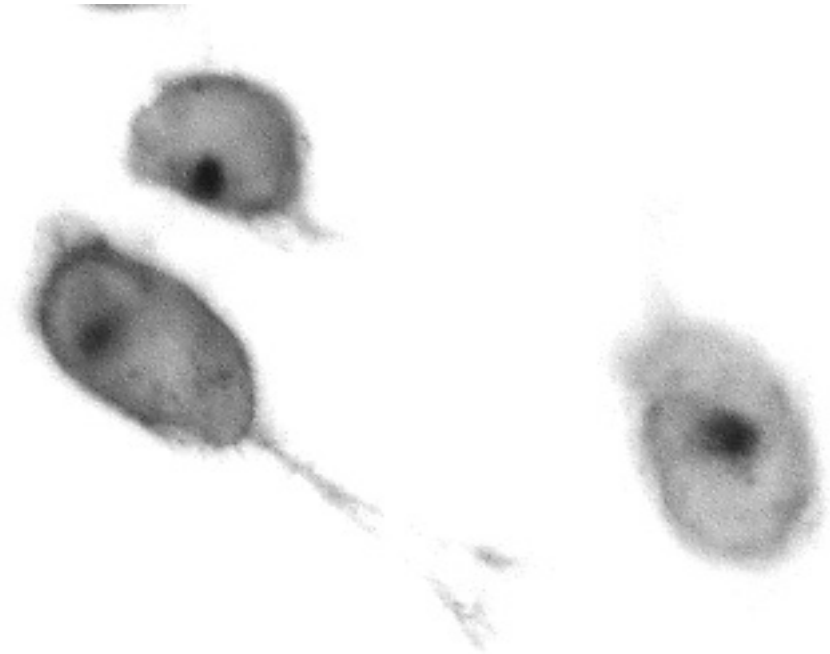
manuscript in preparation



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ABSTRACT

The mouse germ cell lineage originates in the proximal epiblast and is established within nascent mesoderm emerged from the posterior primitive streak in the early embryo. The first primordial germ cells (PGCs) are specified around E6.25 (no/early streak stage) and they are identified by the expression of the transcriptional repressor *Blimp1*. Primordial germ cells form a tight cluster at the base of the allantois until they start dispersing around E7.5 (late head fold stage), and migrate into the hindgut endoderm to finally colonize the future gonads. It is still unclear which signals are necessary for the maintenance of the early gem cell population. We report here that the expression of *Cdx2* around the newly specified PGCs is necessary for proper maintenance of this population at early stages of embryonic development. *Cdx2* starts to be expressed in the posterior part of the primitive streak and the base of the allantois at mid/late-streak stage, where and when the PGC population emerges. In *Cdx2*^{null} embryos the number of PGCs is significantly lower than in wild type littermates. The first noticeable difference in the number of PGCs is visible when *Cdx2* expression is up-regulated in the posterior of wild type embryos. Furthermore, PGCs in *Cdx2*^{null} embryos tend to remain clustered for longer than their wild type counterparts. Given the fact that *Cdx2* was shown to positively act on progenitors for axial tissues via its regulation of the canonical Wnt pathway, we investigated the involvement of Wnt signaling in the *Cdx2* dependent PGC maintenance. We found that *Wnt3a* loss of function decreases the PGC population to the same extent as *Cdx2* inactivation. Moreover, the decrease in PGC number in *Cdx2*^{null} posterior embryonic tissues in in vitro cultures was corrected by adding Wnt3a to the medium. *Cdx2*^{null} embryos also present a phenotype similar to the epiblast specific *Bmp4*^{null} mutant. Furthermore, Bmp4 added to the cultures of posterior explants of *Cdx2*^{null} embryos, recues the PGC population. Since Cdx2 is not expressed in the PGCs themselves, we propose that *Cdx2* expression in posterior embryonic tissues ensures a proper niche for the germ cell progenitors by stimulating canonical Wnt and Bmp.

INTRODUCTION

Primordial germ cells (PGCs) are the precursors of the sperm and oocytes, two types of highly specialized cells, crucial for all sexually reproducing organisms. The germ line is the first to be segregated from the pluripotent epiblast (1). Clonal analysis has shown that PGC precursors are derived from a small cell population in the posterior proximal epiblast at day 6.25 of embryonic development (E6.25), (2). One day later, lineage-restricted germ cells are allocated at the base of the allantois, in the extraembryonic mesoderm. Soon thereafter, about 45 PGCs, strongly expressing tissue non-specific alkaline phosphatase (AP), start migrating towards the hindgut endoderm. During the period of hindgut development the PGC population increases and around E10, PGCs migrate towards the dorsal mesentery and laterally into the forming genital ridges. At E13.5 there are about 25 000 germ cells in the gonads (3). Dependently of the sex of the gonads PGCs start to differentiate into the male or female germ cells to eventually give rise to sperm and oocytes, respectively.

In the mouse, development of the germ line is strictly dependent on the signals provided by the extraembryonic tissues and on the ability of the epiblast to respond to these signals (4). Several members of the Bmp signaling pathway are necessary for specification of the germ line (5-7). *Bmp4* heterozygous embryos have a reduced PGCs population, while *Bmp4*^{null} embryos are completely devoid of germ cells. These latter mutants also lack the allantois (5). *Wnt3* signaling in the early epiblast is a prerequisite for *Bmp4* to induce the cascade of events that will determine PGC specification (4). *Bmp8*^{null} mutants also have an affected PGC population and a delayed outgrowth of the allantois. Although Bmp8b (7) emanating from the ExE and Bmp2 from the visceral endoderm (VE) (6-8) were reported to be involved in the development of the germ line, Bmp4 alone is sufficient to induce PGC formation *in vitro* (4) and this signal is crucial for germ line emergence. As expected, the Smads, effectors of Bmp signaling pathway, are also indispensable in the epiblast for germ cells to develop. *Smad1* and *Smad5* are expressed in a proximo-distal gradient with the highest expression in the proximal epiblast. *Smad1*^{null} and *Smad5*^{null} embryos display defects in PGC specification and allantois formation (40). Furthermore, Smads are not induced in the epiblast of *Wnt3*^{null} embryos, and as a consequence the PGC population is not established, in

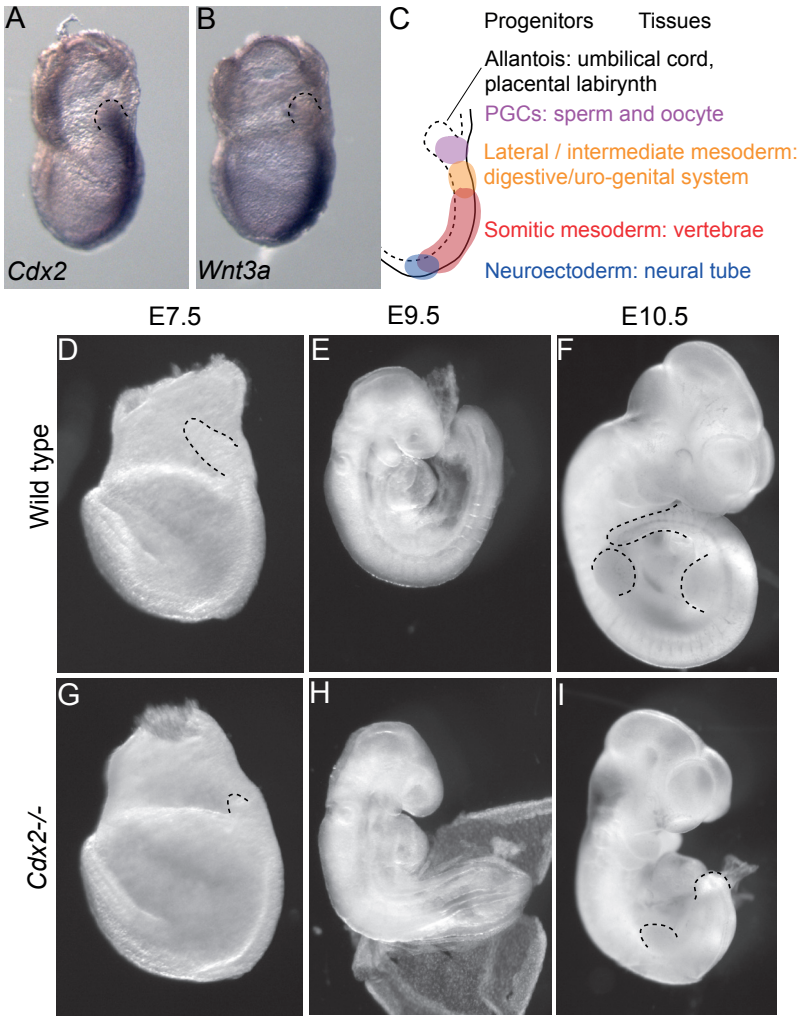


Figure 1 *Cdx2*^{null} embryos are severely truncated and exhibit impaired allantoic growth.
A and **B**-The expression of *Cdx2* and *Wnt3a* (respectively) at the early allantoic bud stage (EB) wild type embryos, shown by in situ hybridization. **C**-overview of the progenitor populations located along the primitive streak at EB stage. **D-I**-The phenotype of *Cdx2*^{null} embryos (**G-I**) in comparison with to wild type littermates (**D-F**) at E7.5 (**D, G**), E9.0 (**E, H**) and E10.5 (**F, I**). Note a severely impaired allantois growth in *Cdx2*^{null} embryos (**G**)

spite of the fact that patent Bmp signals are provided by the ExE. Thus, a specific sequence of signals emanating from the extraembryonic tissues (Bmps) and from the epiblast (*Wnt3*) creates a suitable niche in the posterior proximal epiblast for the emergence of the germ line (4, 41). The importance of these events is supported by transplantation studies, which demonstrated that anterior or distal epiblast cells give rise to PGCs if grafted to the position where this population normally emerges (9). This emphasizes the importance of a suitable niche for PGCs to be maintained.

Bmp4 from ExE induces the expression of *Fragilis*. Epiblast cells expressing *Fragilis* are capable of forming PGCs but also contribute descendants to the allantois and extraembryonic mesoderm (10), thus are not lineage restricted yet. Under the influence of *Wnt3* and Bmp4, a subpopulation of Fragilis-expressing cells starts expressing the transcriptional repressor *Blimp1* (*Prdm1*). Cells expressing both genes will become lineage-restricted germ cells with the expression of germ cell marker *Stella* and strong AP activity (11). *Blimp1* is indispensable for germ line emergence (12). *Blimp1* in PGCs ensures the repression of the previously initiated somatic program by suppressing the expression of genes such as *Hoxa1, Hoxb1, Fgf8, TBra, Lim1* and *Evx1* (13). *Blimp1* also allows

the re-expression of pluripotency genes, including *Sox2* and *Nanog*, and genome wide epigenetic reprogramming during the PGC migration period (13). Independently of the expression of *Blimp1*, *Prdm14* is expressed by PGCs and, is necessary for germ line development as well. *Prdm14* is involved in the re-expression of pluripotency genes and genome wide reprogramming (14). Survival of PGC precursors and PGCs depends on the signals provided by surrounding mesoderm. Fujiwara and colleagues (15), using the tetraploid aggregation of morulas with *Bmp4*^{null} ES cells, showed that Bmp4 produced in the mesoderm is crucial for germ cell survival and correct localization.

Cdx genes belong to the Para-Hox gene family and encode transcription factors controlling several stages of embryonic development. They are involved in embryonic patterning (16) and in ensuring axial extension from progenitors in the posterior growth zone (17). At the pre-implantation stage *Cdx2* is expressed in the trophoectoderm and its derivatives, where it is crucial for the implantation process (18). In the embryo proper the expression of *Cdx2* is initiated in the posterior part of the late streak stage (E7.2). At E7.5 the gene is expressed in the primitive streak area and at the base of the allantois. The time and place of *Cdx2* expression coincide with the increase of the PGC population. *Cdx2* is then down regulated in PGC precursors and up-regulated in the somatic cells. *Wnt3a*, the only canonical Wnt signal present in the window of PGC proliferation and maintenance, is expressed similarly to *Cdx2* and is not expressed by PGCs. Both, *Cdx2*^{null} and *Wnt3a*^{null} mutants present a reduced PGC population. The experiments we describe here lead us to propose that *Cdx2* is crucial to sustain the early germ cell population by maintaining *Wnt3a* and Bmp signaling in its niche in the posterior primitive streak.

RESULTS

Cdx2^{null} embryos are severely truncated and exhibit impaired allantoic growth

The *Cdx2*^{null} mutation is lethal and mutant embryos do not implant in the uterus. We used a *Cdx2* conditional allele (19) and the *Sox2Cre* transgenic strain of mice (20) to by-pass the implantation block and generate *Cdx2*^{null} embryos. The phenotype of these mutants was similar to that of embryos generated by tetraploid aggregation (18). *Cdx2* is expressed in the posterior part of the embryo at the late streak stage (LS) (Figure 1A). This area contains progenitors contributing to posterior tissues of the trunk and tail (Figure 1C), which are affected by the Cdx2null mutation. Mutant embryos can be distinguished at pre-somitic stages by the shortening of the allantoic bud (Figure1 D, G). Posterior axial truncation is visible from the 7/8 somite stage and axial extension terminates

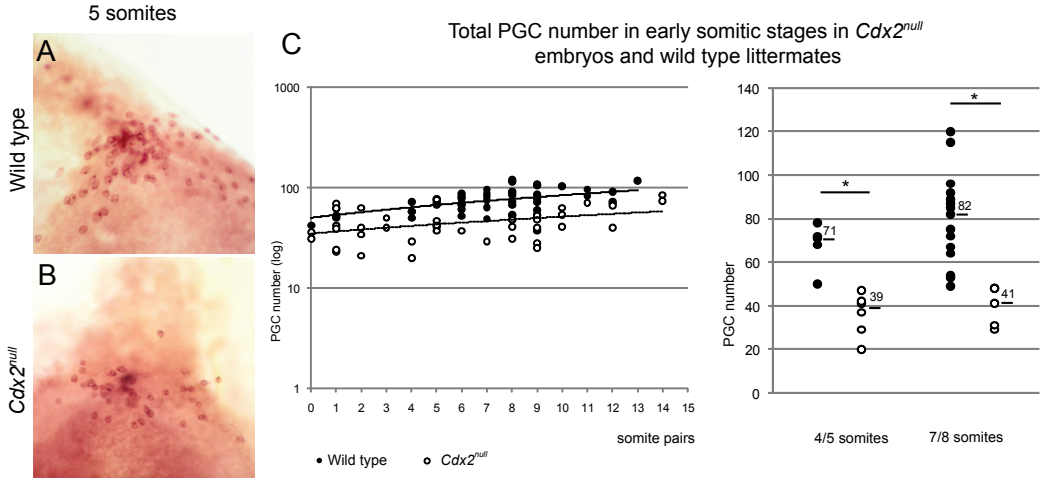


Figure 2 *Cdx2*^{null} embryos have a reduced PGC population at early somatic stages.
A, B-Alkaline phosphatase (AP) staining visualizing the PGC population in 5 somite stage wild type (A) and *Cdx2*^{null} embryos (B). C-quantification of the PGC number at 4/5 somite stage and 7/8 somite stage in wild type and mutant embryos. Note a significant reduction of the PGC number in mutant embryos at both stages of embryonic development, * indicates p<0.001. D-The regression lines drawn for the PGC population in the wild type and *Cdx2*^{null} littermate embryos.

prematurely after generation of a maximum of 17 somites (Figure 1 E, H). *Cdx2*^{null} embryos do not generate posterior endoderm. Due to the impairment of allantoic growth, a functional chorio-allantoic placenta, is never formed and embryos die at E10.5 (Figure 1I).

Cdx2^{null} embryos have a reduced PGC population at early somite stages

In wild type embryos the lineage restricted PGCs are localized within the expression domain of *Cdx2*. Given the role of Cdx genes in tissue progenitors along the primitive streak, we analyzed the PGC population of *Cdx2*^{null} mutants. PGCs can be easily recognized by the high activity of tissue non-specific alkaline phosphatase (AP). Although AP is not exclusive to PGCs and is expressed as well in the surrounding mesoderm, it generates a unique staining pattern in PGCs and is widely used as a PGC marker ever since the identification of these cells in the mouse embryo (21). Using AP as a marker, we analyzed the number of PGCs in early somite embryos. After formation of the hindgut portal, PGCs normally move from the posterior mesoderm of the embryo into the hindgut endoderm, and proliferate at an exponential rate. Since posterior endoderm expansion is affected in *Cdx2*^{null} mutants we did not analyze embryos older than 14 somite pairs to avoid secondary effects that might be confusing for the interpretation of the observations. We did not see any difference in the number of PGCs in *Cdx2* heterozygote vs. wild type littermates (data not shown). However, we observed a clear reduction in the number of PGCs in the *Cdx2*^{null} embryos in comparison with their wild type littermates (Figure 2A, B). In the 4/5 somite stage embryos the number of PGCs was nearly half of that in wild types (39 and 71 respectively, Figure 2C). A very similar situation was observed in slightly older embryos (7/8 somite pairs, Figure 2C) were, the PGC population was reduced by 50% in comparison with wild types. We plotted the number of PGCs against the number of somite pairs formed. The slope of the regression line for mutant embryos did not differ significantly from that for the wild types (Figure 2C), suggesting that survival and proliferation of the established PGC population was not affected at these developmental stages. The elevation of the mutant regression line was significantly lower than that of the wild type, suggesting that the PGC founding population is reduced in *Cdx2*^{null} mutants. We therefore examined the PGC population at earlier stages of embryonic development. In the wild type embryos at early somitic stages

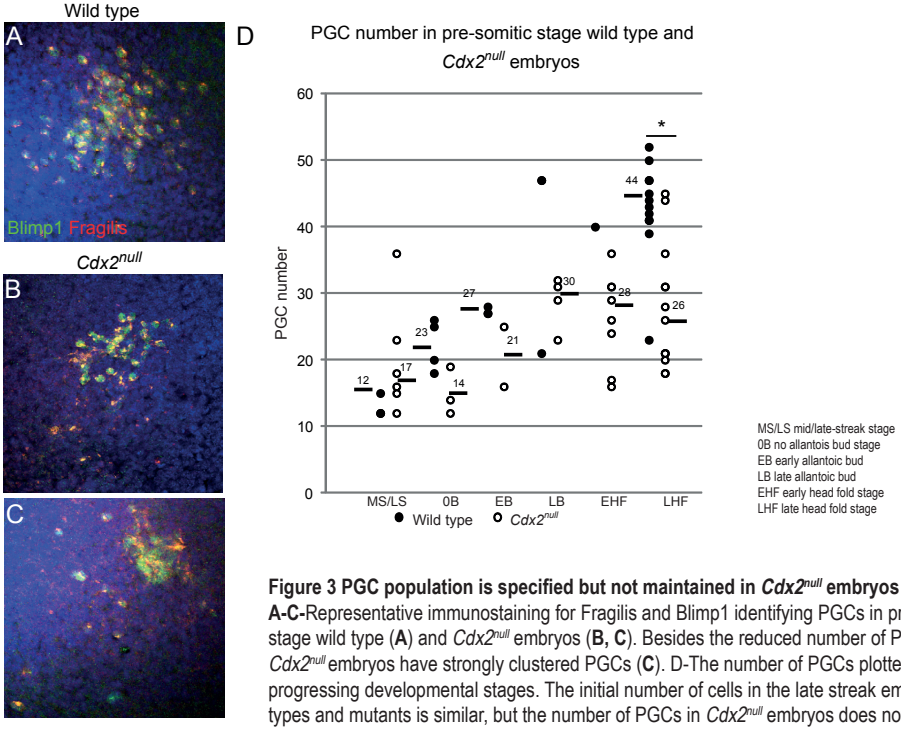


Figure 3 PGC population is specified but not maintained in *Cdx2*^{null} embryos
A-C-Representative immunostaining for Fragilis and Blimp1 identifying PGCs in pre-somitic stage wild type (**A**) and *Cdx2*^{null} embryos (**B, C**). Besides the reduced number of PGCs, some *Cdx2*^{null} embryos have strongly clustered PGCs (**C**). D-The number of PGCs plotted against progressing developmental stages. The initial number of cells in the late streak embryos in wild types and mutants is similar, but the number of PGCs in *Cdx2*^{null} embryos does not increase as much as in the wild type embryos.

around 10 % of the total PGC number localize to ectopic positions, to the yolk sac, allantois and more rarely to the amnion. In some cases in *Cdx2*^{null} embryos we also observed up to 25% of the total PGC population located at the ectopic position, in the yolk sac (data not shown). At pre-somitic stages we did not observe any ectopic PGCs.

PGC specification is unaffected but the early PGC population is not maintained in *Cdx2*^{null} mutant

PGC precursors are located in the posterior proximal epiblast, and the lineage restricted PGCs expressing *Blimp1* and *Fragilis1* reside in the expression domain of *Cdx2*. We used Blimp1/Fragilis1 double immunofluorescence to count the PGCs in early embryos, since AP activity is not detected before the late allantoic bud stage. We determined the number of PGCs at the different stages of embryonic development, between the mid/late streak (MS/LS) stage, when *Cdx2* is up-regulated in the posterior of wild type embryos, and the late head fold stage (LHF) when the founding PGC population of approximately 45 cells is established at the base of the allantois. At the LHF stage, the number of PGCs was significantly reduced in mutants compared to wild types (median values: 31 and 46, respectively) (Figure 3A). However, at earlier stages (LS/0B) the number of PGCs in the *Cdx2*^{null} embryos was very similar in mutants and wild type littermates (around 20 PGCs, Figure 3D). This was suggestive of a normal PGC allocation followed by a defective maintenance of the allocated population in the absence of *Cdx2*. We did not detect any apoptosis in *Cdx2*^{null} embryos making it likely that the PGC the proliferation is affected in those mutants.

In wild type embryos, a cluster of around 20 PGCs is formed at the LS stage and has dispersed by the LHF stage. In 40% of *Cdx2*^{null} embryos, PGCs remained clustered until the LHF stage and in some cases up to the 3- somite stage (Figure 3C). The total number of PGCs in embryos containing this transient cluster was similar to *Cdx2*^{null} embryos without such a cluster. Therefore, *Cdx2*^{null} embryos also present a delay in the breaking down the early PGC cluster.

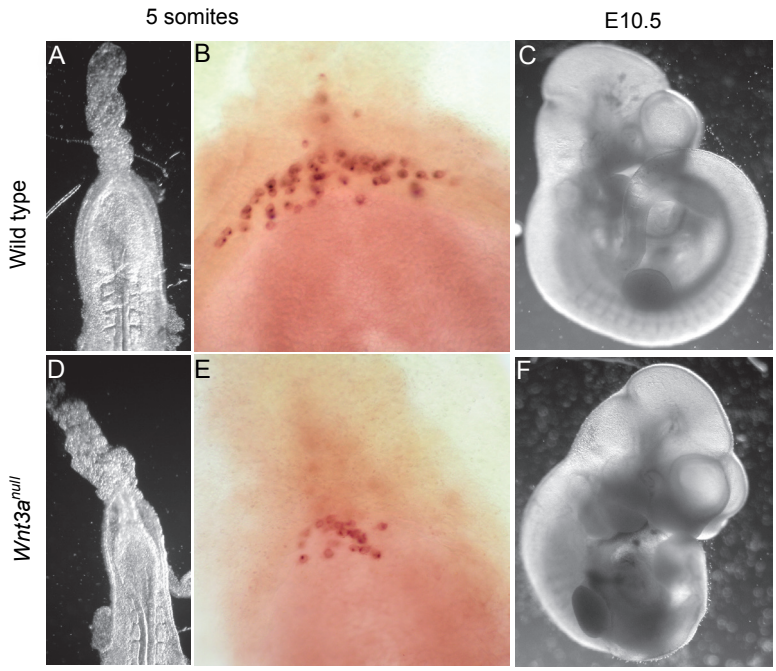
Wnt3a^{null} embryos exhibit the same decrease of the PGC population as *Cdx2*^{null} embryos

We reported earlier that *Cdx2* and *Wnt3a* work in concert to maintain the axial progenitors in the posterior embryonic growth zone, and are crucial for proper axial extension (17, 22, 23). *Wnt3a* is expressed in the primitive streak region of the embryo (Figure 1B) and subsequently in the tail bud, until it is down regulated at approximately E11.5. *Wnt3a*^{null} embryos are truncated in the three germ layers and stop extending their axis after formation of about 20 somite pairs (Figure 4D, F). Unlike *Cdx2*^{null} mutants, *Wnt3a*^{null} mutants do not have a defect in allantoic growth (Figure 4D), and they thus form a functional chorio-allantoic placenta and develop to term. Given the similarity in the expression of *Cdx2* and *Wnt3a* at pre-somitic stages and the fact that Cdx exerts a positive feedback activation of the canonical Wnt pathway (17, 22), we analyzed the PGC population in *Wnt3a*^{null} mutants at early somite stages. We did not observe any change between the PGCs population of Wnt3a heterozygous mutants in comparison to wild type littermates. However, *Wnt3a*^{null} embryos had a greatly reduced number of PGCs (Figure 4E,F). In 4/5 somite stage embryos the PGC population was reduced by half in comparison with the wild type population (Figure 4G).

Wnt3a rescues the PGC population of *Cdx2*^{null} embryos in culture

Axial truncation of *Cdx2*$^{+/-}$*Cdx4*^{null} mutants can be largely rescued by a posterior gain of function of activated Lef1, a downstream effector of the canonical Wnt pathway (22). We set out to investigate, whether the PGC population of *Cdx2*^{null} mutants can also be rescued by the same strategy. We generated *Cdx2*^{null} embryos carrying the *TPLef1* transgene and counted the number of PGCs at the early somite stage. We did not observe any difference in the number of PGCs in *Cdx2*^{null} embryos in the presence or in the absence of the transgene at any of the stages analyzed, suggesting that the *TPLef1* transgene did not rescue the PGC population of *Cdx2*^{null} mutant. The reason why *TPLef1* failed to correct the PGC defects in *Cdx2*^{null} embryos may reside in the fact that these embryos exhibit a much more severe phenotype than *Cdx2*$^{+/-}$*Cdx4*^{null} mutants, as witnessed by their allantois defect and their more severe truncation. Activation of the Wnt pathway by T-dependent expression of the *Lef1* transgene was not sufficient to correct the allantois and axial growth defects, making it assumable that it was also insufficient

to rescue the PGC population of *Lef1* embryos. Therefore we turned to an *in vitro* system and cultured posterior halves of embryos in medium supplemented with Wnt3a at two different concentrations 40ng/ml and 100 ng/ml (Figure 5C). Wnt3a had been reported to stimulate PGCs emergence in in vitro cultures. At LS/0B stages the PGC population is already allocated and PGC numbers increase solely by proliferation (4, 10). The number of germ cells in LS/0B *Cdx2*^{null} mutant explants was significantly lower than in explants from the wild type littermates (Figure 5B, C) and we observed undispersed cluster of PGCs in *Cdx2*^{null} cultures, reproducing the in vivo situation. In cultures with addition of Wnt3a (40ng/ml), the number of PGCs in the mutant explants, was comparable to that in wild type explants (cultured with or without Wnt3a), demonstrating that Wnt3a can rescue the PGC population of the *Cdx2*^{null} embryos. In most cases we still did not observe normal PGC cluster dispersal in *Cdx2*^{null}



G PGC number in wild type and *Wnt3a*^{null} embryos

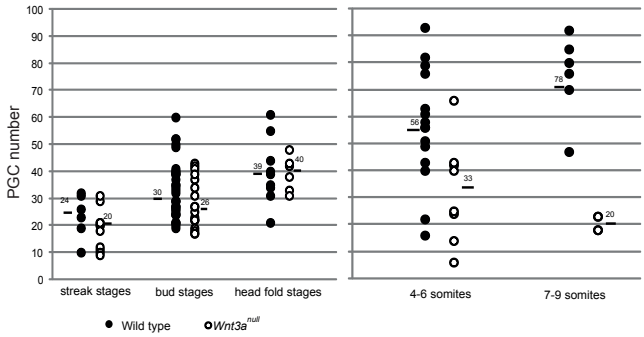


Figure 4 *Wnt3a*^{null} embryos have a reduced PGC population
A-F-The phenotype of *Wnt3a*^{null} embryos. The first manifestation of the mutant phenotype is visible at 5/6 somite stage of embryonic development as a shortening of the posterior (**D**). In contrast to *Cdx2*^{null} embryos the allantois development is not impaired in *Wnt3a*^{null} embryos (**A, D**) and mutant embryos are slightly less severely truncated than *Cdx2*^{null} embryos (**F**). **G**-The number of germ cells of wild type and *Wnt3a*^{null} embryos plotted against the developmental stage. The significant difference in the PGC population is evident from the 4-6 somite stage.

explants cultured with Wnt3a, suggesting that this aspect of the phenotype was not rescued by up-regulating the canonical Wnt pathway in the conditions we used. Similar results were obtained with a higher concentration of Wnt3a (100ng/ml) in the culture medium (Figure 5C). Interestingly the ability of *Cdx2*^{null} explants to respond to the Wnt3a stimulus seems to be developmentally restricted. The PGC population was restored to a wild type level in 4 out of 6 explants (67%) from streak stage embryos. In the explants from bud stage embryos, 50% of cultures had PGC number comparable to wild type explants and in head fold stages Wnt3a had no positive effect of the PGC population of *Cdx2*^{null} explants.

Bmp4 regulates PGC numbers in *Cdx2*^{null} embryos

Bmp4 signaling from the ExE is crucial for PGC specification from the epiblast, however Bmp4 produced by ExM is necessary for maintenance of the early PGC population and correct PGC localization at the base of the allantois (15). Previously we performed a genome-wide transcriptome analysis of *Cdx2*^{null} embryos in comparison to wild type embryos (23). Microarrays performed at two developmental stages revealed that Bmp4 expression was down regulated by 1.6 factor in *Cdx2*^{null} embryos. In situ hybridization with a *Bmp4* probe performed on *Cdx2*^{null} and wild type embryos at head fold stages confirmed that finding (Figure 6A, B). The reduction of PGC numbers

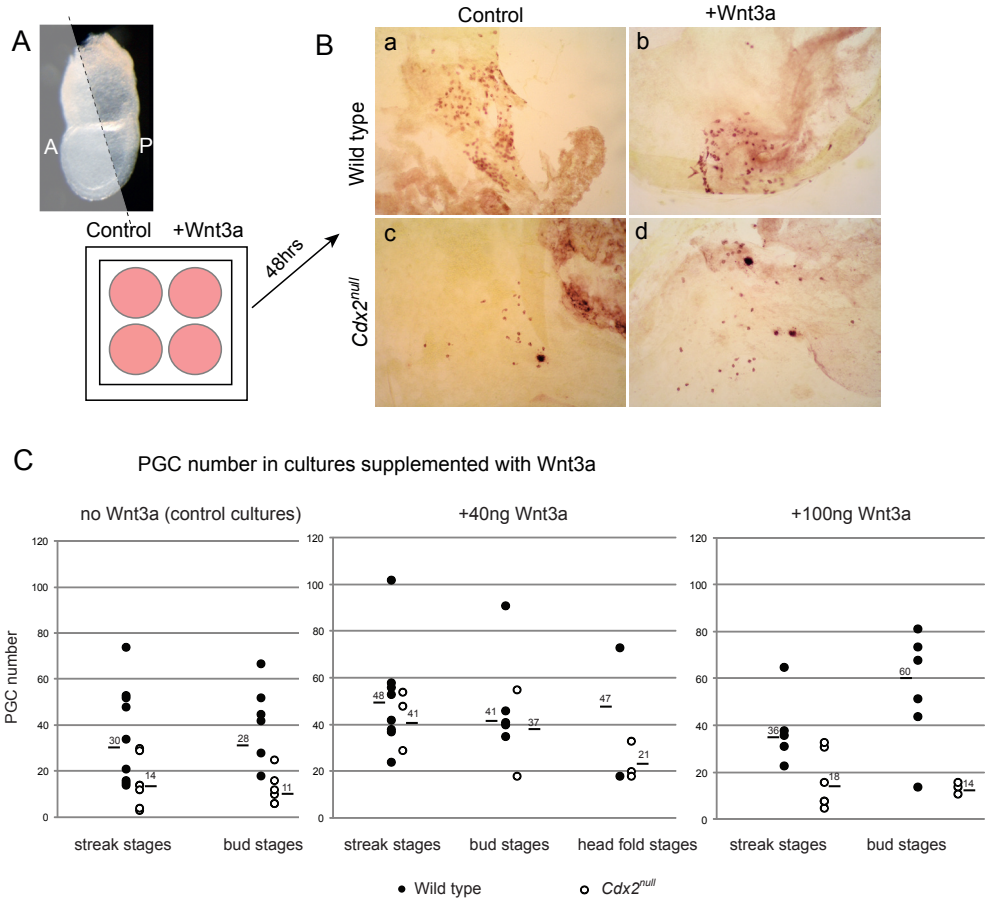


Figure 5 Wnt3a rescues the defective PGC population of *Cdx2*^{null} mutants in in vitro cultures
A-The scheme of the culture experiments. Embryos at different developmental stages were cut as shown in the figure **A** and posterior halves were cultured with or without the addition of Wnt3a for 48 hrs. **B**-representative pictures of control cultures (**a, c**) and cultures supplemented with Wnt3a (**b, d**). **C**-the number of PGC obtained in in vitro cultures in control cultures and in cultures supplemented with 40 and 100ng/ml of Wnt3a.

from early stages on, in *Cdx2*^{null} embryos, and a tendency for PGCs to localize to the yolk sac at early somitic stages could therefore be a consequence of the reduced levels of *Bmp4* in *Cdx2*^{null} embryos. To validate this hypothesis we cultured posterior halves of *Cdx2*^{null} embryos, in medium supplemented with 50ng/ml of Bmp4. The number of germ cells in LS/0B *Cdx2*^{null} explants cultured with the addition of Bmp4 was comparable to explants from the wild type littermates (Figure 6C, E-G). Explants from *Cdx2*^{null} embryos at allantoic bud stages also had higher numbers of PGCs than control cultures when supplemented with Bmp4. We did not observe the dispersal of the PGC cluster in any of the cultures of Cdx2null explants supplemented with Bmp4 suggesting that that process is not dependent on the niche factors like Bmp4 or Wnt3a. Rescue of the PGC population in *Cdx2*^{null} embryos by Bmp4 suggest a link between Cdx and the Bmp pathway.

DISCUSSION

Cdx2 maintains the early PGC population via canonical Wnt signaling

A role of *Wnt3* in the emergence of the germ cells has been demonstrated at very early stages, before Cdx genes are expressed in the embryo. The canonical Wnt signaling has not been involved so far in the subsequent maintenance of the germ line.

We now show that loss of *Cdx2* or *Wnt3a* has a profound effect on the germ cell population. In both mutants we observed a reduction of the PGC numbers by half. Rather than defect of PGC allocation, the early PGC population was not maintained in the mutant situations. The addition of Wnt3a to the medium of embryonic explants from *Cdx2*^{null} mutants restored the PGC population to nearly wild type levels.

At the time of PGC emergence, the primitive streak area contains progenitors for trunk and tail tissues. *Cdx2* and *Wnt3a* are expressed in this embryonic area and are crucial for the maintenance of these progenitors. We showed earlier by genetic and grafting experiments (22, 24), that the maintenance of axial progenitors in Cdx mutants is corrected by a wild type environment. Since the PGC population is also situated in the most posterior part of the primitive streak, and given the rescuing effect of Wnt3a for the rise of the PGC population, the role of *Cdx2* and *Wnt3a* must be relevant as well for these progenitors. Given the inability of a T-dependent gain of Lef1 function to correct the PGC deficiency of *Cdx2*^{null} mutants, and the success of such a rescue for axial growth of *Cdx2*$^{+/-}$*Cdx4*^{null} embryos we assume that the required intensity of Wnt signaling to ensure an adequate niche of

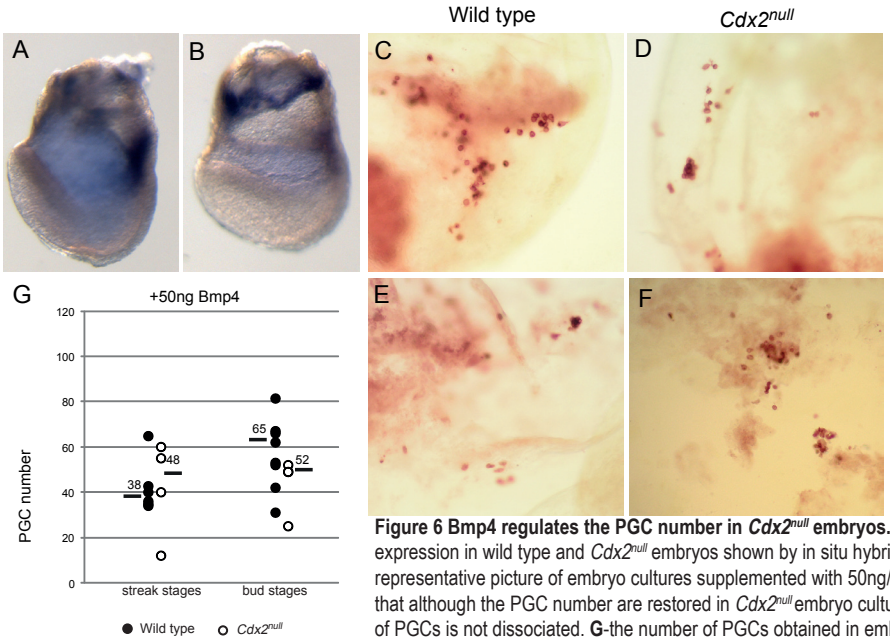


Figure 6 Bmp4 regulates the PGC number in *Cdx2*^{null} embryos. A, B-Bmp4 expression in wild type and *Cdx2*^{null} embryos shown by in situ hybridization. **C-F**-representative picture of embryo cultures supplemented with 50ng/ml of Bmp4. Note that although the PGC number are restored in *Cdx2*^{null} embryo cultures, the cluster of PGCs is not dissociated. **G**-the number of PGCs obtained in embryo cultures supplemented with Bmp4.

the posterior progenitors including the germ line, is higher for *Cdx2*^{null} than for *Cdx2*$^{+/-}$*Cdx4*^{null} mutants. This is in agreement with the *Wnt3a* and Cdx (23) dosage dependence of posterior development. These data reinforce the emerged concept that Cdx transcription factors exert a sustaining function on tissue progenitors in the posterior growth zone, mediated by the canonical Wnt pathway. They demonstrate that this function applies to the germ line as well as to the somatic trunk and tail tissues

Cdx and the Bmp pathway generate a suitable microenvironment for the early PGC population

Before Cdx expression, PGC progenitors are recruited from the posterior epiblast in response to early Bmp4 signals from the extraembryonic tissues. At the time *Cdx2* becomes expressed in posterior embryonic tissues, *Bmp4* is expressed by the extraembryonic mesoderm (ExM) in the forming and extending allantoic bud, around the PGCs (5). At E8.5 *Bmp4* is expressed is the lateral mesoderm. Fujiwara and colleagues (15) have shown that this later onset of Bmp4 expression is crucial for PGC survival and correct localization, as well as for proper development of the allantois. *Cdx2*^{null} mutants have defects in both the PGC population and the allantois, and the PGC developmental step impaired is at the level of the population maintenance. This phenotype resembles the *Bmp4* epiblast insufficiency phenotype. *Bmp4* was among the down-regulated genes when the transcriptome of early *Cdx2*^{null} embryos was compared to that of wild types (23). Our study provides a link between Cdx and the Bmp pathway, placing Bmp4 as a downstream target of *Cdx2* in PGC maintenance. Bmp4 supplied from the ExM is necessary for the PGC survival only at the earliest stages, shortly after PGC emergence from the epiblast. In our cultures Bmp4 was supplied to the embryonic explants at the stage when epiblast already lost the competence to give rise to new PGCs, therefore the rescuing effect that we see cannot the attributed to the PGCs recruitment from the epiblast, but is a result of correcting the microenvironment for the maintenance of the previously allocated population.

A signaling principle has been proposed for PGC emergence from the epiblast (4). Our data provide more insight into the maintenance of the established PGC population and its requirements for proper expansion. *Wnt3* supplied in the medium in epiblast cultures allows formation of PGCs. (4). The expression of *Wnt3a* latter is initiated when *Wnt3* expression extinguishes in the posterior of the embryo. Therefore *Wnt3a* provides continuity of canonical signaling in the posterior through out different stages of embryonic development and is crucial for maintaining the posterior progenitors, including the PGC population. In *Wnt3a*^{null} embryos posterior truncation phenotype is visible later than in *Cdx2*^{null} embryos. This difference may result from the fact that Cdx2 expression extends into the base of the allantois at the primitive streak stage, whereas *Wnt3a* is not expressed there. Once PGCs enter endoderm they are unlikely to be dependent on *Wnt3a* signaling since this gene is not expressed in the hindgut endoderm. Possibly this transition is be reflected in our cultures of head fold stage *Cdx2*^{null} embryos. At the end of the culture period, PGCs are developmentally equivalent to migratory PGCs thus might be insensitive to stimulation by Wnt3a and explain the lack of its rescuing effect.

Other factors essential for PGC maintenance are the Steel factor and its receptor c-kit, shown by many studies to be essential for PGC survival (25-28) and proliferation (28, 29). c-kit is a tyrosine-kinase receptor expressed at the surface of PGCs from head fold stages on, while steel is expressed in PGCs and along the path of PGC migration, creating a "travelling niche" (30, 31). It has been shown that as early as E7.5, the PGC population is dependent on Steel (30)

***Cdx2* affects dispersal of the early PGC cluster**

Cell-cell contact is important for PGC behavior. E-cadherin (32), and Fragilis1, are involved in cell-cell mediated specification of PGC precursors (33). Inactivating E-cadherin with blocking antibodies in epiblast culture, effectively inhibits emergence of Stella positive PGCs. Once PGCs normally become lineage restricted, their E-cadherin is down regulated allowing them to migrate and start proliferating. In 40% of *Cdx2*^{null} embryos we observed a delay in PGC cluster dispersion. This defect is not corrected by addition of Wnt3a or Bmp4 to the posterior embryonic explant cultures. We are investigating whether the down-regulation of E-cadherin is delayed in PGCs. Recent experiments in other contexts have shown that cell-cell interactions are disturbed in *Cdx2*^{null} mutants. These mutants present disturbances in their neural epithelium (23) as well as multiple lumens in their gut epithelium (34).

Future experiments will reveal whether the expression and distribution of Cadherins is altered in Cdx mutants.

MATERIALS AND METHODS

Animals and genotyping

All mice were in the C57Bl6j/CBA mixed background and were housed according to "law on animals in experiments", under the licenses required in the Netherlands. Generation and genotyping of the strain carrying *Cdx2conditional* allele was described by (19). The *Wnt3a* strain was obtained from S. Takada (35). TPLef1 transgene was described earlier (17).

AP staining

Dissection of embryos and AP staining was performed according to (10). Briefly, embryos at early somatic stages with intact yolk sac were dissected from the deciduas in PBS and immediately fixed in 4%paraformaldehyde (PFA) in PBS for 2hrs on ice and then washed 3x 10 minutes in PBS. The fixed embryos were incubated in 70% ethanol for at least 1 hour, followed by 3 washing steps water before staining, 5 minutes each. The TNAP staining solution was prepared freshly and was composed of 0.5% sodium 5'5'diethyl barbiturate (Veronal), 0.6% MgCl2, 0.1 mg/ml α-naphthyl phosphate, and 0.5 mg/ml Fast Red TR (Sigma). The staining reaction was monitored from time to time and stopped by rinsing the embryos in water as soon as PGCs were detected. The stained embryos were stored in 70% glycerol. The anterior piece of each embryo was removed for genotyping before flat mounting the rest of the embryo under glass coverslip. PGCs were identified as single cells with a stained ring and an intracellular intense dot stained under a 20x objective of a compound microscope. All steps of the staining procedure were done under gentle shaking at 4°C except the enzymatic reaction

Immunofluorescence

Pre-somitic embryos were dissected and fixed as described above. For identification of PGCs we used a rat anti-Blimp1 antibody (Santa Cruz) at the dilution 1:50 and a rabbit anti-Fragilis antibody (Abcam) at the dilution of 1:500. Appropriate secondary antibodies were obtained from Jackson Immunoresearch and used in the dilution 1:500. Before incubation with primary antibodies embryos were treated with 0.1% Triton X-100 (Sigma) for 10-15 min, depending on the size of the embryos and blocked in 1%BSA solution for 5hrs at room temperature. Incubation with primary antibodies was carried out for 3 days followed by 8 washes (30min each, at 4°C) and overnight for secondary antibodies followed by washes. DAPI (Invitrogen) was used for counterstaining the nuclei. Embryos were analyzed by confocal laser scanning microscope equipped with Leica software.

Statistics on PGC counting

The data distribution for each group was analyzed with the Kolmogorov-Smirnov test. The Z value ≤ 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≤0.05 was considered statistically significant.

In vitro culture

Pre-somitic embryos were stages according to (36). Embryos at late streak-no allantois bud (LS-0B) stages were isolated and dissected in a cold medium with 7.5% fetal bovine serum (FBS) and 25mM HEPES (Sigma). Posterior pieces were cultured in 4well dishes (Nunc) on glass coverslips in GMEM based standard medium with 15%FBS on a monolayer of mouse embryonic fibroblasts for 46-48 hours in the incubator at 37°C with 5%CO_2 in the atmosphere, for details see (8). Cultures were fixed and subjected to AP staining as described above.

In situ hybridization

Embryos were dissected out of deciduas and fixed in 4% parafolmaldehyde overnight at 4°C, washed in PBS0 and stored in 100% methanol in -20°C. Whole mount in situ hybridization with *Cdx2* and *Wnt3a* riboprobes (37, 38) was performed as described in (39).

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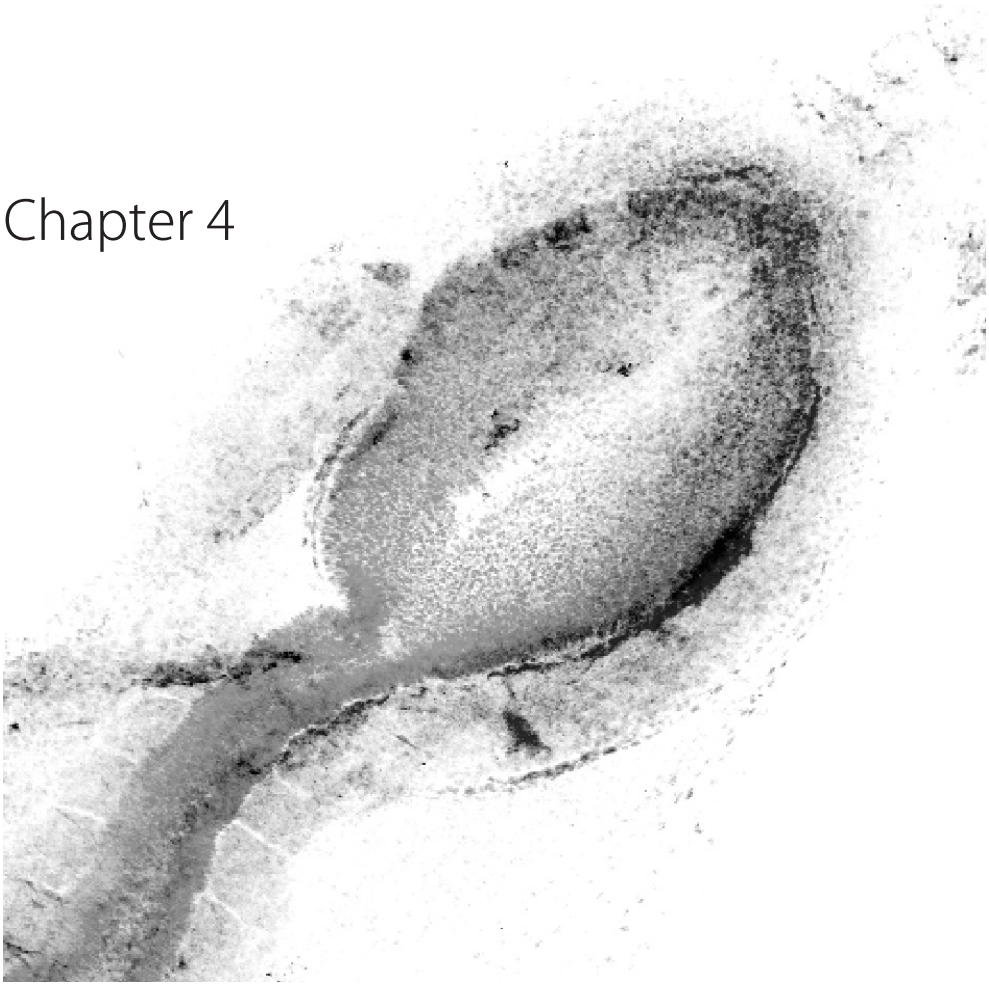
PGC population in *Cdx2* and *Wnt3a* mutants

Chapter 4



Cdx/Hox genes and Wnt signalling in morphogenesis of derivatives from the posterior growth zone

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

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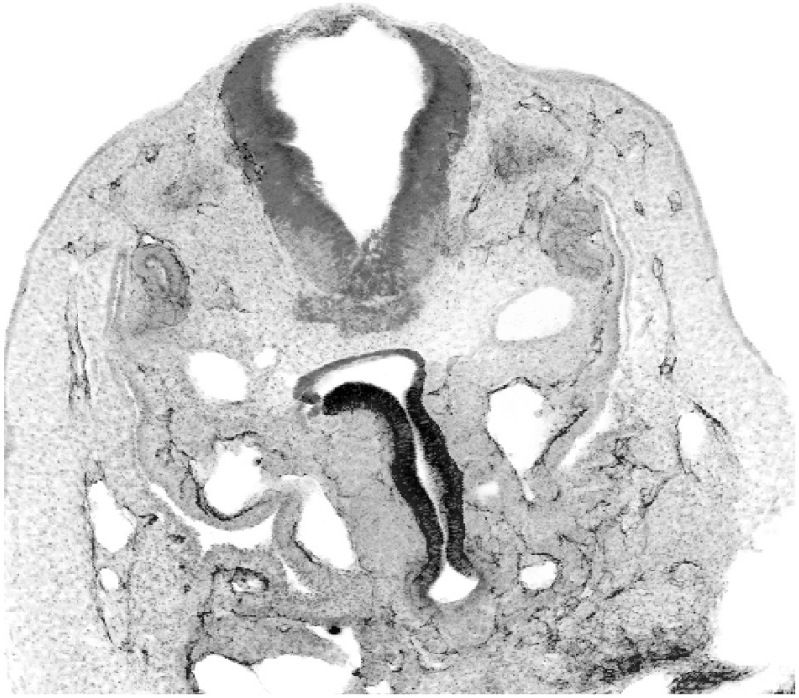

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ABSTRACT

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

INTRODUCTION

The *Drosophila* gene *Caudal* (*Cad*) (1) 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 both mice and humans. In the mouse, all three genes are expressed in embryos at the primitive streak stage of development. *Cdx1* transcripts appear at embryonic stage (E) 7.2 in the posterior part of the streak, extending posteriorly to the base of the allantois; *Cdx2* and *Cdx4* start transcription at about the same stage in an overlapping region extending into the base of the allantois (2). All three genes remain highly expressed in and along the primitive streak and later in the tailbud until E10.5 (*Cdx4*), E11.5 (*Cdx1*) and E12.5 (*Cdx2*). All three genes are also expressed in the developing hindgut endoderm but only *Cdx1* and *Cdx2* remain expressed there into late gestation and postnatally (3).

All three Cdx genes are involved in antero-posterior patterning of the embryonic axis. *Cdx1*^{null} mice exhibit anterior homeotic shifts in vertebral identity involving the upper cervical vertebrae (4) while *Cdx2*$^{+/-}$ animals manifest similar homeotic defects more posteriorly, in the lower cervical and upper thoracic regions (5). *Cdx2*^{null} embryos fail to implant because the gene is essential to trophectoderm development (6). *Cdx4*^{null} mice exhibit only a mild anterior transformation at a particular thoracic position with a very low penetrance and no other abnormality. In *Drosophila Cad* has been found to be the homeotic gene specifying the identity of the last abdominal segment, the analia (7).

Cdx genes have been shown to play an essential role in posterior axial elongation in the mouse (8-10) and in several insect and arthropod species that also extend their body axis by posterior addition of tissues (11). This mode is not used by long germ band insects like the fruit fly. Although each of the three Cdx genes contributes to posterior axial extension, the contribution of *Cdx2* is the most obvious, since heterozygote *Cdx2* mutants have a slightly shorter axis. Using a *Cdx2conditional* allele (10, 12, 13) and an epiblast restricted *Cre* transgene (14), it was possible to specifically inactivate *Cdx2* in the embryo proper. E10.5 *Cdx2*^{null} embryos are posteriorly truncated in a similar manner to the *Cdx2*^{null} embryos that were rescued by tetraploid fusion following intercrossing of *Cdx2* heterozygotes (15). These *Cdx2*^{null} embryos do not develop a chorio-allantoic labyrinth and die in utero at E10.5. They lack axial tissues posterior to the forelimbs. Conditional *Cdx2* mutants at E8.5 bypass the placental failure, and allows the embryos to develop up to birth (10). Inactivation of *Cdx2* exclusively in the endoderm from its initial specification using *Foxa2Cre* leads to blunt ending of the gut at the ceacum (13) and demonstrates a role of *Cdx2* in posterior endoderm expansion. Compound mutants carrying one *Cdx2*^{null} allele and homozygous null for *Cdx4* (referred to later as *Cdx2/4* mutants), fail to generate their posterior tissues caudal to the hindlimbs (9). Most of these embryos die around E10.5 from deficient placental labyrinthine development (16), but the pen-

etrance of the phenotype is variable, leaving about 10 % of the *Cdx2/4* embryos to progress to full term. These latter animals die shortly after birth.

The Caudal Regression syndrome, also called Caudal Dysplasia (17) encompasses a range of congenital defects of varying severity. These may involve malformations of the lumbar vertebrae, partial or complete sacral agenesis, caudal neural tube defects and abnormalities of cloacal derivatives. The latter include recto-anal atresia, recto-urinary or recto-vaginal fistulae and abnormalities of the bladder outflow tract. Animal models have been described for Caudal Regression or related syndromes such as Anorectal Malformations (ARMs) and were found to involve *Sonic hedgehog* (18), *Retinoic acid* (RA) (19), and the non canonical *Wnt5a* (20, 21). A role for Gdf11 and its associated pro-protein convertase Pcsk5 has been suggested as well since inactivating the latter causes the VACTERL-like phenotype (22) comprising vertebral and anorectal anomalies. Recently, anorectal malformations induced by ethylenethiourea in rat embryos were reported to be accompanied by downregulation of *Cdx1* (23).

We analysed the morphogenetic defects of an allelic series of Cdx mutants, at different stages of embryogenesis and found them to exhibit (besides posterior vertebral truncations) phenotypical traits mimicking Caudal Regression defects with respect to uro-rectal morphogenesis and neurectoderm patterning. We previously reported that the caudal vertebral truncation of *Cdx2/4* mutants was corrected by a posterior gain of *Lef1* expression activating the canonical Wnt pathway (9). We now have carried out a detailed study of the relationship between Cdx and Wnt in posterior neural and anorectal tissue morphogenesis. Rescue of the *Cdx2/4* vertebral truncation phenotype by a gain of activated *Lef1* re-established development of a separated urogenital sinus and rectum in mutant animals. We also analysed posterior tissues in a novel hypomorph *Wnt3a* mutant, and found that it exhibits a vertebral truncation phenotype of intermediate severity between the *Wnt3a*^{null} mutants (24) and the mild *Wnt3a*^{hypomorph} mutants *Vestigial tail (Vt)* (25). In this mutant we found similar defects in the uro-rectal region and in the caudal neural tube as those we saw in *Cdx2/4* and *Cdx2* mutants (induced at stages to bypass placentation defects). We also observed similar defects in posterior neural tube patterning and in urorectal septation in transgenic embryos precociously expressing Hox13 genes. These findings point to the participation of Cdx genes and canonical Wnt signalling not only in the generation of the vertebrae, but also in posterior neural tube morphogenesis, and cloacal development. The data also stresses the importance of the correct timing of Hox gene expression for these events. We propose that the site of action of this network centered on Cdx and Wnt resides in the posterior growth zone in the tail bud, and reveals a unifying function of Cdx genes in posterior morphogenesis of tissues in the three germ layers. This suggests that the etiological nature of human Caudal Dysplasia and anorectal malformations (ARMs) may often be the result of a shortage of growth stimulation of the progenitors of posterior tissues in the tail bud at earlier stages of development.

RESULTS

Anorectal malformation in Cdx mutants and in embryos precociously expressing Hox13 genes.

Fetuses lacking *Cdx4* and one allele of *Cdx2* (*Cdx2/4* mutants) usually (90 %) exhibit lethal placental defects (16). Ten percent overcome this defect and develop further but all die within a few days of birth (9). Inspection of neonates with this genotype revealed an imperforate anus. Morphogenesis of anorectal and urethral tissues was analysed further. Surviving *Cdx2*$^{+/-}$*Cdx4*$^{0/-}$ full term male were growth retarded with short or absent tails. The anal opening was absent in all animals. The abdomen was often distended and on transillumination this was seen to be due to an enlarged fluid filled bladder (Figure S1). There was no evidence of defecation and post-natal survival was not possible. On serial sectioning the bladder was dilated and thin walled. The urethral outflow tract was patent but appeared somewhat distorted, and probably not functional due to pressure from the dilated hindgut. The hindgut ended blindly at the level of the bladder neck and a fistula between the urinary and intestinal tracts was present in all the adult specimens examined (n=4). The region at which the gut terminated was variable (see Figure 1D-F for E10.5 embryos). We also studied serial sections of *Cdx2*$^{+/-}$*Cdx4*$^{0/-}$ male embryos at E15.5. These exhibited similar features to those described for full term specimens with the exception that the urinary outflow tract failed to open externally (Figure 1A, B). We conclude that in the newborn male (*Cdx2*$^{+/-}$*Cdx4*$^{0/-}$) compound

mutants described above, 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 on the lower surface of the penile shaft. This re-establishment is, however, defective and insufficient to relieve the accumulation of urine in the bladder. We also examined serial sections of a *Cdx2*$^{+/-}$*Cdx4*$^{-/-}$ female mouse fetuses at E18.5. Once again, we found anal atresia, though a recto-urinary fistula did not develop due to interposition of the utero-vaginal canal and its mesentery. The bladder in these animals was enormously dilated and the urinary tract did not open to the exterior. There was no evidence of hydronephrosis or of hydroureter. We examined the genital system in all the serially sectioned animals and found no abnormalities in either the gonads or in the gonadal ducts of either sex (not shown).

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

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

Some of the *Cdx2PHoxb13* transgenic founder mice expressing the *Hoxb13* gene precociously, under the *Cdx2* promoter (9), were found to manifest anal atresia. We generated transgenic fetuses expressing *Hoxc13* under the *Cdx2* promoter, and observed that they all exhibit ano-rectal agenesis and abnormal communication between the bladder and the hindgut (Figure 1G, H). Premature expression of Hox13 genes thus leads to a phenotype similar to that resulting from a decrease in Cdx activity.

Uro-rectal septation, Cdx and Wnt signaling

Posterior axial defects of *Cdx2/4* mutants are partially corrected by a transgene expressing an activated form of the downstream effector *Lef1* from the *TBrachyury* promoter (9). This posterior Wnt gain of function rescued the morphogenesis of the hindgut and bladder outflow tracts in 8 out of 9 cases analysed (89%) (example in Figure 2A, B). Given this functional rescue of Cdx mutants by an activated *Lef1* transgene, we also analysed the cloacal derivatives of *Wnt3a* mutant embryos. *Wnt3a*^{null} embryos fail to generate tissues posterior to the forelimbs, preventing such an analysis, but embryos homozygous for an hypomorphic *Wnt3a* allele (26) exhibit a vertebral truncation phenotype of intermediate severity between the *Wnt3a*^{null} and the mild *Wnt3a* hypomorph *Vestigial tail (Vt)* mutants (25). All examined embryos (n=8) homozygous for the new *Wnt3a* hypomorphic allele arrest their axis extension at sacral levels (n=8), thereby resembling *Cdx2/4* compound mutants, and *Cdx2*^{null} embryos generated with the conditional *Cdx2* allele and Cre recombinase induction at E7.5. The severity of the phenotype of homozygous *Wnt3a* hypomorph mutants was variable, and some embryos displayed sirenomelia (fused hindlimbs). This latter phenotype, observed in 1 out of the 4 mutants, was the most severe and was accompanied by bladder agenesis. All the other mutants (75%) were deficient in cloacal development at E10.5 similarly to *Cdx2/4* mutants. They failed to undergo complete septation of the urogenital and anorectal tracts (Figure 2C versus A).

Cdx2 and *Cdx4* are normally expressed in all three germ layers at the tail end of embryos, and in overlapping domains in the gut endoderm at E9.0 (Figure 2D-G). *Cdx2* remains expressed in the endoderm at later stages, with a maximum in the para-ceacal region, decreasing in both directions (3). It is thus expressed at a much lower level in the cloaca than more anteriorly at E12.5 (Figure S2). We analysed the expression of *Wnt3a*, and of *Axin2*, a read out of canonical Wnt signaling, during cloacal septation and anorectal morphogenesis in wild types and Cdx mutants. *Wnt3a* and *Axin2* are expressed in the posterior growth zone at the tail end of the embryo,

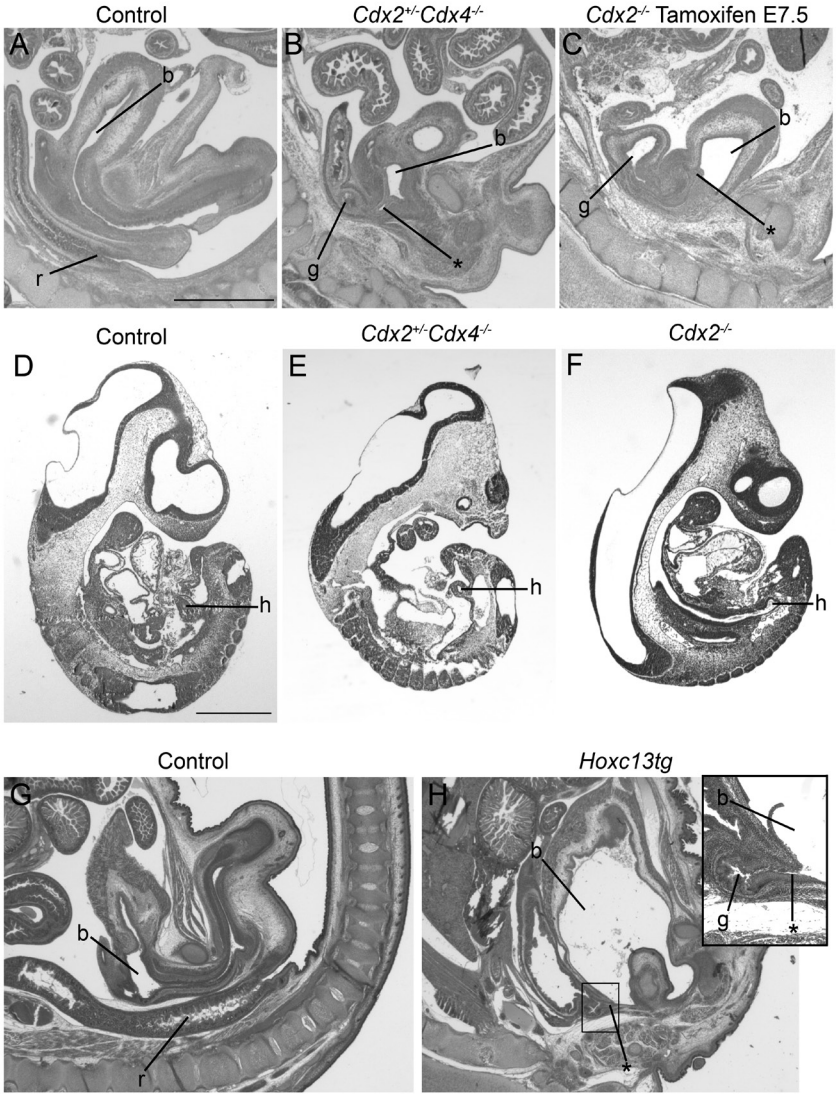


Figure 1 Phenotypes of Cdx mutants and *Hoxc13* transgenic embryos in the urorectal region. **A-C**, Sagittal sections of the urorectal region of a E15.5 embryos control (**A**), *Cdx2*$^{+/-}$*Cdx4*$^{-/-}$ mutant (**B**) and *Cdx2*^{null} (upon *Cdx2* inactivation at E7.5 by tamoxifen induction of *Rosa26CreER*^{T2}); **D-F**: E10.5 sagittal sections of a control (**D**), *Cdx2*$^{+/-}$*Cdx4*$^{-/-}$ mutant (**E**), and a *Cdx2*^{null} mutant (**F**), showing the blunt-ending hindgut. **G,H**, Sagittal sections though the urorectal region of a E18.5 control (**G**), and transgenic fetus expressing *Hoxc13* from the *Cdx2* promoter (**H**). The insert in **H** is a blow up of part of an adjacent section showing the fistula between the bladder and the gut. h, hindgut; b, bladder; g, gut; r, rectum; *, communication between bladder and gut. Scale bar in **A** (**A-C**): 1mm; in **D** (**D-F**): 0,5mm.

but are not expressed in either the endodermal lining of the cloacal cavity, or in the mesoderm of the urorectal septum at E10.5 (Figure 2H-O). The same restriction to tailbud tissues applies to the activity of the *TBrachyury* promoter driving the rescuing *Lef1* transgene. This promoter is the "primitive streak" promoter fragment shown previously to be active exclusively and transiently in the mesoderm emerging from the primitive streak during gastrulation, and in the tailbud thereafter (27). The *TPLef1* transgene is thus not active in the anlage of ano-rectal and urethral tissues at E10.5. These data suggest that the defect in urorectal septation in *Wnt3a* mutants and the rescue effect of *TPLef1* in Cdx mutants must originate from the progenitors of cloacal structures at a time when they still resided in the posterior growth zone.

Wnt pathway components in Cdx mutants

In searching for a mechanism underlying the impaired development of posterior tissues in Cdx mutants, in particular with respect to Wnt signaling, attempts were made to identify Cdx transcriptional targets. Transcriptional analysis was performed in posterior tissues of *Cdx2*^{null} mutants versus controls. This analysis had to be performed at early somite stages (Figure S3) when *Cdx2* is active in the wild type, but before posterior morphogenesis is

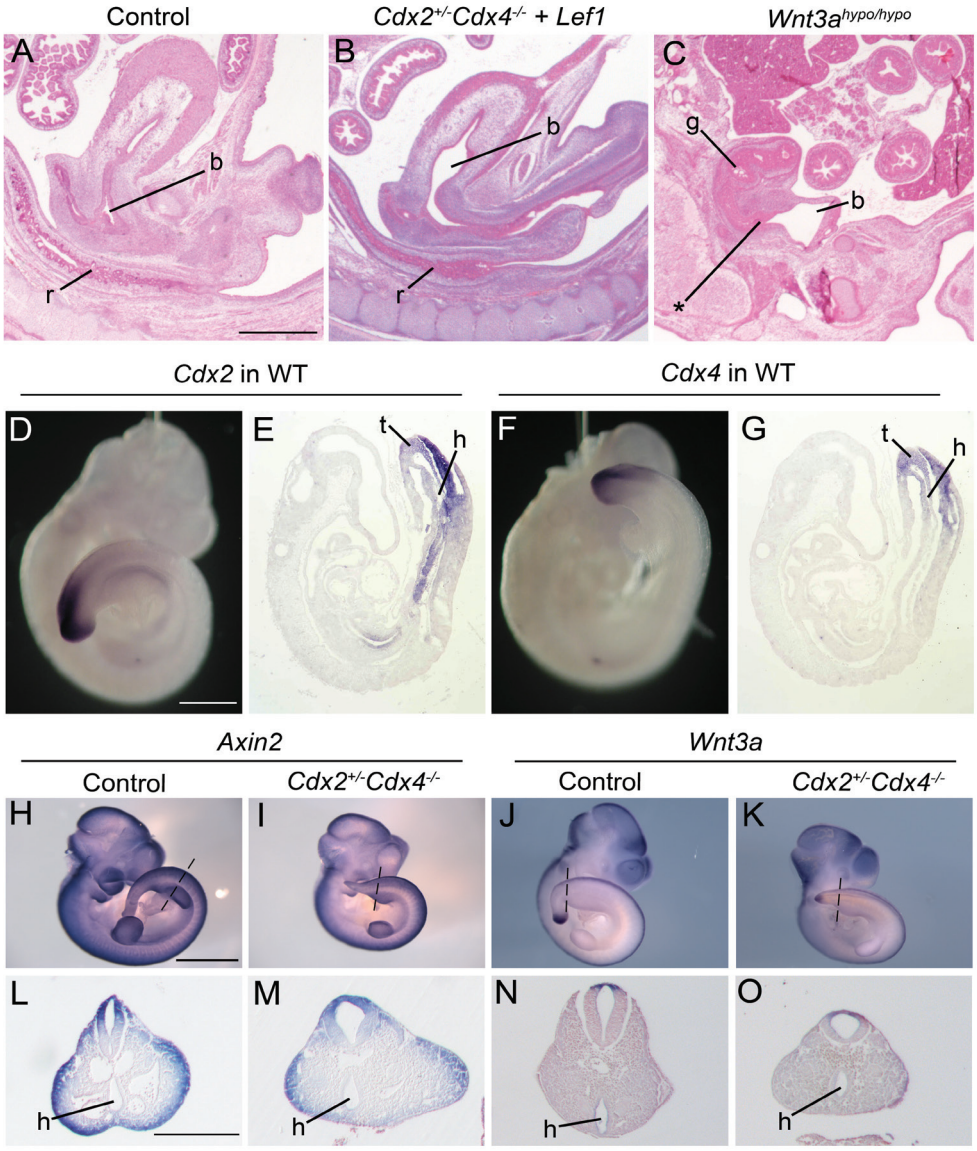


Figure 2 Phenotypes of *Lef1* rescued Cdx mutant and *Wnt3a* mutants, and gene expression in the urorectal region. A-C, sagittal sections of the urorectal region of a E15.5 control (**A**), *Cdx2/4* rescued by the *TPLef1* transgene (**B**), and *Wnt3a*$^{hypo/hypo}$ (**C**); the section in **C** is not exactly mid-sagittal but shows the bladder-rectum fistula. **D-G**, in situ hybridization of E9.5 whole mount wild type embryos with a *Cdx2* (**D**) and *Cdx4* (**F**) probes, and sagittal sections through E9.0 embryos hybridized with the same probes (**E** and **G**, respectively); **H,I,L,M**, In situ hybridization with an *Axin2* probe on a whole mount E10.5 control (**H**) and *Cdx2/4* embryo (**I**) and cross sections (**L,M**) of these whole mounts at the level indicated by the dashed line in **H** and **I**, respectively. **J,K,N,O**, In situ hybridization with a *Wnt3a* probe on a whole mount E10.5 control (**J**) and *Cdx2/4* mutant (**K**) embryos, and corresponding transverse sections (**N,O**). b, bladder; r, rectum; t, tailbud; g, gut; h, hindgut; *, communication between bladder and gut. Scale bars in **A**: 1mm, in **D**: 0,5mm, in **H** and L: 0.5mm.

heavily altered in the mutants. Two microarray screens, performed at slightly different stages and in duplicate (Supplemental Table S1) did reveal a slight downregulation of *Wnt3* (by a factor of 1.43 at the stage of 7/8 somites) whereas no change in expression of *Wnt3a* and *Axin2* was detected. *Axin2*, a readout of canonical Wnt signalling, was thus not downregulated in *Cdx2* mutants at early stages, when posterior tissues in the mutant can still be compared to wild type counterparts. Given the rescue of the *Cdx2/4* mutant phenotype by *TBrachyury* promoter-driven expression of an activated *Lef1 (TPLef1)*, we performed quantitative PCR analysis of *Axin2* expression in early *Cdx2/4* mutant transgenic for *TPLef1*, compared with non transgenic mutants. We found that Axin2 was not significantly upregulated in the *TPLef1* transgenic embryos. These data suggest that the activity of canonical Wnt signaling is not modulated extensively, either in Cdx mutants or in their *TPLef1* rescued counterparts, at these early stages. It is possible that a subtle modulation of this pathway increases in amplitude with time, a possibility that cannot be cleanly investigated because the posterior tissue of Cdx mutants becomes increasingly affected.

In addition to *Wnt3*, two other genes concerned with canonical Wnt signaling were affected in early *Cdx2* mutants. *Frzb1*, a Wnt antagonist at the level of receptor binding (28), also found to facilitate diffusion of Wnt ligands in *Xenopus* embryos (29), was found to be downregulated in both transcription screens, by a factor of 1.97 and 1.65, respectively (Supplemental Table S1). *Nkd1*, encoding a protein negatively interacting with Dishevelled (Dvl) (30) was found to be upregulated in both arrays, by a factor of 1.50 and 1.77, respectively (Supplemental Table S1). Dvl is a central mediator for both the canonical and non canonical Wnt pathways. Altered transcription levels of *Frzb1* and *Nkd1* in our transcriptome analysis of E8.5 *Cdx2*^{null} mutant embryos were validated with RNA from an independent pool of embryos at the same stage. In situ hybridization revealed that these genes are expressed in caudal embryonic tissues but not in the cloacal area at the time of urorectal septum development (not shown). We conclude from the phenotypes of *Wnt3a*, and *TPlef1*-rescued Cdx mutants, and from the gene expression analysis, that Cdx genes and the canonical Wnt pathway are involved in the morphogenesis of cloacal tissues, and that they play their essential role in the posterior growth zone at the tail end of the embryo at the time this zone generates cell descendants that contribute to the urorectal septum mesoderm.

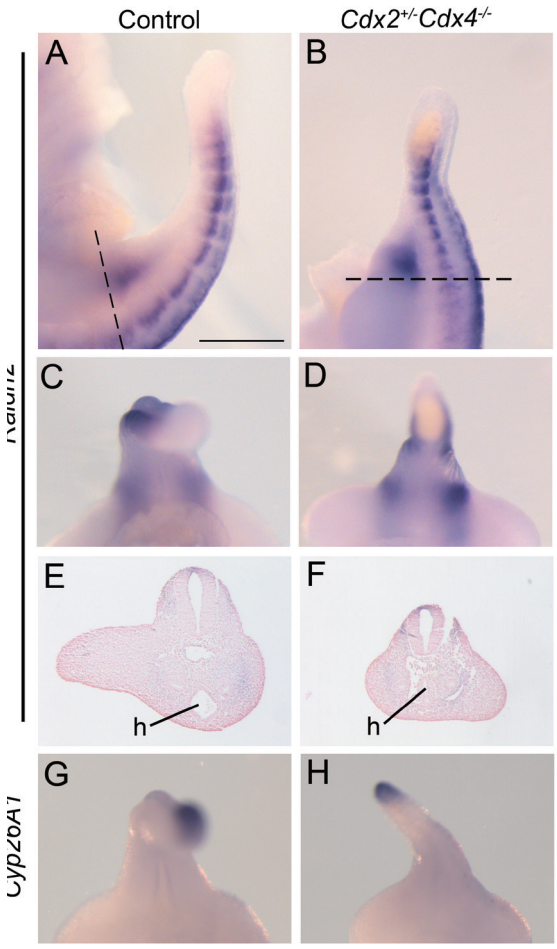
Retinoic acid signaling in cloacal development of Cdx mutants

Exposure of E9.5 embryo to excess RA in utero (31-33) and inactivation of the RA-degrading enzyme Cyp26a1 (34, 35) lead to caudal defects similar to Cdx and Wnt mutations, and to premature expression of Hox13 genes. *Cyp26a1* is expressed exclusively in caudalmost tissues in the tailbud during trunk and tail axial extension, and Cdx loss of function mutants and transgenic embryos expressing *Hoxb13* or *Hoxc13* precociously were found to transcribe it at lower levels in these tissues (9, 10). *Cyp26a1*, which was shown to be a direct Cdx target (10); it is not expressed at all in cloacal derivatives (Figure 3G, H). The impact of inactivating *Cyp26a1* on cloacal septation must therefore occur early through the function of the gene in caudal progenitors of cloacal tissues residing in the growth zone.

Transcription of the gene encoding the RA biosynthetic enzyme Raldh2 takes place in the somites and in two lateral areas in the cloacal region at E10.5, the time of cloacal septation (Figure 3A-F). *Raldh2* expression is absent in the cloacal tissues themselves but is localized in lateral ventral mesenchyme, probably associated with the anlagen of the genital tubercle that develops at a later stage. Ventral Raldh2 expression was slightly increased in *Cdx2/4* mutants compared to controls (Figure 3A-D). The transcriptome analysis of *Cdx2* mutants at early somite stages also revealed up regulation of *Raldh2* in posterior tissues at stages earlier than the first manifestation of the posterior axial defects (Supplemental Table S1). This potential increase of diffusing RA together with lower *Cyp26a1* in the posterior growth zone from which cloacal descendants are generated may causally contribute to anorectal malformations in Cdx mutants.

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

Disruption of Shh signaling has long been associated with the etiology of anorectal malformations (36-38). Shh signaling is required in different and successive phases of cloacal and genital development (39). *Shh* is expressed in the notochord and floor plate of the neural tube, and in the gut endoderm (40). At E12.5, *Shh* is expressed in the endoderm lining of the cloaca and its derivatives, but not in the mesenchyme of the urorectal septum (39)



gure 3 Expression of genes of the biosynthesis and gradation of RA in control and *Cdx2/4* mutants. A-F, In situ bridization with a *Raldh2* probe on a whole mount E10.5 con- l (**A,C,E**), and *Cdx2/4* (**B,D,F**), viewed laterally (**A,B**) and from ventral side (**C,D**), and transverse sections thereof (**E,F**). **H**, Ventral view of a E10.5 control (**G**) and *Cdx2/4* embryo (**H**), bridized with a *Cyp26a1* probe; h, hindgut. Dashed lines in **A** d **B**, indicate the level of sections **E** and **F**, respectively. Scale r: 0,5mm.

(Figure 4A, D, G). Transcriptome analysis of posterior tissues of early embryos suggested that *Shh* was slightly downregulated in *Cdx2* mutants (see supplemental Table S1). However, gene down regulation was not evident in the cloacal region of E10.5 *Cdx2/4* mutant embryos hybridized as whole mounts with *Shh* probes (Figure 4A, B and data not shown, n=4). We examined the expression of *Shh* in the cloacal region of E12.5 *Cdx2/4* mutants, which all exhibit incomplete septation, and could not identify any difference in expression level in the endoderm relative to control embryos (Figure 4G, H). These experiments argue against a major direct impact of Cdx gene products on *Shh* expression in the cloacal region in this crucial E10.5-E12.5 window. *Shh* expression was not downregulated either in *Wnt3a* hypomorph mutants (Fig. 4C ,F ,I versus A, D, G).

Hoxa13 and *Hoxd13* are expressed in the endoderm and mesoderm of the cloacal tissues in both mice and chicks (41-45). Double inactivation of these genes also leads to a defect in partition between the urogenital and rectal outflow tracts (46). The expression of Hox13 genes was monitored in *Cdx2/4* mutants and controls at E10.5 and E12.5. *Hoxc13* and *Hoxb13* are not expressed in the cloacal region at E10.5. *Hoxa13* and *Hoxd13* are expressed in the cloacal area at this stage, but no difference was found in their expression levels between mutants and controls (Figure 5A-H). In situ hybridization on sagittal sections of E12.5 embryos revealed that *Hoxa13, Hoxb13* and *Hoxd13* are expressed at the same level both in the cloacal endoderm and in the urorectal septum mesoderm in *Cdx2/4* embryos and in controls (Figure 5I-P). We could not detect *Hoxc13* expression in these tissues, although the gene was expressed in the tail bud mesoderm and neurectoderm (not shown). We therefore conclude that Cdx mutants are not causing urorectal septum defects by doenregulating Hox13 genes.

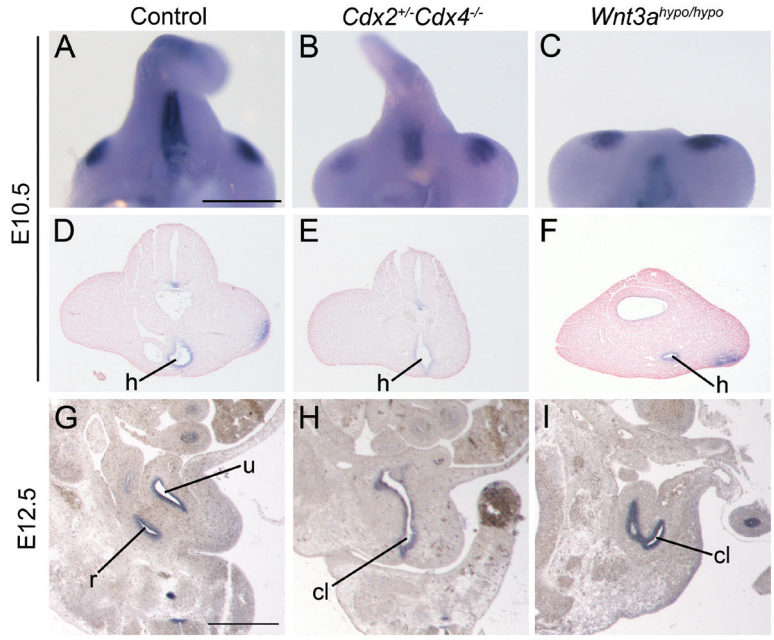


Figure 4 Expression of *Shh* in *Cdx2/4* mutants and Wnt3a hypomorph mutants. A-F In situ hybridization with a *Shh* probe of a whole mount E10.5 control (**A**), *Cdx2/4* (**B**) and *Wnt3a*$^{hypo/hypo}$ (**C**) embryos, and transverse sections thereof (**D-F**); **G-I**, in situ hybridization on sagittal sections of the urorectal region of a E12.5 control (**G**), *Cdx2/4* (**H**), and *Wnt3a*$^{hypo/hypo}$ (**I**) mutant embryos. h, hindgut; r, rectum; u, urethra; cl, cloaca. Scale bars: 0,5mm.

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

An additional phenotypic feature indicates the relevance of Cdx mutations to the human Caudal Regression syndrome. Not only do mouse and human conditions exhibit anorectal septation and posterior skeletal defects, but both also manifest abnormalities in the caudal neural tube. Transverse sections of *Cdx2/4* compound embryos at axial levels cranial to the truncation revealed Sox2 positive ectopic tubular structures ventral to the neural tube, and irregularities in the cellular arrangement in the neurepithelium in all cases (Figure 6A, D and Figure S4 A, B for the axial levels of these defects; n=6). Ventral ectopic neural structures (ens) were also found in *Cdx2*^{null} (n=4) and in *Cdx2/4* double null (n=3) mutant embryos obtained after epiblast-restricted inactivation of the *Cdx2* conditional allele (Figure 6B ,E and and C, F, respectively). We characterized the posterior neurepithelium of the latter mutant embryos at E10.5 with a number of antibodies on transverse sections. Alpha6 integrin is normally expressed on cell membranes in the ventral neural tube and in the gut endoderm (47). It was either not expressed (n=1) or expressed considerably less (n=2) in the mutant neural tube (Figure 6C ,F), in spite of the fact that expression was observed in the mutant gut endoderm (not shown). The neural cell arrangement was disrupted in the mutant at these caudal levels, and the lumen of the neural tube was irregular in shape (Figure 6F). Neural tube morphogenesis was analysed in sections of *TPLef1*- rescued *Cdx2/4* mutants, and found to be similar to that in wild types even at posterior levels (not shown).

Transgenic embryos precociously expressing Hoxb13 under the transcriptional control of the *Cdx2* promoter were examined for neural tube morphogenesis. These embryos were reported earlier to exhibit an axially truncated vertebral column resembling in that to embryos with decreased Cdx expression (9). The truncation due to homozygocity for the *Cdx2PHoxb13* transgene, though relatively mild (axial arrest after 25 sacral and caudal vertebrae, instead of the normal 35, Figure S4 G,H) was found to be more severe than in hemizygotes. Cross sections of E10.5 embryos of this genotype revealed in all cases a disturbed cellular arrangement of the neurepithelium, and the presence of ectopic tubular structures expressing the neural marker Sox2, similar to those in Cdx mutants (Figure 6G,J) (n=4). Alpha6 integrin staining labelled the adherens junctions between cells of the ventral aspect of the neural tube in both *Hoxb13* transgenic and wild type embryos, attesting to correct dorso-ventral patterning of the mutant and transgenic neural tubes. Analysis of the posterior neural tube of embryos transgenic for *Cdx2PHoxc13* (Figure S4 I,J) revealed similar ectopic neural structures at E10.5 (Figure 6H, K), and a split in the neural tube posteriorly at E18.5 (Figure 6I, L).

The ectopic neural structures of Cdx mutants and of transgenic embryos prematurely expressing Hox13 genes are reminiscent of features reported in *Wnt3a*^{null} mutants (24, 48) (Figure 6N, P and S4 K, L), and we analysed transversal sections of *Wnt3a*^{null} and *Wnt3a* hypomorph homozygous embryos (Figure 6M, O and S4 M, N). These also revealed ectopic neural structures and neurepithelial irregularities of a severe type (Figure 6O), resembling the *Cdx2PHoxc13* split neural tube.

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

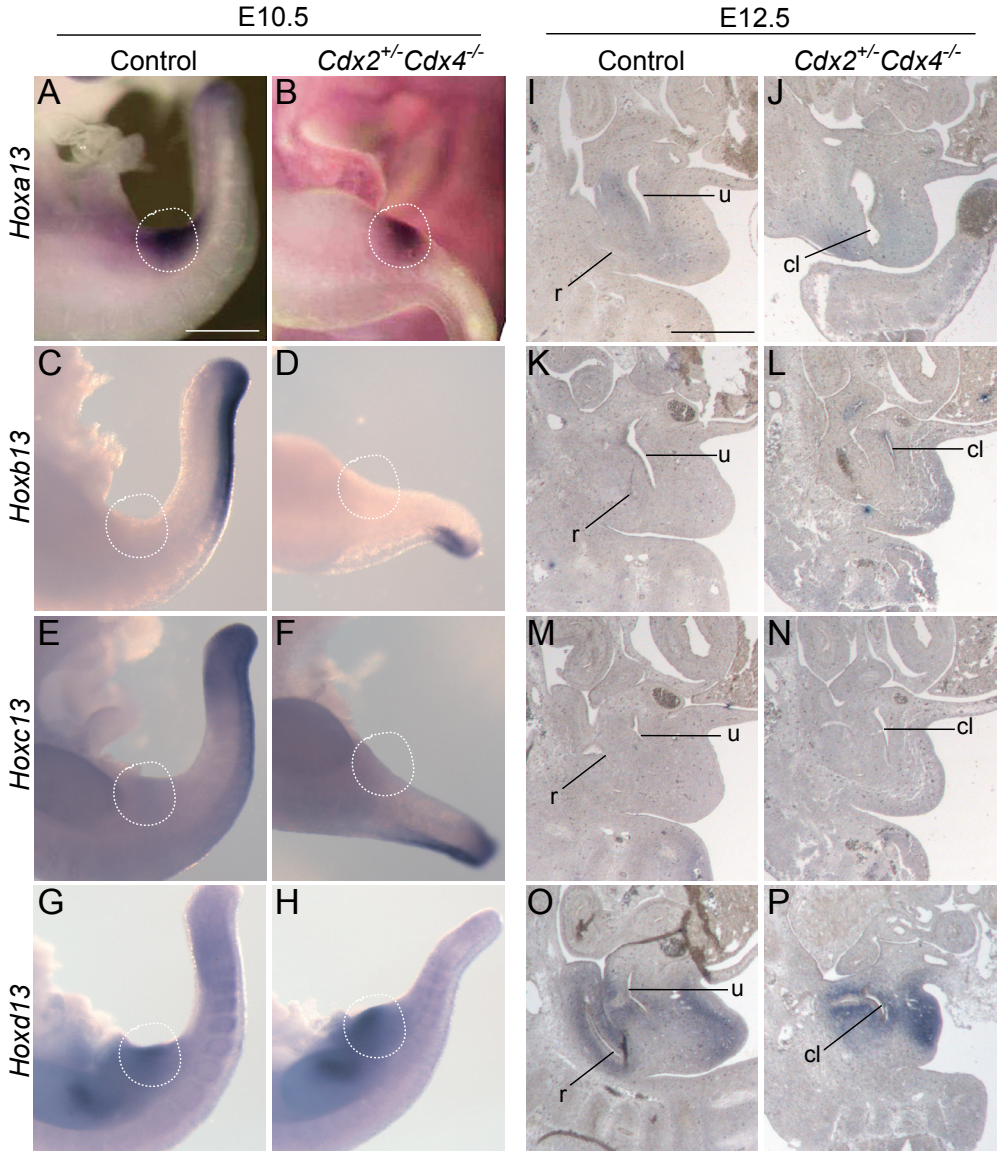


Figure 5 Expression of the four Hox13 genes in wild types and *Cdx2/4* mutants at E10.5 and E12.5. A-H, In situ hybridization with probes for *Hoxa13* (**A,B**), *Hoxb13* (**C,D**), *Hoxc13* (**E,F**) and *Hoxd13* (**G,H**) on whole mount E10.5 control (**A,C,E,G**) and *Cdx2/4* mutant (**B,D,F,H**)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

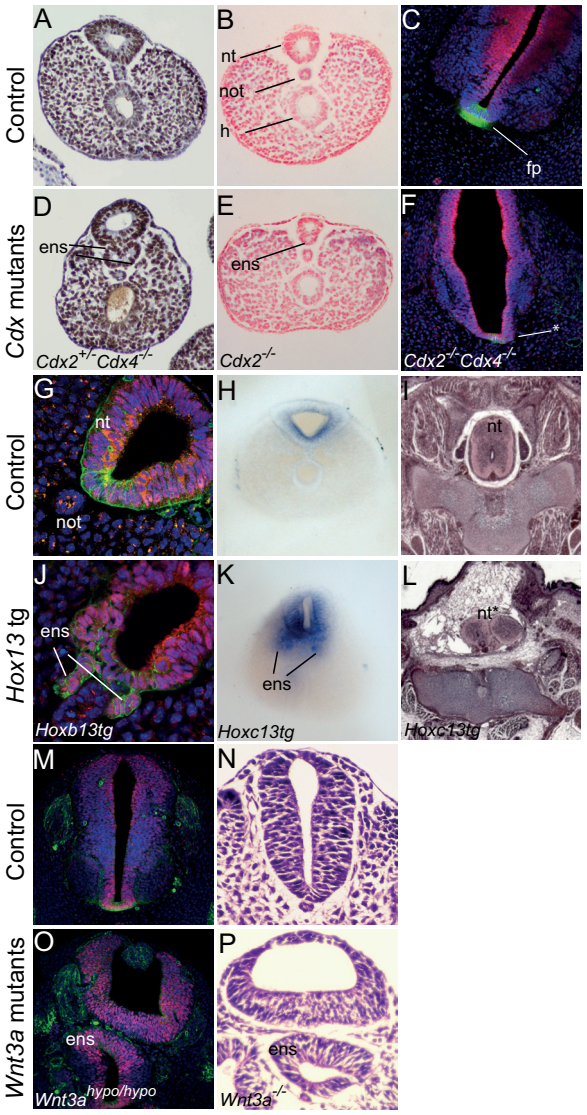


Figure 6 Characterization of the posterior neural tube of wild types, Cdx mutants, Wnt3a mutants and transgenic embryos precociously expressing Hox13 genes. The axial levels of the sections analysed for the different genotypes are indicated in Supplemental Figure S4. **A-F**, controls and Cdx mutants. Transverse sections of a E10.5 control (**A**) and a *Cdx2/4* mutant (**D**) embryos immunostained for the proliferation marker Ki67; **B,E**, neutral red stained histological section of a control (**B**) and *Cdx2*^{null} (**E**) embryo; **C, F**, immunofluorescence for alpha6 Integrin (green) and Sox2 (red) on transverse sections at posterior levels of a control (**C**) and *Cdx2/4* double null mutant embryo (**F**) Note the ectopic neural structures –ens- ventral to the neural tube in the mutants in **D** and **E**, and the arising ens on the left side –asterisk- of the very weak alpha6 integrin staining in **F. G-L**, Controls and transgenic embryos expressing a *Cdx2PHox13* transgene. Immunofluorescence for alpha6 Integrin (green) and Sox2 (red) on transversal sections at posterior levels of a control E10.5 (**G**) and a *Cdx2PHoxb13* transgenic embryo (**J**). Cross sections through the posterior part of a E10.5 control (**H**) and a *Cdx2PHoxc13* transgenic embryo (**K**) after hybridization with a *Sox2* probe. Cross sections in the posterior region of a E18.5 wild type (**I**) and *Cdx2PHoxc13* transgenic embryo (**L**); **M-P**, Controls and *Wnt3a* mutants. Staining of transversal sections of a E10.5 wild type (**M**) and a *Wnt3a*$^{hypo/hypo}$ mutant (**O**) with anti Sox2 (red) and anti alpha6 integrin (green) antibodies; hematoxylin/eosin stained sections through a E10.5 wild type (**N**) and a *Wnt3a*^{null} embryos (**P**). ens, ectopic neural structures; h, hindgut, nt*, split neural tube; not, notochord," in F, emerging ectopic neural structure.

DISCUSSION

Cdx, Hox and Wnt and posterior morphogenesis

The data in this work reveal that in addition to the extension of the vertebral axis, development of posterior neurepithelium and of cloacal derivatives also depends on the activity of Cdx genes, on precisely timed, sequential Hox gene expression, and on persisting canonical Wnt signaling in the posterior embryonic growth zone. Alteration of any of these parameters arrests the skeletal, neural and cloacal development programme and mimics human congenital Caudal Regression or Caudal Dysplasia. The involvement of a shortage of growth stimulation in the progenitor zone of posterior tissues at early stages in the tail bud therefore sheds a new light on the aetiology of these syndromes, and possibly on anorectal malformations (ARMs) more generally.

Other genetic factors have been considered in the etiology of the Caudal Regression syndrome, such as *Shh* and its proposed downstream signaling effectors *Wnt5a* and *BMP4* (20, 21, 33, 49). *Shh* plays multiple essential roles during embryogenesis. Its inactivation in the mouse leads to agenesis of distal limbs and caudal axial structures in addition to midline patterning defects (50). *Shh* was shown to contribute to morphogenesis

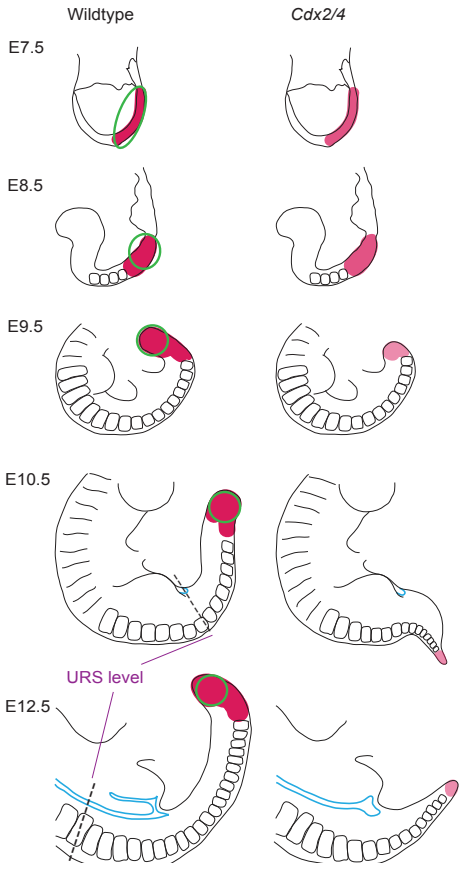


Figure 7 Scheme summarizing the morphology and gene expression in the posterior growth zone of wildtypes (surrounded by a green circle) and *Cdx2/4* mutants. The growth zone is along the primitive streak at E7.5, E8.5, and in the tail bud region at E9.5, E10.5 and E12.5. The expression of *Cdx2*, *Wnt3a*, and *Cyp26a1* is found in posterior tissues including the growth zone of the wildtype (red color), and of *Cdx2/4* mutants (less intense red or pink color), but is absent in the region where the urorectal septum (URS) region develops between E10.5 and E12.5. The situation in *Cdx2/4* mutants is the same as that in transgenic embryos precociously expressing Hox13 genes, and in *Wnt3a* hypomorph mutants. The cloacal endoderm (in blue) expresses *Cdx2* at very low levels, but this is not shown here (not stained in red). The dashed lines indicate the axial level where the uro-rectal septum mesoderm develops between E10.5 and E12.5.

of uro-rectal structures by a specific function in the endodermal lining of the cloacal cavity, from where it signals onto the growing urorectal septum mesenchyme (39). We did not observe a downregulation of *Shh* expression in posterior endoderm derivatives of Cdx mutants during the time window of uro-rectal septation. Cdx loss of function therefore does not appear to impair urorectal septum development by downregulating Shh in the endoderm. Furthermore it is unlikely that Cdx involvement in anorectal development is exerted at the level of the endoderm. Grainger and colleagues (51) report that endoderm-specific inactivation of *Cdx2* using the VillinP-Cre does not lead to imperforate anus. These data, together with our observations on the Wnt3a mutant phenotype, the TPLef1 rescue of Cdx mutants, and the growth zone-restricted expression of the canonical Wnt pathway components, emphasize an essential role of the Cdx and Wnt at the level of the posterior growth zone that supplies progenitors for the cloacal structures earlier than urorectal septum generation (see graphic scheme in Figure 7).

Timing of Hox expression is crucial for cloacal development

A posterior gain of function of the trunk Hox gene *Hoxb8*, rescues *Cdx2/4* mutant defects (9), including uro-rectal septation failure. This rescue must occur early in the progenitor region in the tail bud since the promoter used on the rescuing transgene is the *Cdx2* promoter which is not active in urorectal mesoderm. While microarray screens had not revealed a downregulation of Hoxb8 in early *Cdx2/4* mutants, transcriptome analysis in the severely impaired $Cdx2^{null}$ mutants, revealed a down regulation of this Hox gene (2.75 fold at the 4/5 somite stage, and 2.47 fold at the 7/8 somite stage, Table S1). Phenotypical rescue of the *Cdx2/4* mutation by posterior gain of Hoxb8 function therefore probably results from the correction of a slight reduction of *Hoxb8* transcription, even though this slight reduction is not detectable at stages before tissue are visibly affected.

Expression of Hox13 genes begins in the posterior growth zone of the embryo (remnants of the primitive streak) at around E9.5. The function of Hox genes during embryogenesis has been proven to be critically

dependent on a correct timing of their expression (52-55). Hox13 genes normally control cloacal development after more anterior axial structures have been generated. Precocious expression of Hox13 genes negatively interferes with the trunk developmental program as shown for the axial skeleton (9, 56). Precocious expression of Hox13 genes also jeopardizes the development of cloacal mesoderm. Transgenic embryos expressing Hox13 genes under the *Cdx2* promoter do generate the Hox13 protein prematurely since the *Cdx2* promoter is active in posterior embryonic tissues at E7.2. *Cdx2PHoxb13* transgenic mice were found to manifest anal atresia in some of the cases, and transgenic fetuses expressing Hoxc13 under the *Cdx2* promoter exhibit ano-rectal agenesis and abnormal communication between the bladder and the hindgut. These deleterious consequences of premature expression of Hox13 genes prove that sequential temporal control of Hox gene expression is a prerequisite for balanced morphogenesis of urorectal tissues, in a similar manner to that indicated for axial skeletal structures (9). Precociously expressed Hox13 genes would functionally antagonize the action of earlier Hox genes, a phenomenon observed in different tissues and called Posterior Prevalence (9, 56-58). The impairment of urorectal septation in Hox13 transgenic embryos indicates that expression of these genes needs to be delayed in order not to interfere with earlier Hox genes in the control of cloacal progenitors in the posterior growth zone.

Defects of the urorectal septum of Cdx and Wnt mutants must trace back to their impaired progenitors in the posterior growth zone

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

Lateral plate mesoderm is generated in the gastrulating mouse embryo from the posterior 1/3 of the primitive streak, whereas somitic and midline mesoderm (notochord) emerge from the anterior 2/3 and the anterior extremity of the streak, respectively (59-62). At later stages, after the posterior neuropore closes, the anterior primitive streak, node-streak border and node region become internalized and form the chordoneural hinge (CNH) (63, 64), while the rest of the primitive streak is curved along the ventral outer surface of the tailbud, becoming the ventral ectodermal ridge (VER). Mesoderm emergence from the VER, regulated by Bmp (65, 66), contributes some cells to the posterior tailbud until E9.5 (61, 65) and has completely ceased by E10.5. The trunk lateral mesoderm at axial levels of the urorectal septum therefore must be laid down from progenitors that have emerged from the posterior growth zone earlier than when cloacal septation takes place.

Cdx, Hox, Wnt and neurepithelium expansion and patterning

Cdx and *Wnt3a* loss of function mutations and precocious expression of Hox13 genes impair elongation and morphogenesis of the caudal neurepithelium. Phenotypic similarity between the neural tube of Cdx mutants, of transgenic embryos precociously expressing Hox13 genes and of *Wnt3a* and other Wnt pathway mutants (48, 67, 68) adds to the mutual resemblance of these mutants with respect to axial and lateral mesoderm. It consolidates the emerging concept that Cdx and Hox genes function in the same pathway as Wnt signaling in controlling generation and patterning of posterior tissues in the three germ layers.

Mild tail truncation defects such as those in transgenic embryos prematurely expressing *Hoxb13* are accompanied by severe neurepithelium patterning defects at levels anterior to the truncation. This points to an intrinsic disturbance in the Cdx/Hox network during patterning of the caudal neurepithelium, rather than to a mere consequence of axial growth arrest. The abnormalities observed in the posterior neurepithelium arrangement in Cdx mutants and transgenic embryos precociously expressing Hox13 genes are in some way reminiscent of aberrant gut endoderm histology observed recurrently at posterior axial levels on sections of *Cdx2/4* mutants (69) and in transgenic embryos prematurely expressing *Hoxb13* (data not shown). Another study (70) also reported that $Cdx2^{null}$ embryos exhibit multiple lumens, with disturbed apico-basal polarity in the endoderm epithelium. Abnormal apico-basal position of nuclei in *Cdx2* mutant intestinal epithelium has also been described (51), reminiscent of the irregular polarization of the neurectoderm in Cdx mutants described here.

Genotypes	Posterior vertebral truncation[1] (level of the truncation)	Cloacal septation and anorectal defects[2]	Posterior neural tube dysmorphology[3]
Cdx1^{null} (n>10)	-	-	-
Cdx4^{null} (n>10)	-	-	-
Cdx2$^{+/-}$ (n>10)	+ (tip of the tail)	-	-
Cdx2$^{+/-}$ Cdx4^{null} (n>10)	+ (hindlimb level)	+	+
Cdx2^{null} Sox2Cre (n=10)	+ (forelimb level)	ng	+
Cdx2^{null}RosaCreERT2 Tam E7.5 (n= 3)	+ (hindlimb level)	+	+
Cdx2^{null} Cdx4^{null} Sox2Cre (n=4)	+ (forelimb level)	ng	+
Cdx2$^{+/-}$ Cdx4^{null}TPLef1Tg (n=7)	- (largely rescued)	-	-
Wnt3a^{null} (n=10)	+ (forelimb level)	ng	+
Wnt3a$^{hypo/hypo}$ (n=10)	+ (hindlimb level)	+	+
Cdx2PHoxb13 homozygous (n=5)	+ (caudal level)	+ a	+
Cdx2PHoxc13 hemizygous (n=4)	+ (sacral level)	+	+

[1]Analysed at E10.5 or new born; [2]Analysed at E12.5, E14.5 or E18.5; [3] Analysed at E10.5;ng region not generated; [a] (9)
Table 1 Genotype and phenotype of Cdx and Wnt mutants and Hox13 transgenic embryos.

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

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

Another transcription factor expressed in the posterior growth zone is *TBrachyury*. *TBrachyury* mutants were the first mutants isolated from a mutagenesis screen in the mouse (71). The *TBrachyury* transcription factor is required for embryonic axial elongation, and *TBrachyury* was shown to be a direct target of *Wnt3a* (72), and to exert its activity on axial extension by the maintenance of canonical Wnt signaling (73-75). T is expressed in the epiblast and mesoderm of the primitive streak, and strongly in the notochord. Ectopic neural structures have been observed as well in *TBrachyury* mutants (72).

Mutants in another Tbox gene, *Tbx6*, form ectopic neural structures more severe in extent than Hox13 transgenics, and Cdx, *Wnt3a* and *TBrachyury* mutants. Additional neural tubes form in this mutant at the expense of somitic mesoderm, as a result of upregulation of *Sox2* expression in descendants of posterior progenitors in the growth zone (76). The *Sox2* expression domain is also expanded to include regions outside the neural tube at posterior axial levels in Cdx mutants, and in transgenic embryos precociously expressing Hox13 genes, suggesting an overlap in the mechanistic impairment of the partition of mesoderm and neurectoderm in these mutants.

In Cdx, Wnt , *TBrachyury* and Tbx6 mutants, and in transgenic embryos precociously expressing Hox13 genes, the ectopic neural structures form at the ventro-lateral side of the neural tube. This area is normally colonized by descendants of the anterior primitive streak and later chordo-neural hinge (CNH) (59, 62), making it assumable that these mutations affect the activity of the population of long term neural/mesodermal progenitors (77).

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

TBrachyury^{null} mutants are posteriorly truncated in the three germ layers, and anal atresia was reported for heterozygotes for *Tcurtailed*, one of the *TBrachyury* mutant alleles (78), suggesting that interfering with *TBrachyury* function leads to abnormalities in the urorectum as well as to axial truncation. *TBrachyury* remains strongly expressed in the embryonic tailbud during cloacal development but it is not expressed in the uro-rectum septal mesoderm and endoderm (data not shown) again suggesting that its involvement in anal atresia origi-

nates in its earlier function in the progenitor area in the tail end of the embryo. *TBrachyury* mutants thus exhibit a largely overlapping spectrum of posterior defects with the Cdx and Wnt mutants described here. The similarity in impact of Cdx and *TBrachyury*, mediated in both cases by Wnt signaling strengthens even further the hypothesis that the canonical Wnt pathway is the central player in the balanced morphogenesis of the derivatives of the posterior growth zone during emergence of tissues from the different germ layers. Our data so far do not establish whether or not Cdx and Hox genes operate independently of *TBrachyury* to sustain growth in the posterior growth zone.

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MATERIALS AND METHODS

Mice

All mice were in the C57Bl6j/CBA mixed background. *Cdx2* heterozygotes and *Cdx4null* mutant mice as well as the protocols to genotype them have been described previously (16, 79). As *Cdx4* is X-linked, *Cdx2*$^{+/-}$*Cdx4*$^{-/0}$ and *Cdx2*$^{+/-}$*Cdx4*$^{+/-}$ embryos and pups were generated by crossing *Cdx2* heterozygote and *Cdx4*^{null} mice. *Cdx2*$^{+/-}$*Cdx4*$^{-/-}$ female embryos and pups were generated by crossing *Cdx2*$^{+/-}$*Cdx4*$^{+/-}$females with *Cdx4*$^{-/0}$ males.

Transgenic mouse lines and embryos expressing Hoxb13 and Hoxc13 under the control of the *Cdx2* promoter (80) were described earlier (9). Embryos and animals were analyzed at embryonic stages E8.5, E9.5, E10.5, E12.5, E15.5, E18.5, at birth (P0), and two days after birth (P2). After the first paragraph of the Results section, *Cdx2*$^{+/-}$*Cdx4*$^{-/0}$ male and *Cdx2*$^{+/-}$*Cdx4*$^{-/-}$ female animals will be referred to as *Cdx2/4* compound mutants.

The generation of *Cdx2* conditional mutants has been reported (12) and will be described in detail elsewhere. Epiblast specific *Cdx2*^{null} mutants were obtained by crossing *Cdx2* conditional homozygotes and *Cdx2*$^{+/-}$*Sox2Cre* transgenic mice (14). Post-placentation inactivation of *Cdx2* was achieved by using the *Rosa26CreER*^{T2} (Generous gift of Austin Smith) and tamoxifen intraperitoneal injection at E7.5. The time delay in effective action of tamoxifen in inducing the Cre recombinase in the embryos in our experiments allowed the embryos to develop beyond placentogenesis. This Cre allele was genotyped using the following Cre primers: forward CCGGGCTGCCACGACCAA, reverse GGCGCGGCAACACCATTTTT (fragment size: 445bp). *Wnt3a*^{null} mice were obtained from S. Takada (24). A new *Wnt3a* loss of function hypomorph mutant was recently described (26). Mice were treated according to the "Law on animals in experiments", under the licences required in the Netherlands.

Tissue treatment

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

In situ hybridization

Whole mount In situ hybridization of mutant and control embryos was performed according to Young et al., 2009. Probes were generated against *Cdx2, Cdx4, Axin2, Wnt3a, Wnt5a, Raldh2, Cyp26a1, Shh, Ihh, Hoxa13, Hoxb13, Hoxc13 and Hoxd13* (40, 46, 81-88). Hybridization on sections from paraffin embedded embryos was according to (16).

Antibody staining

Antibody staining on 50 µm vibratome sections of agarose embedded embryos were performed with anti Sox2 (Millipore cat #AB5603) and anti α6 integrin (47). Counterstaining was with DAPI (Invitrogen cat #D3571).

Genome-wide transcriptome analysis of Cdx2 mutants versus controls

Micro-array screens of downregulated and upregulated genes in *Cdx2*^{null} mutant versus wild type embryos were performed at

the 4/5 somite and 7/8 somite stage. RNA was isolated from the posterior part of the embryos (20 embryos of each genotype and stage), dissected at the same axial levels by using the last somite boundary and the base of the allantois as landmarks. Treatment of tissues and micro-array hybridization and analysis were performed as described in (9)

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SUPPLEMENTARY MATERIAL

Supplementary material contains four figures and one table.

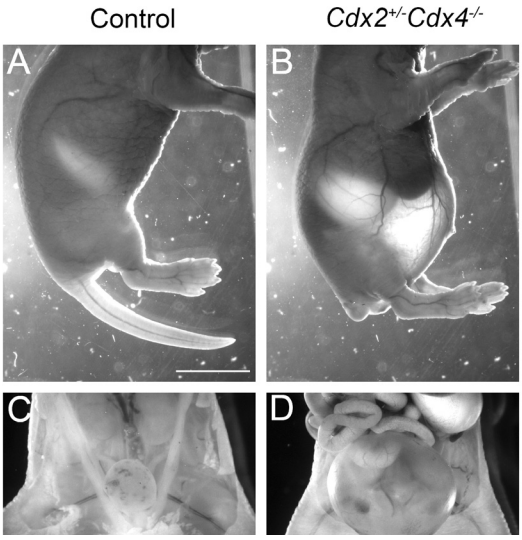

Figure S1 Dilated bladder in *Cdx2/4* mutants at birth. A-D Control (A,C) and *Cdx2/4* (B,D) new born mice shown laterally (A-B) and ventrally after skin removal (C,D), showing the normal bladder (A,C) and the dilated bladder of Cdx mutants (B,D).

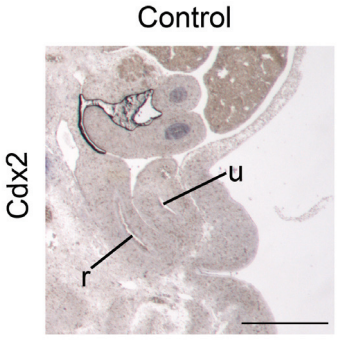

Figure S2 Expression of Cdx2 at E12.5. A-I In situ hybridization of transversal sections of the urorectal region of E12.5 Control (A,B,D,F,H) and *Cdx2/4* mutant (C,E,G,I) embryos with a *Cdx2* probe (A) and all four Hox13 genes (B-I). Scale bar: 0.5mm. u, urethra; r, rectum; cl, cloaca. Note that the expression in the cloacal endoderm is much weaker than in more anterior gut endoderm seen on the section.

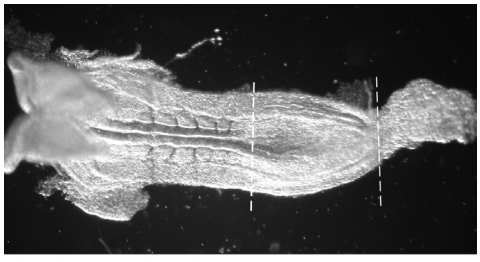

Figure S3 Dissected embryonic tissues used in the microarray experiments. Whole mount 5 somite embryo. Dashed lines indicate landmarks for dissection for tissue used in the micro-array (between the base of the allantois and one somite length posterior to the last somite).

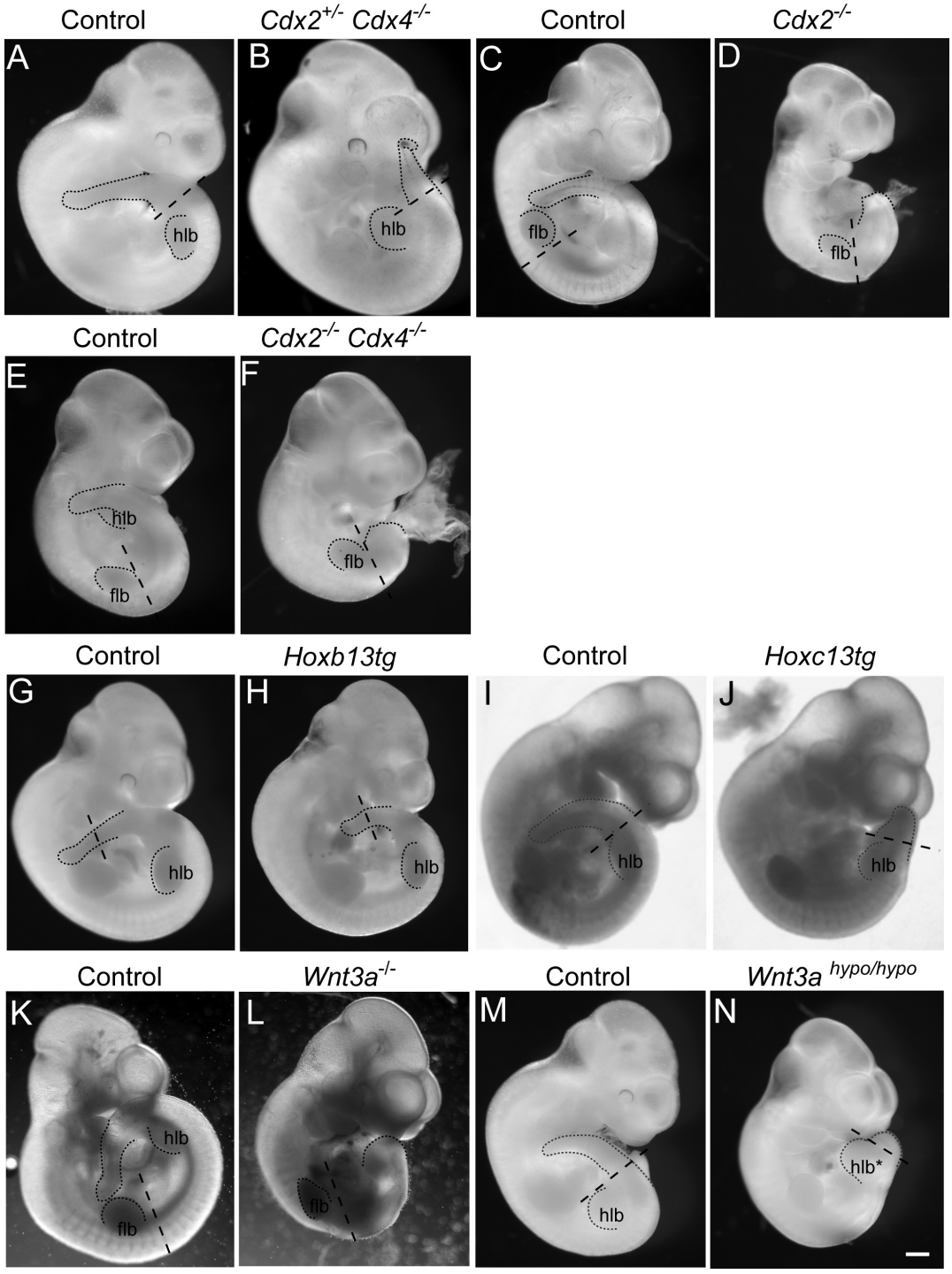

Figure S4 Photographs of E10.5 embryos of all genotypes analysed, with indication of the axial level where the neural defects of Figure 6 were found. For each genotype, an E10.5 mutant embryo and a matched control are shown, with a dashed line at the axial level that was analysed in Figure 6.

Table S1A Top 20 and potentially interesting genes upregulated in *Cdx2*^{null} relatively to control embryos at the 4/5 somite stage

Agilent ID	Gene Symbol	Gene title	Fold Change	
A_51_P218335	Tbx1	T-box 1	7,56	up
A_52_P428654	Zic1	zinc finger protein of the cerebellum 1	7,00	up
A_52_P171166	BC048679	cDNA sequence BC048679	6,69	up
A_52_P385594	Tal2	T-cell acute lymphocytic leukemia 2	6,19	up
A_52_P118560	Kynu	kynureninase (L-kynurenine hydrolase)	5,97	up
A_52_P437792	A730017C20Rik	RIKEN cDNA A730017C20 gene	5,69	up
A_51_P333438	TC1651696	Filamin-interacting protein L-FILIP, partial (22%)	5,66	up
A_51_P146970	Dmrt2	doublesex and mab-3 related transcription factor 2	4,85	up
A_51_P448167	Cer1	cerberus 1 homolog	4,51	up
A_52_P671132	Dner	delta/notch-like EGF-related receptor	3,86	up
A_51_P403705	2610100L16Rik	product:hypothetical protein, full insert sequence. [AK011787]	3,81	up
A_51_P227222	Adamts2	a disintegrin-like and metallopeptidase (reprolysin type)	3,72	up
A_52_P270429	2200001I15Rik	RIKEN cDNA 2200001I15 gene	3,65	up
A_51_P423709	Fam84a	family with sequence similarity 84, member A	3,62	up
A_52_P136782	Rgs5	regulator of G-protein signaling 5	3,61	up
A_52_P455428	Glra2	glycine receptor, alpha 2 subunit	3,29	up
A_51_P417720	Itga11	integrin alpha 11	3,24	up
A_52_P122649	Dmrta1	doublesex and mab-3 related transcription factor like family A1	3,21	up
A_52_P60194	C4bp	complement component 4 binding protein	3,19	up
A_52_P425667	Adcyap1	adenylate cyclase activating polypeptide 1	3,16	up
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A_51_P202440	Rarb	retinoic acid receptor, beta	2,18	up
A_52_P552665	Fzd7	frizzled homolog 7	2,10	up
A_52_P58145	Aldh1a2	aldehyde dehydrogenase family 1, subfamily A2	1,96	up
A_51_P361220	Fzd4	frizzled homolog 4	1,77	up
A_51_P511015	Fzd9	frizzled homolog 9	1,70	up
A_51_P124285	Nkd1	naked cuticle 1 homolog	1,50	up

Table S1C Top 20 and potentially interesting genes upregulated in *Cdx2*^{null} relatively to control embryos at the 7/8 somite stage

Agilent ID	Gene Symbol	Gene title	Fold Change	
A_51_P194230	Zic1	zinc finger protein of the cerebellum 1	12,82	up
A_51_P423709	Fam84a	family with sequence similarity 84, member A	8,61	up
A_52_P437792	A730017C20Rik	RIKEN cDNA A730017C20 gene	8,09	up
A_51_P218335	Tbx1	T-box 1	7,38	up
A_51_P397876	En2	engrailed 2	6,52	up
A_51_P227222	Adamts2	a disintegrin-like and metallopeptidase (reprolysin type)	5,63	up
A_51_P287198	Krt23	keratin 23	5,63	up
A_52_P224348	Kctd12b	potassium channel tetramerisation domain containing 12b	4,82	up
A_51_P455166	Prl4a1	prolactin family 4, subfamily a, member 1	4,57	up
A_52_P60194	C4bp	complement component 4 binding protein	4,07	up
A_52_P661412	Adora1	adenosine A1 receptor	3,86	up
A_52_P127925	Tcfec	transcription factor EC	3,83	up
A_51_P403705	2610100L16Rik	RIKEN full-length enriched library, clone:2610100L16	3,80	up
A_52_P270429	2200001I15Rik	RIKEN cDNA 2200001I15 gene	3,70	up
A_51_P276305	Stra8	stimulated by retinoic acid gene 8	3,55	up
A_51_P317141	Col2a1	collagen, type II, alpha 1	3,52	up
A_52_P39083	Ccbe1	collagen and calcium binding EGF domains 1	3,50	up
A_52_P739568	AK082480	RIKEN full-length enriched library, clone:C230053P15	3,47	up
A_52_P478025	Smpd3	sphingomyelin phosphodiesterase 3	3,40	up
A_52_P63892	Itga4	integrin alpha 4	3,32	up
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A_52_P58145	Aldh1a2	aldehyde dehydrogenase family 1, subfamily A2	2,30	up
A_52_P552665	Fzd7	frizzled homolog 7	1,94	up
A_51_P124285	Nkd1	naked cuticle 1 homolog	1,77	up
A_51_P202440	Rarb	retinoic acid receptor, beta	1,65	up
A_51_P511015	Fzd9	frizzled homolog 9	1,49	up
A_51_P361220	Fzd4	frizzled homolog 4	1,37	up

Table S1D Top 20 and potentially interesting genes downregulated in *Cdx2*^{null} relatively to control embryos at the 7/8 somite stage.

Agilent ID	Gene Symbol	Gene title	Fold Change	
A_51_P337308	Saa3	serum amyloid A 3	19,57	down
A_52_P973575	Hoxb9	homeo box B9	10,41	down
A_52_P591310	Hoxd13	homeo box D13	9,50	down
A_51_P370458	Krtap17-1	keratin associated protein 17-1	8,05	down
A_51_P487818	Fabp1	fatty acid binding protein 1	7,78	down
A_51_P172502	Cxcl12	chemokine (C-X-C motif) ligand 12	6,23	down
A_51_P103406	Vwf	Von Willebrand factor homolog	5,45	down
A_52_P665240	Krt83	keratin 83	5,12	down
A_51_P398525	Fn3k	fructosamine 3 kinase	4,85	down
A_51_P137322	Cck	cholecystokinin	4,79	down
A_52_P525317	Gja5	gap junction membrane channel protein alpha 5	4,79	down
A_51_P309530	Nepn	nephrocan	4,72	down
A_52_P556448	Krt86	keratin 86	4,59	down
A_51_P456208	Tff3	trefoil factor 3	4,50	down
A_51_P181297	Serpinb1a	serine (or cysteine) peptidase inhibitor, clade B, member 1a	4,49	down
A_52_P648524	Dio3	deiodinase, iodothyronine type III	4,31	down
A_51_P466285	Hoxa10	homeo box A10	3,97	down
A_51_P210510	Sparcl1	SPARC-like 1	3,97	down
A_51_P502437	Cacna2d3	calcium channel, voltage-dependent, alpha2/delta subunit 3	3,93	down
A_51_P113784	Prpf19	PRP19/PSO4 pre-mRNA processing factor 19 homolog	3,77	down
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A_51_P102911	Hoxa11	homeo box A11	3,03	down
A_51_P260265	Hoxd4	homeo box D4	2,69	down
A_52_P346987	Hoxc9	homeo box C9	2,49	down
A_51_P402686	Hoxb8	homeo box B8	2,47	down
A_51_P509263	Hoxa7	homeo box A7	2,46	down
A_51_P117924	Hoxa9	homeo box A9	2,13	down
A_52_P973575	Hoxb9	homeo box B9	1,92	down
A_51_P496245	Hoxc6	homeo box C6	1,81	down
A_52_P382149	Cyp26a1	cytochrome P450, family 26, subfamily a, polypeptide 1	1,70	down
A_52_P49014	Shh	sonic hedgehog	1,69	down
A_51_P286748	Frzb	frizzled-related protein	1,65	down
A_52_P686760	Gdf11	growth differentiation factor 11	1,65	down
A_51_P479444	Ihh	Indian hedgehog	1,57	down
A_52_P544043	Pcsk5	proprotein convertase subtilisin/kexin type 5	1,55	down
A_51_P372853	Rarg	retinoic acid receptor, gamma	1,48	down
A_51_P485542	Hoxd8	homeo box D8	1,43	down
A_52_P258116	Wnt3	wingless-related MMTV integration site 3	1,43	down
A_51_P383741	Bmp4	bone morphogenetic protein 4	1,41	down
A_51_P137991	Wnt5b	wingless-related MMTV integration site 5B	1,39	down

Table S1B Top 20 and potentially interesting genes downregulated in *Cdx2*^{null} relatively to control embryos at the 4/5 somite stage

Agilent ID	Gene Symbol	Gene title	Fold Change	
A_51_P337308	Saa3	serum amyloid A 3	18,72	down
A_51_P309530	Nepn	nephrocan	7,82	down
A_51_P103406	Vwf	Von Willebrand factor homolog	7,42	down
A_51_P370458	Krtap17-1	keratin associated protein 17-1	6,85	down
A_52_P685021	Cxcl12	chemokine (C-X-C motif) ligand 12	5,23	down
A_51_P509263	Hoxa7	homeo box A7	5,13	down
A_52_P556448	Krt86	keratin 86	5,13	down
A_52_P665240	Krt83	keratin 83	4,83	down
A_52_P201106	4930473A06Rik	RIKEN cDNA 4930473A06 gene	4,14	down
A_51_P181297	Serpinb1a	serine (or cysteine) peptidase inhibitor, clade B, member 1a	4,14	down
A_52_P346987	Hoxc9	homeo box C9	4,12	down
A_52_P880457	Ampd1	adenosine monophosphate deaminase 1	4,04	down
A_52_P973575	Hoxb9	homeo box B9	3,93	down
A_51_P496245	Hoxc6	homeo box C6	3,79	down
A_52_P591310	Hoxd13	homeo box D13	3,63	down
A_52_P441294	Chl1	cell adhesion molecule with homology to L1CAM	3,61	down
A_52_P590168	Tg	thyroglobulin	3,55	down
A_52_P350950	Dennd2c	DENN/MADD domain containing 2C	3,11	down
A_51_P466285	Hoxa10	homeo box A10	3,03	down
A_51_P117924	Hoxa9	homeo box A9	2,96	down
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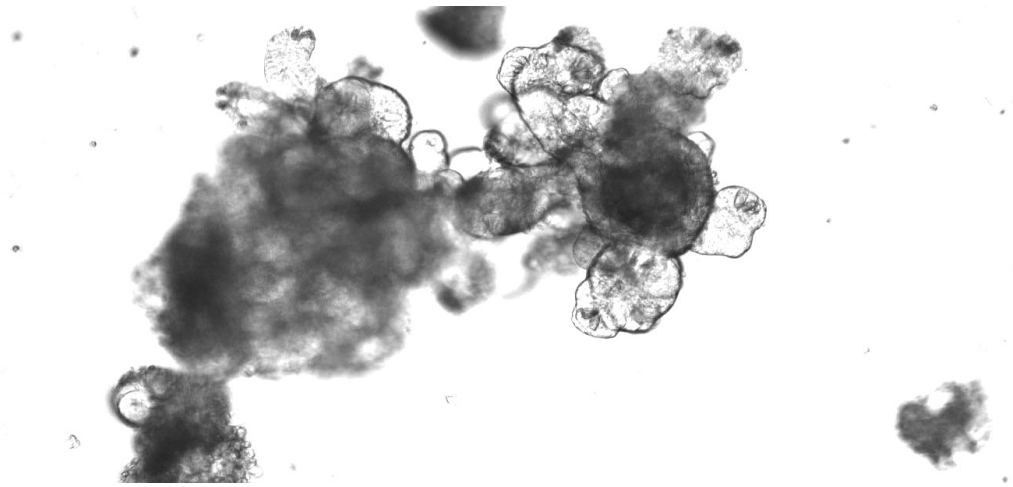
A_51_P402686	Hoxb8	homeo box B8	2,75	down
A_51_P260265	Hoxd4	homeo box D4	2,29	down
A_51_P102911	Hoxa11	homeo box A11	2,26	down
A_51_P479444	Ihh	indian hedgehog	2,16	down
A_52_P85805	Wnt5b	wingless-related MMTV integration site 5B	2,07	down
A_51_P286748	Frzb	frizzled-related protein	1,97	down
A_51_P485542	Hoxd8	homeo box D8	1,93	down
A_52_P49014	Shh	sonic hedgehog	1,87	down
A_52_P382149	Cyp26a1	cytochrome P450, family 26, subfamily a, polypeptide 1	1,81	down
A_51_P372853	Rarg	retinoic acid receptor, gamma	1,61	down
A_51_P383741	Bmp4	bone morphogenetic protein 4	1,59	down
A_52_P686760	Gdf11	growth differentiation factor 11	1,50	down
A_52_P544043	Pcsk5	proprotein convertase subtilisin/kexin type 5	1,48	down



Chapter 5

Cdx2 is crucial for maintenance of the small intestinal stem cells

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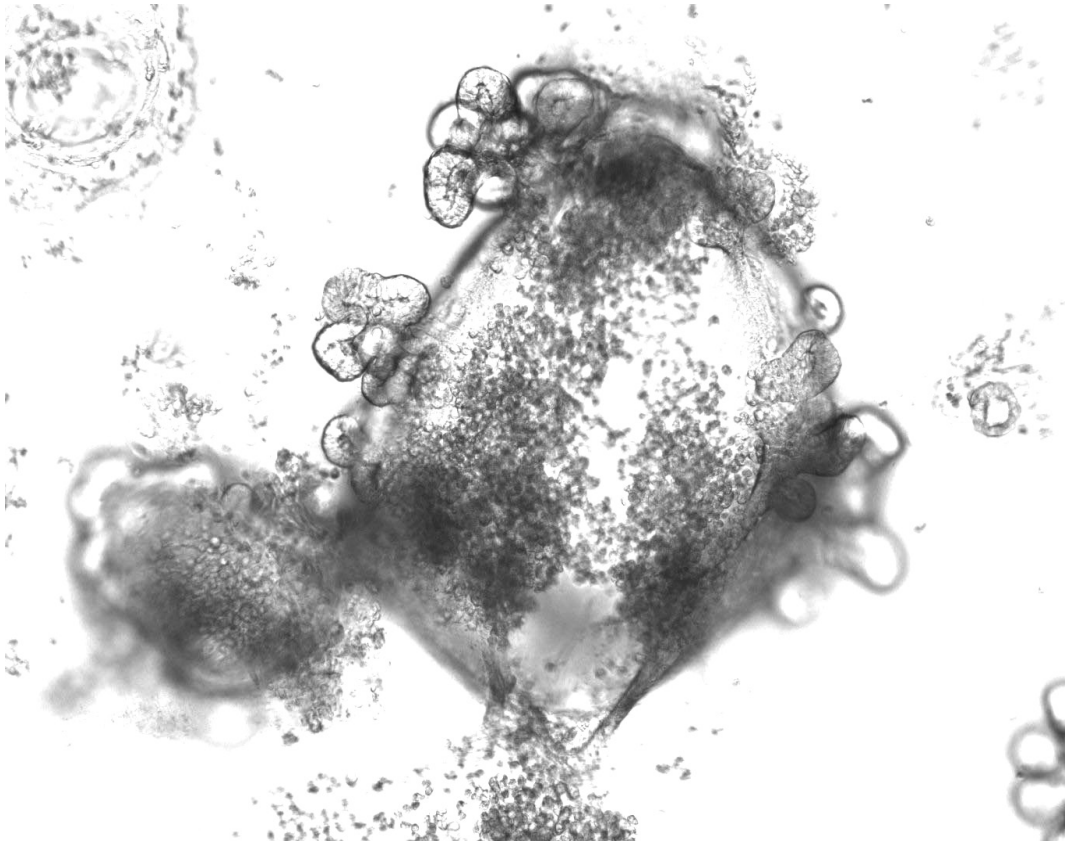

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ABSTRACT

Upon *Cdx2* inactivation in adult intestine by the stem cell specific *Lgr5CreER^{T2}*, *Cdx2* negative crypts produced subsurface cystic vesicles that express gastric markers and do not contribute to populate the crypt surface any more. We show that, in parallel with this in vivo situation, *Cdx2* inactivation in intestinal stem cells in culture abolishes their ability to form long term growing intestinal organoids when grown in intestine specific conditions in matrigel. These Lgr5^{+ve} *Cdx2^{null}* intestinal stem cells were rescued once cultured in conditions appropriate for pyloric tissues, and generated organoids that could be passaged over a long period of time. These organoids expressed gastric genes instead of intestinal markers, contained some *Lgr5* expressing cells and failed to express the gastric endoderm-specific transcription factor *Sox2*. *Cdx2* therefore partly transforms the intestinal into gastric epithelium in a cell autonomous fashion. Future work will tell whether the gene expression profile of these *Cdx2^{null}* Lgr5^{+ve} progenitors is intermediate between the normal intestinal and pyloric signature, and which genetic step is missing to transform the *Cdx2^{null}* intestinal into genuine pyloric cultures.

INTRODUCTION

Mouse Cdx genes are ortologues of *Drosophila Caudal* and have been shown to play important roles in embryonic axial patterning and in generation of the trunk and tail tissues (1-3). All three genes: *Cdx1*, *Cdx2* and *Cdx4* are expressed in the embryo proper in the primitive streak area at E7.2 and later on in the tailbud. *Cdx4* is downregulated around E10.5. However the expression of *Cdx1* and *Cdx2* is maintained in the hindgut endoderm and persists until adulthood. Apart from mild vertebral homeotic transformation, *Cdx1^{null}* animals have no apparent abnormal phenotype either in axial skeleton or gut endoderm. *Cdx2^{null}* embryos fail to implant due to the impairment of the trophectoderm development and thus die in the peri-implantation period (4). *Cdx2* heterozygous animals are viable and fertile and present anterior homeotic shift of cervical and thoracic vertebrae accompanied by rib abnormalities (4). In adult mice *Cdx2* expression is confined to the intestine and varies along the rostrocaudal axis with the strongest expression level observed around the cecum area (5). *Cdx2$^{+/-}$* animals present multiple adenomatous polyps predominantly found in the proximal colon and more rarely in the small intestine (4). Within these polyps metaplasia is evident with keratinized epithelium characteristic of oesophageal epithelium. Embryonic gut undergoes histodifferentiation into the stomach and intestine by 14 days of gestation. The endoderm of the stomach expresses *Sox2* and the glandular morphology of the organ is acquired as a result of a cross talk between the endoderm and underlying mesoderm, which expresses *Barx1* (6). *Cdx2$^{+/-}$* embryos at E14.5 and E16.5 present patchy *Sox2* expression in the embryonic small intestine and cecum with *Barx1* expression in the adjacent mesoderm (7). When expressed ectopically in the glandular gastric mucosa, *Cdx2* induces trans differentiation of gastric epithelium into the intestinal epithelium, including the expression of intestine associated genes (8).

Cdx2 overexpresion under the *Villin* promoter causes imbalance of differentiated cell types in the small intestine (9). *Villin-Cdx2* transgenic animals have low survival rate and their intestine is devoid of mature Paneth cells, or the Paneth cell number is severely reduced. As a consequence, there is an increase in the number of goblet cells, which share a common progenitor cell with Paneth cells, and cells with intermediate morphology between goblet and Paneth cells are present. This suggests that an appropriate level of *Cdx2* protein governs the development of different lineages in the small intestine. In addition, crypt development is accelerated and morphologically normal crypts appear two days after birth, instead of the two weeks necessary in wild type animals. This finding suggested that *Cdx2* level possibly modulates Wnt/ β catenin activity, as crypt development is highly dependent on canonical Wnt pathway. Inactivation of *Cdx2* in adult mice with *Villin-CreER* causes death within 3 weeks from inactivation due to loss of enterocytes (10).

In the work presented here we show that, in adults, homeotic transformation of intestinal epithelium into stomach epithelium is takes place at least partially after inactivation of *Cdx2* in the epithelium of the intestine (via *AhCre*) or specifically in the Lgr5+ve stem cell population (via *Lgr5CreER^{T2}*). Loss of *Cdx2* abolishes the self-re-

newing properties of stem cells in vivo and impairs their ability to feed new cells into the villi surface. Furthermore we show that the identity of the intestinal epithelium is partially changed into stomach-like. Cell differentiation is converted from intestinal into stomach derivative not expressing *Sox2*. Single *Cdx2*^{null} intestinal stem cells are not able to form intestinal organoids and cannot be maintained in in vitro culture. Interestingly, if *Cdx2*^{null} stem cells are cultured in medium designed for pyloric cultures they can be maintained and passaged for long period of time and adopt the stomach morphology. This suggests that *Cdx2* ablation partially hampers small intestinal epithelium and partially transforms it into stomach–like epithelium.

RESULTS

Cdx2 determines the identity of the adult intestinal epithelium

In collaborative studies with Felix Beck (Leicester) we found that conditional inactivation of *Cdx2* in the intestinal epithelium as well as specifically in the Lgr5^{+ve} intestinal stem cells of adult mice allows survival of the animals, but prevents the differentiation of stem cells of the *Cdx2*^{null} crypts into the various lineages present in the healthy intestine. After 1 week from *Cdx2* inactivation by *AhCre* (*P450-Cre*) or *Lgr5CreER*^{T2}, *Cdx2*^{null} patches were observed along the villi surface and in crypts. These areas expressed several stomach specific markers without change of epithelial morphology. Pepsinogen C, normally expressed in the stomach, was detected along the villi surface within the *Cdx2*^{null} areas. Alkaline phosphatase expression from the brush border was lost and Claudin 18, which normally is specifically expressed in the stomach epithelium, was expressed instead. Four weeks post *Cdx2* inactivation, the intestine of animals with *Cdx2* inactivated by *AhCre*, exhibited misshaped villi morphology with over-representation of mucous secreting cells and abnormally looking Paneth cells. In the *Lgr5CreER*^{T2} inactivated *Cdx2* intestine, *Cdx2*^{null} patches remained deep in the mucosa and did not contribute cells to the villi surface indicating their inability of repopulating the villi. Intestinal function was maintained by compensatory growth of unaffected crypts, which in both type of experiments rapidly restored the homeostasis of the intestine so that after 8 weeks phenotypically wild type epithelium was present in the whole intestine. However, the *Cdx2*^{null}crypts were not lost from the intestinal epithelium but persisted as cysts within the mucosa. The *Cdx2*^{null} cysts were devoid of Paneth cells and of any proliferating cells, as demonstrated by absence Ki67. Some cells lining the cyst expressed Pepsinogen C and Claudin18, characteristic for the stomach epithelium and did not express any marker of differentiated small intestine. The *Cdx2* deprived adult intestinal lining was unique in that it lead to gastric gene expression without altering the intestinal architecture, presumably because of the absence of *Barx1* expression in the endoderm. This work has been accepted for publication in Development.

Inactivation of *Cdx2* in intestinal crypts cultured in vitro abolishes their ability to form long term growing intestinal organoids.

We inactivated *Cdx2* in intestinal crypts cultured in matrigel in vitro. Intestinal crypts were isolated from mice carrying one *Cdx2*^{null} and one *Cdx2flox* allele and *Lgr5CreER*^{T2} (11) and dissociated into very small clumps of cells. These clumps were seeded in 25 μl drops of Martigel in 24-well pate (12). In fully-grown organoids (Figure 1B), the Cre recombinase was induced with 4-hydroxy tamoxifen by adding to the culture for overnight. The induced and non-induced organoids were then washed, mechanically dissociated, and the cells were plated in matrigel in culture conditions used for growing intestinal crypts in vitro (12). While the non induced cultures gave rise to the rapidly growing and budding intestinal organoids previously described (12), the tamoxifen induced cultures consisted of empty cysts (30%) among normal looking intestinal organoids (Figure 1D, E). Empty, thin-walled cysts devoid of Paneth cells appeared from day 3 of the culture and persisted through the 7-day culture period (Figure 1G-N). These cysts did not survive passage and cultures consisted of 100% normal intestinal organoids after passaging. These observations were confirmed in 3 independent experiments. From each experiment, cystic and normal-looking organoids from tamoxifen induced cultures were picked manually at day 5 after passage and genotyped individually, confirming that the empty cysts were *Cdx2* negative whereas the phenotypically normal intestinal organoids still contained the wild type *Cdx2* allele (Figure 1G, F). We embedded a collection of cysts and normal organoids in paraffin and submitted them after sectioning to immunostaining with anti-lysozyme,

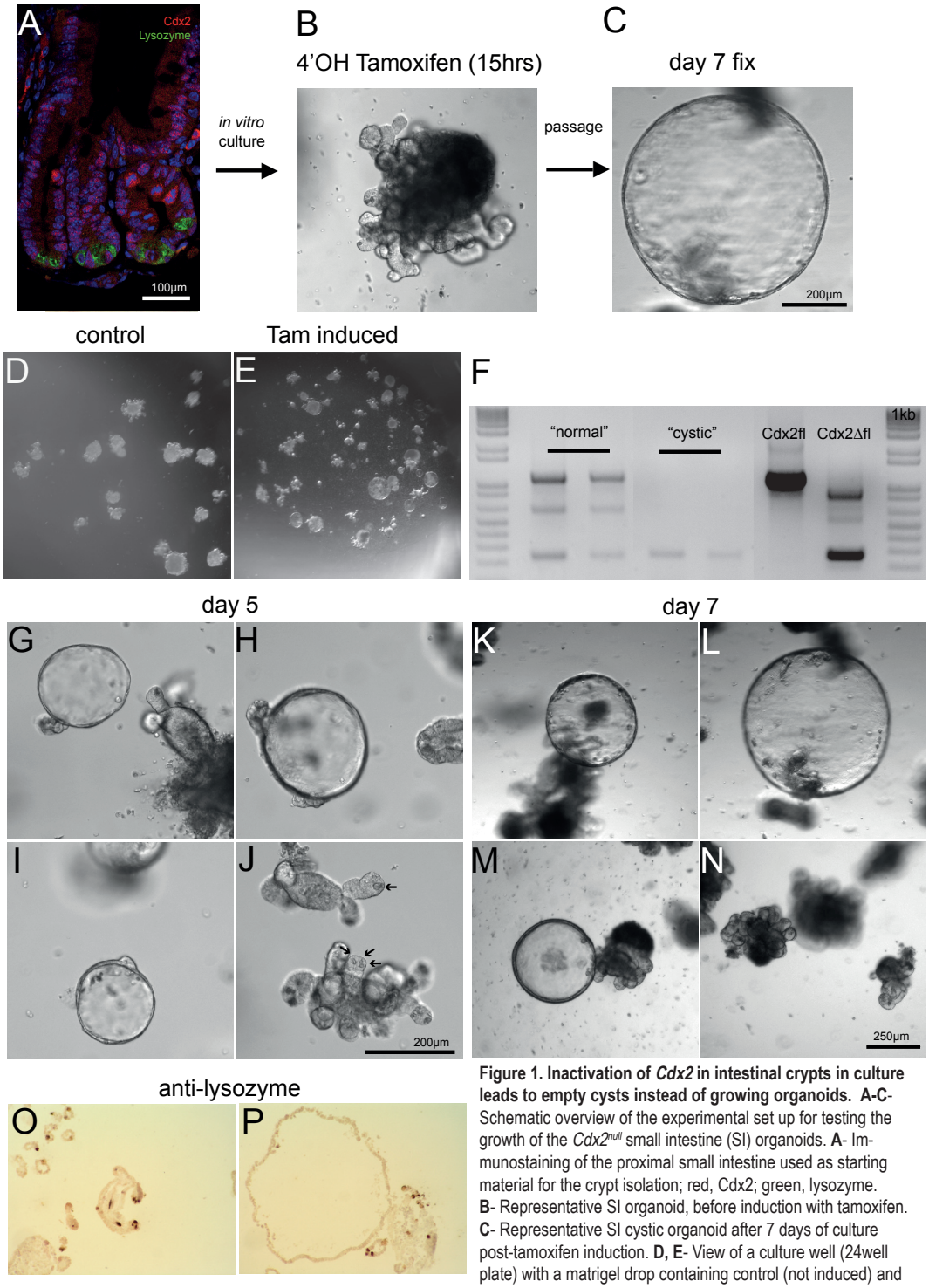

Figure 1. Inactivation of *Cdx2* in intestinal crypts in culture leads to empty cysts instead of growing organoids. A-C- Schematic overview of the experimental set up for testing the growth of the *Cdx2*^{null} small intestine (SI) organoids. **A**- Immunostaining of the proximal small intestine used as starting material for the crypt isolation; red, Cdx2; green, lysozyme. **B**- Representative SI organoid, before induction with tamoxifen. **C**- Representative SI cystic organoid after 7 days of culture post-tamoxifen induction. **D, E**- View of a culture well (24well plate) with a matrigel drop containing control (not induced) and tamoxifen induced intestinal organoids, respectively. **F**- Genotyping of intestinal "normal-looking" and "cystic" organoids after they were manually picked from the tamoxifen-induced culture. For each lane of "normal" and "cystic", 5 organoids were pooled; the

control *Cdx2*^{fl} is from a *Cdx2*$^{flox/flox}$ DNA sample; the control *Cdx2*$^{\Delta fl}$ is from a *Cdx2*$^{+/null}$ DNA sample; a 1kb plus DNA ladder is shown on each side. The lowest band in the normal and cystic organoids corresponds to the KO allele, and the highest band, only present in the normal looking organoids, corresponds to the non-deleted floxed allele; the highest band in the *Cdx2*$^{\Delta fl}$ control lane corresponds to the wild type *Cdx2* allele. **G-J**-Representative SI organoids after 5 days of culture post tamoxifen induction; **G- I,**"cystic" *Cdx2*^{null} organoids, **J**, phenotypically normal intestinal organoid with visible Paneth cells indicated by arrows. **K-N**- Representative SI organoids after 7 days of culture post-tamoxifen induction. **K-M** "cystic" *Cdx2*^{null} organoids. **N**- Normal looking, intestinal organoid. Note that even after 7 days of culture "cystic" organoids do not bud and remain empty. **O, P**- Sections of organoids and cysts from 7 day cultures post tamoxifen induction, stained for the Paneth cell marker-lysozyme (brown staining).

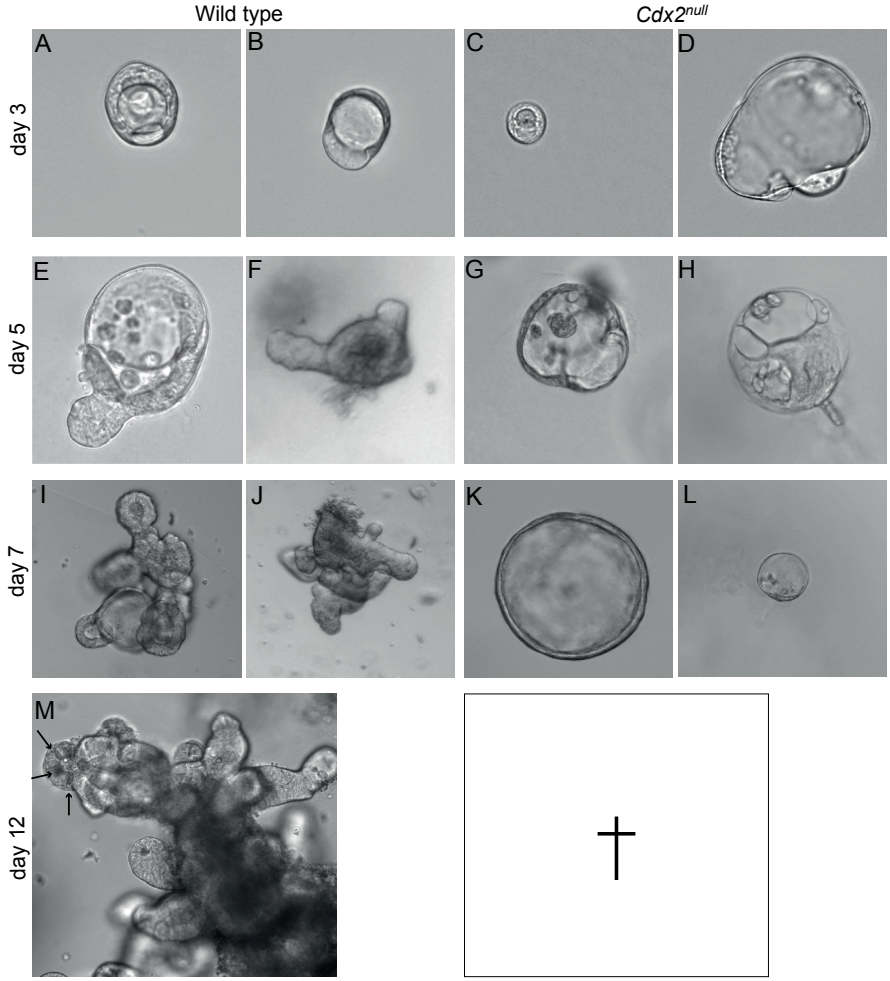

Figure 2. Single Lgr5^{+ve} intestinal stem cells fail to give rise to small intestinal organoids. - Time course of organoid formation form single, FACS sorted wild type (**A-B, E-F, I-J, M**) and *Cdx2*^{null} (**C-D, G-H, K-L**) Lgr5^{+ve} intestinal stem cells shown from day 3 to day 12. Note that at day 5 of culture budding structures are visible in wild type cultures (**E-F**) but not in the *Cdx2*^{null} cultures (**G-H**). At day 7 of culture wild type cultures formed typical small intestinal organoids with multiple budding structures (*I-J*). *Cdx2*^{null} cultures formed thin walled cysts (**K**) or failed to grow (**L**). After 12 days of culture wild type organoids contained multiple buds with clearly visible Paneth cells (arrows) (**M**) while *Cdx2*^{null} cultures were devoid of living materials.

a marker of Paneth cells. The *Cdx2*^{null} cysts did not express lysozyme, which confirms morphological observation (Figure 1J), whereas the control organoids did (Figure 1O, P). Cystic organoids were thus devoid of Paneth cells. They and could not be passaged, unlike the long term growing intestinal organoids produced from control crypts. These in vitro observations therefore support the conclusion from the in vivo experiments that *Cdx2* negative stem cells are unable to self renew and generate mature intestinal endoderm and Paneth cells.

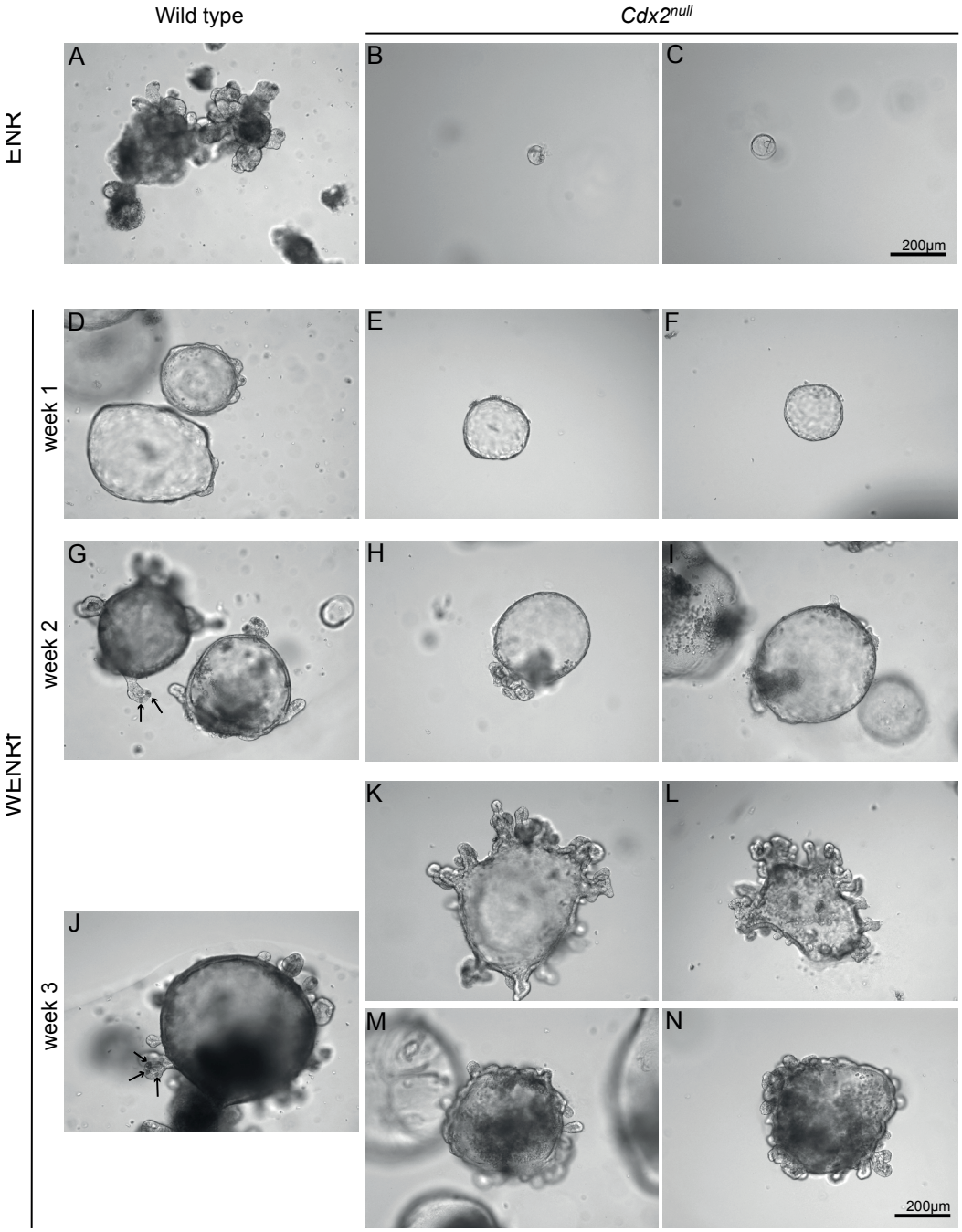

Figure 3. *Cdx2*^{null} small intestinal organoids from *Cdx2*^{null} intestinal stem cells can be cultured in medium designed to grow pyloric organoids. **A-C-** Comparison of organoids obtained from single FACS sorted wild type Lgr5^{+ve} intestinal stem cell (**A**) and from *Cdx2*^{null} intestinal stem cell (**B-C**) cultured in standard ENR conditions for intestinal organoids. **D-N**- Growth of wild type (**D, G, J**) and *Cdx2*^{null} (**E-F, H-I, K-N**) small intestinal organoids in medium designed for gastric organoids during 3 week of culture. Organoids of both genotypes at the beginning of the culture are thin walled cysts and within 2 weeks of culture they begin to form budding structures (**G-I**). At this time point of the culture, Paneth cells are present in the wild type organoid buds (arrows) (**G, J**). *Cdx2*^{null} organoids form budding structures without Paneth cells (**K-N**).

Single Lgr5GFP^{+ve} *Cdx2^{null}* stem cells fail to give rise to growing organoids in standard small intestinal culture conditions

We induced fully-grown organoids isolated from intestinal crypts with tamoxifen as described above, dissociated the cells, FACS-sorted Lgr5GFP^{+ve} stem cells and plated them in matrigel-based culture (around 100 cells in 25 μl drop of matrigel). *Cdx2^{null}* intestinal stem cells failed to grow in intestinal culture conditions (ENR). In a wild type stem cell culture after 5 days small organoids with buds were visible. In contrast, *Cdx2^{null}* stem cells did not give rise to budding organoids and only 25% of stem cells formed small cysts, without evidence of budding structures. Many of these cysts were empty and dying (Figure 2 G, H). By day 7 of the wild type culture organoids formed, cyst-like structures with lumen filled with cell debris and several buds as well as clearly visible Paneth cells in the tip of the buds (Figure 2M). Most of the *Cdx2^{null}* cultures did not contain any cyst, suggesting that ablation of *Cdx2* is incompatible with stem cell survival. Very few *Cdx2^{null}* cysts remained, were lost upon further passage.

Cdx2^{null} stem cells grow into organoids and can be maintained if cultured in medium optimal for gastric organoids

Supplying Wnt3a conditioned medium to the ENR medium resulted in a slight improvement of the survival rate of the FACS sorted *Cdx2^{null}* Lgr5^{+ve} stem cells. *Cdx2^{null}* cultures in ENR medium with Wnt3a formed empty, thin walled cysts, larger then those grown in ENR medium alone and could be cultured for two weeks (not shown). Each passage however caused a loss of the living material. Paneth cells were absent from these cultures at all time.

Survival of *Cdx2^{null}* stem cells was possible if FACS-sorted Lgr5GFP^{+ve} cells were cultured in medium designed for culturing pyloric glands in the matrigel based system (11). Sorted cells were placed in ENR medium containing Wnt3a conditioned medium and fgf10 (WENRf) at the same density as previously described. During the first week of culture, *Cdx2^{null}* stem cells formed large round organoids, which could be easily passaged without loss of material or morphology. After 2 weeks (approximately 3 passages) *Cdx2^{null}* organoids had changed morphology. Their walls thickened from flat thin cells to squamous epithelium and small budding structures became evident (Figure 3 H, I). These organoids could be passaged without further change in morphology for at least 20 weeks, after which the cultures were terminated.

Cdx2^{null} organoids cultured in WENRf medium resembled organoids made from pyloric glands (11), which normally do not express *Cdx2*. To directly compare these organoids we isolated pyloric glands and cultured them in conditions described earlier (11). The wild type pyloric organoids were similar to those described by (11) and they could be passaged over long periods of time (Figure 4). When we compared *Cdx2^{null}* intestinal organoids to pyloric organoids at different time points of their growth after passage, we found that their morphology was very similar (Figure E, F vs. G, H; also Figure 4D vs. Figure3 K, L).

Cdx2^{null} organoids express some of the gastric markers

The similarities in morphology between *Cdx2^{null}* intestinal organoids grown in stomach conditions and wild type pyloric organoids lead us to speculate that *Cdx2^{null}* intestinal organoids could revert to a stomach phenotype. We collected material from wild type pyloric glands and pyloric organoids, wild type and *Cdx2^{null}* small intestinal organoids and performed RT-PCR to compare the gene expression profiles of *Cdx2^{null}* organoids and wild type pyloric organoids. Wild type stomach endoderm expresses *Sox2* but not *Cdx2*; conversely small intestine expresses *Cdx2* but not *Sox2*. Thus we used these master transcription factors as markers to determine the identity of the organoids (Figure 5). We also compared the expression of several stomach specific (*gastric intrinsic factor somatostain, pepsinogen and gastrin*) and intestine specific markers (*Muc2*) by RT-PCR. Wild type pyloric glands and wild type pyloric organoids expressed genes characteristic for stomach identity and function: s*omatostatin, gastrin, gastric intrinsic factor, pepsinogen* and *Sox2* (Figure 6). Wild type intestinal organoids did not express any of these genes but expressed *Muc2* and *Cdx2* associated with intestinal identity. All samples expressed *Lgr5*, confirming the presence of stem cells in these organoids. *Cdx2^{null}* organoids had lost the expression of *Muc2*. We detected the expression of pepsinogen and gastric intrinsic factor but not somatostatin or gastrin. We never detected the presence of *Sox2* either by immunofluorescent staining (Figure 5) or RT-PCR (Figure 6). This suggests that *Cdx2^{null}* intestinal organoids grown in gastric conditions are at an intermediate state between stomach and

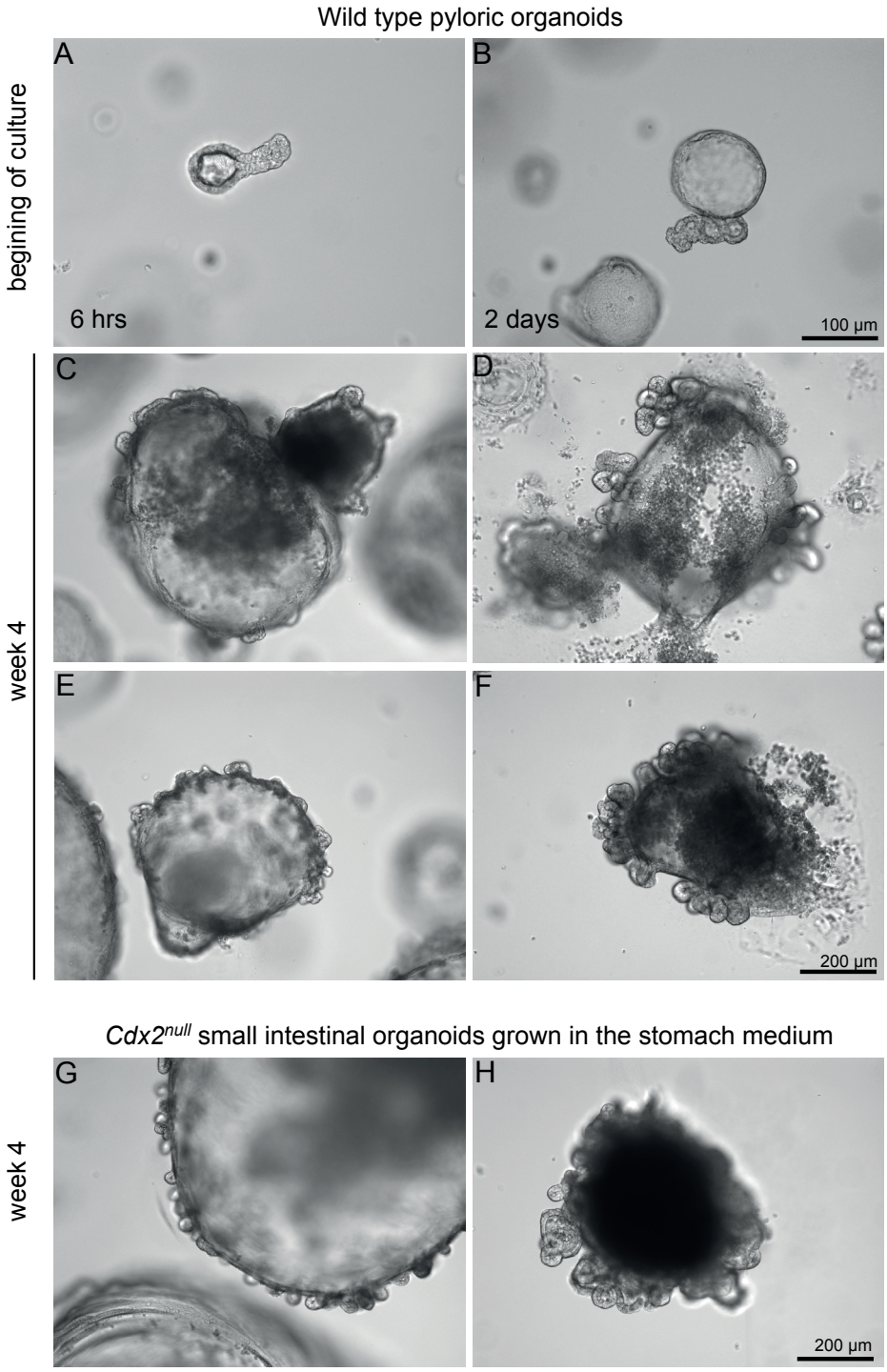

Figure 4. *Cdx2*^{null} small intestinal organoids grown in gastric conditions morphologically resemble wild type pyloric organoids. **A-F**- Overview of a culture of pyloric organoids. **A**- Pyloric glands 6hrs after isolation in a matrigel based culture. **B**- Two-days old pyloric organoids. **C-F-** Representative pyloric organoids after 4 weeks in culture. **G-H-** *Cdx2*^{null} small intestinal organoids grown in gastric conditions after 4 weeks of culture, note the morphological resemblance to gastric organoids (**E-F**).

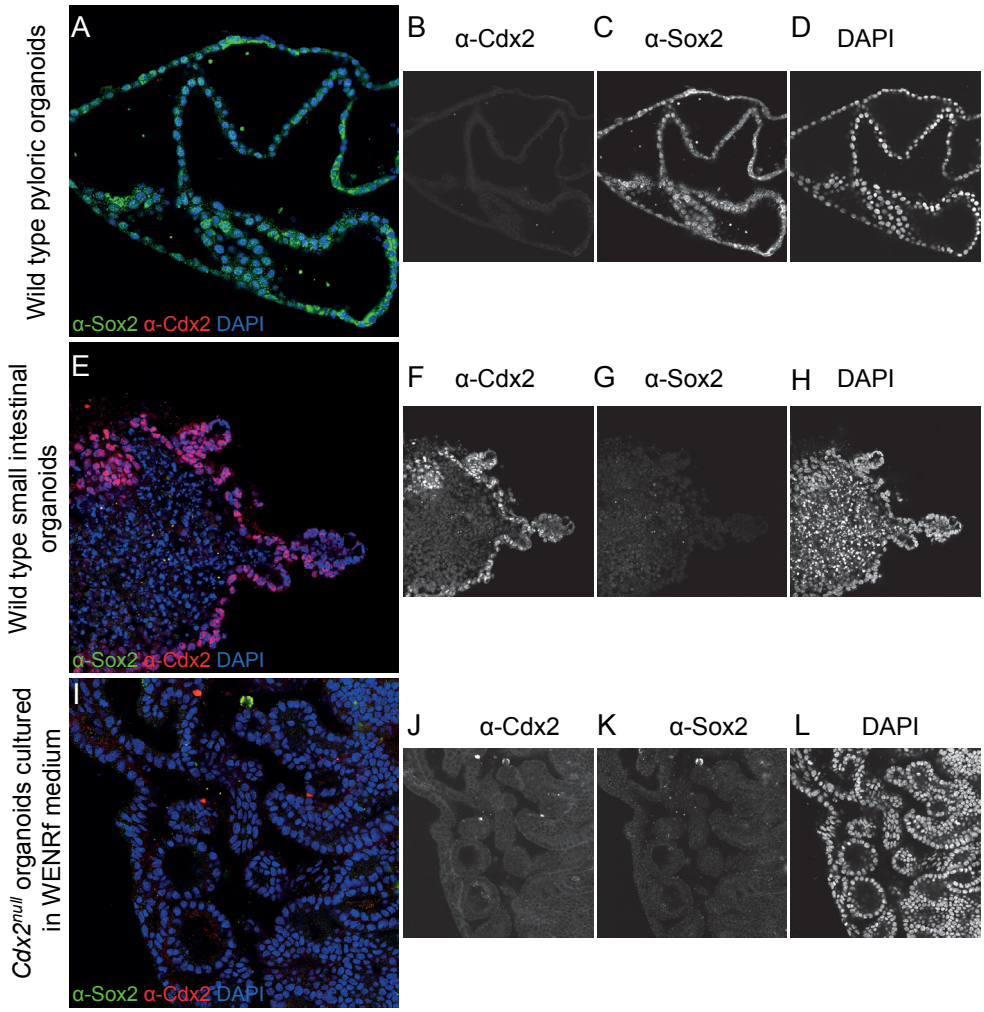

Figure 5. *Cdx2*^{null} small intestinal organoids grown in stomach conditions do not express the stomach marker Sox2. Comparison of Cdx2 and Sox2 expression in wild type pyloric organoids (A-D), wild type small intestinal organoids (SI),(E-H) and Cdx2null small intestinal organoids grown in gastric conditions (I-L). Note that *Cdx2*^{null} organoids do not express Sox2 at the protein level suggesting that the transformation into the gastric phenotype is not complete.

intestinal identity.

DISCUSSION

Loss of *Cdx2* in intestinal stem cells abolishes their self-renewing capacity

Adult *Cdx2*^{null} intestinal stem cells have lost their capacity to self renew, and no organoid can be maintained and grown in culture conditions appropriate for wild type intestinal stem cells. Loss of *Cdx2* in the stem cells of adult intestine also prevents the generation of Paneth cells and of all other intestinal differentiated derivatives, and leads to the induction of stomach markers both in vivo and in culture. However, *Cdx2*^{null} intestinal stem cells do survive and self renew in growth conditions optimal for gastric stem cells in matrigel cultures. They then give rise to organoids containing derivatives expressing stomach markers but do not express the stomach endodermal marker *Sox2*. Inactivation of *Cdx2* in adult intestinal stem cells therefore does not completely transform the epithelium into stomach epithelium.

Additional cues are required together with the absence of *Cdx2* to transform intestina into stomach epithelium.

The establishment of intestinal versus stomach identity normally takes place during embryonic life. The expression of master controlling transcription factors in the endoderm epithelium belongs to the key events for this establishment. While *Sox2* is expressed in the stomach endoderm, *Cdx2* becomes transcribed in the more posterior digestive tract and governs the establishment of intestinal identity. *Cdx2* is able to transform gastric into intestinal epithelium when ectopically expressed in the stomach (8). However, another crucial event for the specification of anterior versus posterior organogenesis in the digestive tract is the mesenchymal gene expression pattern. *Barx1* expression in the mesenchyme is a prerequisite for correct stomach morphogenesis. Full transformation of intestine into stomach occurs following rare events of loss of heterozygocity in mice carrying one $Cdx2^{null}$ allele, leading to *Cdx2* loss from early embryonic stages on, and accompanied by *Barx1* expression in the mesenchyme (7). This *Barx1* requirement in the mesoderm is not met when *Cdx2* is knocked out in adult intestinal stem cells in vivo (our work by Stringer et al., in revision for development) or in vitro (this work). The function of *Barx1* may be one of the missing elements underlying the difference between the morphology and gene expression features of the normal stomach, and that of the *Sox2* negative stomach-like epithelium in the $Cdx2^{null}$ areas after inactivation *Cdx2* using $Lgr5CreER^{T2}$ in vivo and in vitro. Finding culture conditions able to mimic the action of Barx1 on the Sox2 expressing status of the endoderm may reveal the identity of intermediate molecules involved in the specification of genuine stomach epithelium. In parallel to this approach, transcriptome analysis of the $Lgr5^{+ve}$ cells from normal intestinal and pyloric organoids, and of organoids resulting from culturing $Cdx2^{null}$ intestinal stem cells cultured in stomach conditions should pave the way to obtaining the signature of the different stem cells present in organoids and in vivo.

MATERIALS AND METHODS

Animals

All animals used to generate organoids were in the C57Bl6j/CBA mixed background and were housed according to the "law on animals in experiments", under the licenses required in the Netherlands. Generation of the *Cdx2* conditional allele was described in (14). To increase the efficiency of targeted knock out of Cdx2 in intestinal stem cells we crossed $Lgr5GFPkiCreER^{T2}$ mice (13) with mice carrying one $Cdx2^{null}$ ($Cdx2^{+/-}$) allele and one conditional allele ($Cdx2^{flox}$) to generate $Cdx2^{-/flox}Lgr5GFPkiCreER^{T2}$ mice used in all experiments.

In vitro cultures

The organs of $Cdx2^{-/flox}Lgr5GFPkiCreER^{T2}$ mice were used to generate stomach organoids and small intestinal organoids in matrigel based culture (12). Isolation of SI crypts from proximal small intestine, culture conditions for organoids (ENR medium), and FACS sorting of $Lgr5^{+ve}$GFP stem cells have been described previously by (12). Stomach organoids were made from pyloric glands of the stomach and cultured in medium (WENRf) described previously (11). Whole mount organoids were induced with 100nM 4'OH tamoxifen for 15 hours. FACS-sorted $Lgr5^{+ve}$ cells were plated at the density of 100 cells per well in 25 μl of matrigel in 24 well plates. Medium was changed every other day to replenish exhausted growth factors. In the experiment where whole mount organoids were induced, passaged and cultured in ENR, medium 5 cystic and 5 normal-looking organoids were picked up manually at day 5 of culture, pooled and genotyped to confirm that they were $Cdx2^{null}$. Continuous cultures of Cdx2null organoids in WENRf medium were genotyped by randomly collecting 25 organoids from one well, and genotyping them individually. This procedure was performed at least 3 times during the first 8 weeks of culture to exclude the possibility of amplifying organoids that had escaped tamoxifen induction.

Immunostaining

Organoids were freed of matrigel by 30 min incubation with matrigel breaking reagent (BD Sciences) on ice, fixed in 4%paraformaldehyde for 30 min at room temperature and washed in PBS0. All samples were permeabilzed with 0.1% Triton X-100/Tris buffered saline (TBS) for 5min, at room temperature. Blocking was done with 0.5% BSA for 1hr, at room temperature followed by overnight staining with primary antibodies at 4°C, washed with TBS and incubated with appropriate secondary antibodies at 4°C, overnight. The counterstaining was done with DAPI (Invitrogen). In some cases tissues or organoids were paraffin embedded and antibody staining was performed on 7 μm sections in standard conditions. The antibodies used were: anti-Lysozyme, DAKO, 1:1500; anti-Cdx2, Biogenex, 1:200; anti-Sox2, Chemicon, 1:200.

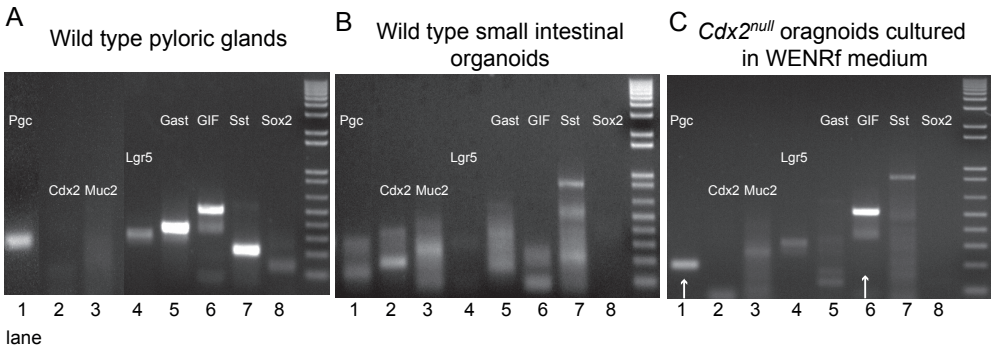

Figure 6. *Cdx2*^{null} intestinal organoids grown in gastric conditions express some of the gastric markers. Comparison of expression of gastric and intestinal specific markers in wild type pyloric glands (**A**), wild type small intestinal organoids (SI, **B**) and *Cdx2*^{null} organoids grown in gastric conditions (**C**). Note that *Cdx2*^{null} organoids lost most of the expression of *mucin2* (*Muc2*, **C**, lane 3), normally expressed in wild type intestinal organoids (**B**, lane 3). Expression of the gastric markers *pepsinogen* and *gastric intrinsic factor* (*Pgc*, **C**, lane 1 and *Gif*, lane 6, indicated by the arrows) was observed in SI organoids grown in stomach conditions. We did not detect the expression of *Gastrin, Somatostain* or *Sox2*, (**C**, lanes 5, 7 and 8 respectivley). All three samples expressed *Lgr5* indicating the presence of stem cells (lane 4 in **A, B** and **C**).

RT-PCR

Organoids from one well were pooled together and RNA was isolated with the use of the RNeasy Kit (Quiagen).10 μ g of total RNA was subjected to a reverse transcriptase reaction. PCR was performed on a cDNA template in standard conditions, with the use of the following primer sets:

Somatostatin-mSstfw:GAGGCAAGGAAGATGCTGTC, mSstrv:GGGCATCATTCTCTGTCTGG;

Gastrin-mGastfw:GCCAACTATTCCCCAGCTCT, mGastrv:GGCTCTGGAAGAGTGTTGCT;

Gastric intrinsic factor-mhGiffw:TGAATCCTCGGCCTTCTATG, mhGigrv:CAGTTAAAGTTGGTGGCACTTC;

Pepsinogen-mPgcfw:CCAACCTGTGGGTGTCTTCT, mPgcrv:TTAGGGACCTGGATGCTTTG;

Mucin2-mhMuc2fw:GAACGGGGCCATGGTCAGCA, mhMuc2rv:CATAATTGGTCTTGCATGCC;

Cdx2-mhCdx2fw:CTTGCTGCAGACGCTCAAC, mhCdx2rv:TCTGTGTACACCACCCGGTA;

Lgr5-mhLgr5fw:GGAAATGCTTTGACACACATTC, mhLgr5rv:TTATAATAACCTTGATGAATTCC;

Sox2-mSox2fw:CCTTTTCTCTTCTCGCCGAA, mSox2rv:TAG5CACTTGTTGCCAGAACG.

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Discussion

Chapter 6

General discussion

In the embryo of a fruit fly, *Drosophila melanogaster*, the body is formed by compartmentalization of the syncytial blastoderm. Graded expression of maternally deposited *Caudal (Cad)* mRNA is responsible for establishing the posterior part of the body(1). The loss of *Cad* results in the transformation of annalia, the most posterior segment into anterior structure (2). In the short and intermediate germ band insect embryos like Artemia and Tribolium only the anterior structures are formed from the syncytial blastoderm and the posterior is sequentially generated from the posterior growth zone under the control of *Cad*. Knock-down of *Cad* causes loss of all structures posterior to the head (3).

Mouse orthologues of *Caudal*, Cdx genes are also crucial for the formation of the posterior body (4-7). This property of Cdx genes is evolutionary conserved in all bilaterians (8).

In **chapter 2** we showed that Cdx genes do not impair the activity of axial progenitors in a cell autonomous way (9); however they affect the niche for posterior progenitors contributing descendants to the trunk and tail tissues. The *Cdx2*$^{+/-}$*Cdx4*^{null} mutant node-streak border area, harboring the stem cell-like population of axial progenitors, has the potential to adapt to the new microenvironment after being grafted into a wild type recipient embryo and contribute descendants to two germ layers, mesoderm and neurectoderm. It is a question whether there is a transcriptional signature for axial stem cells. So far there is no endogenous genetic marker that could be used to trace descendants of these cells. Interestingly it has been shown previously (10) that posterior progenitors from slightly more posterior sites of the primitive streak, migrate along the streak and find themselves in the NSB area, changing their fate accordingly. It is likely therefore that axial stem cells do not have an endogenous genetic marker distinguishing them from the other, short-term progenitors in the primitive streak area. It is presumably the unique niche generated by a combination of transcription factors and signaling pathways, that make the NSB/CNH, favorable for the cells to remain in the undifferentiated state, self renew and contribute descendants to the axial tissues over a long period of time. This is supported by the results of serial transplantation of cells from the CNH area from older to younger embryos (11). Removing components of the niche most probably causes an imbalance, which also affects the choice between differentiation programs of the progenitors descendants. This could be the mechanism underlying the formation of ectopic neural structures in the posterior of the embryo. Such ectopic neural structures are observed in *Cdx2*$^{+/-}$*Cdx4*^{null} and other Cdx mutants (12). Ectopic neural tissue is also observed in *Wnt3a*^{null} and *Wnt3a*^{hypomorph} mutants as well as in embryos with precocious expression of *Hoxb13* (12). This phenomenon is described in the chapter 3. T-box mutants, like *TBrachyury*^{null} and *Tbx6*^{null} mutant also exhibit ectopic neural structures (13, 14). Thus imbalance in posterior signaling causes descendants of posterior progenitors to adopt the neural state rather than to become mesoderm. Cdx genes and their downstream program including Wnt signaling govern the differentiation choice of cells generated in the posterior growth zone.

Cdx genes do not only affect the long-term axial progenitors. In **chapter 4** (12) we described defects of Cdx mutants in the uro-rectal system in tissues, which arise from the descendants of the short-term progenitors of the lateral plate mesoderm. These defects are reminiscent of a congenital disease in human: the Caudal Regression Syndrome. Malformation of lumbar vertebrae, sacral agenesis, and defects in the neural tube and in cloacal derivatives with a varying severity are characteristic for this syndrome. Cdx genes normally are not expressed in the tissues that are directly affected in the Caudal Regression Syndrome, and the phenotype in Cdx mutants was understood to arise from the impaired posterior growth zone at the time before it delivers descendants in the cloacal derivatives.

The germ cell progenitors are another population affected in Cdx mutants. *Cdx2* is not expressed in the PGCs themselves but the primordial germ cell population is transiently located within the Cdx expression domain. The location of the early PGC population in the mouse embryo is unique and it is not evolutionary conserved among the animal kingdom. In many vertebrates, like fish and chicken, formation of the germ cells is pre-determined by germ granules in different places in the embryo. In the mouse, the PGCs are specified from the pluripotent epiblast and subsequently migrate to the extraembryonic mesoderm and later on to the hindgut, before colonization of the genital ridges. as described in **chapter 3**, in *Cdx2*^{null} embryos PGC population is affected from early stage, when *Cdx2* is normally up-regulated in the surrounding tissue. The dependence of PGCs on the Cdx genes is thus imposed by their location at the time of specification. During migratory stage before reaching the genital ridges, PGCs are confined within the hindgut, which in the wild type embryos expresses *Cdx2*. Since

Cdx2^{null} embryos are severely truncated and do not generate their posterior endoderm correctly it is not possible to define the PGC dependence on *Cdx2* at migratory stage. It seems unlikely that *Cdx2* has a direct effect on the PGCs themselves. Cdx genes presumably sustain the appropriate signaling for the PGC population to grow. *Cdx2* could however also contribute to the establishment of the "travelling niche" for PGCs. this could be verified in a co-culture system. The established PGC population can be isolated from the *Cdx2*^{null} embryos carrying the *Stella-GFP* or *Blimp1-Venus* transgene, and put into co-culture with the wild type endoderm. The behavior of the PGCs could be followed for several days in the in vitro culture. If *Cdx2* contributes to the establishment of the suitable microenvironment for the PGC population to migrate, defects in PGC proliferation, survival or motility will become evident.

Although unlikely, it will be interesting to test weather in other animals PGC are dependent on Cdx genes. Since in the other species PGC are specified in random positions in the embryo it is not expected that Cdx genes play a role at early stages, just after the PGC specification. Nevertheless at some point of embryonic development, germ cells need to enter the genital ridges via the hindgut endoderm, leaving a window in which PCGs could rely on signals downstream of Cdx genes.

In **chapter 5** we described the role of *Cdx2* in the adult intestine. *Cdx2* is a master transcription factor regulating intestinal identity. Ablation of *Cdx2* expression in vivo, in the stem cells of the adult intestine result in the loss of the stem cell ability to self renew and generate the progeny that normally differentiate into all types of cells of the intestinal epithelium. In in vitro cultures single *Cdx2*^{null} intestinal stem cells fail to proliferate and form intestinal organoids like wild type stem cells do, in the standard intestinal culture conditions. Interestingly if single *Cdx2*^{null} intestinal stem cells are cultured in the medium designed for the gastric organoids they gave rise to organoids similar in morphology to pyloric organoids. We determined that *Cdx2*^{null} intestinal stem cells grown in the stomach medium formed organoids, which express some of the stomach markers. However they do not express the major transcriptional regulator of the stomach identity, *Sox2*. A genome wide transcription profiling of *Cdx2*^{null} intestinal organoids grown in stomach medium will help to define their state. Based on the expression of a few markers we defined them as possessing the identity intermediate between that of the small intestine and stomach. In vivo, the character of the stomach epithelium is established as a result of the interaction of the endoderm with underlying mesoderm. This situation is not mimicked in in vitro cultures. Additional factors are probably needed for the full transformation of *Cdx2*^{null} intestinal organoids into the stomach organoids. It is possible that adjusting the culture conditions of *Cdx2*^{null} intestinal organoids will allow to fully transform them into organoids with the stomach characteristic gene expression. We detected the stem cell marker, *Lgr5* in the stomach-like organoids proving that the stem cells are still present in these organoids. Out results so far show that stem cell of the small intestine a have certain plasticity and can adopt a new fate in changed conditions.

The investigations described in this thesis demonstrate that Cdx genes are key players in maintaining the self renewing properties of embryonic stem cell-like progenitors and at least some of the adult stem cells, as well as the differentiation program of these progenitors.

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Addendum

NEDERLANDSE SAMENVATTING

De drie Cdx-genen hebben belangrijke functies tijdens de embryonale ontwikkeling van de muis. Eén van deze genen, *Cdx2*, is onmisbaar voor implantatie van het embryo in de baarmoederwand. Alle drie genen zijn actief tijdens gastrulatie en zijn essentieel voor het verlengen van de lengte-as van het embryo. Cdx-mutanten hebben dan ook een verkorte lichaamsas en missen posterieure structuren. De ernst van deze defecten hangt af van welke Cdx-gen ontbreekt. **Hoofdstuk 1** geeft een overzicht van de rol van Cdx genen in muizen embryonale ontwikkeling.

In Hoofdstuk 2 wordt een hypothese over het werkingsmechanisme van de Cdx genen tijdens verlenging van de lichaamsas getest. Het was al bekend dat de aanleg van achterste weefsels in muizenembryo's afhankelijk is van stamcel-achtige voorlopers, die zich in een posterieure groeizone van het embryo bevinden. We vroegen ons af of het deze voorloper-cellen waren die getroffen worden door de muaties in de Cdx-genen. Daarom werden kleine stukjes weefsel uit het gebied waar deze voorlopers zich bevinden, van embryo's met de Cdx-mutatie naar normale embryo's getransplanteerd. Het resultaat was dat de getransplanteerde cellen, ondanks de mutatie, in een omgeving van gezonde cellen, normaal aan verlenging van de lichaamsas konden deelnemen. De conclusie is dat Cdx genen zorgen voor het behoud van een geschikte omgeving (niche) voor de precursors van axiale weefsels.

De Cdx genen, en in het bijzonder *Cdx2*, beginnen tot expressie te komen op de plaats waar (de posterieure groeizone) en tijdens het stadium dat de voorlopers van de kiemcellen gespecificeerd worden. In **Hoofdstuk 3** tonen we aan dat expressie van *Cdx2* nodig is voor het in stand houden van de kiemcel voorlopers net nadat ze gespecificeerd zijn. Vanaf dat moment hebben *Cdx2*^{nul} mutanten veel minder kiemcellen dan het normaal aantal. Inactivatie van *Wnt3a* leidt tot hetzelfde fenotype als inactivatie van *Cdx2*, en het toevoegen van *Wnt3a* aan een in vitro kweek van het embryonale deel waar de kiemcellen zich bevinden corrigeert het aantal kiemcellen. Een kleinere kiemcelpopulatie is ook een kenmerk van mutanten die een andere groeifactor missen in de omgeving van de kiemcellen, Bmp Bmp4 toevoegen aan de kweek van posterieur embryo gedeelten voldoet ook om het aantal kiemcellen op het normaal niveau terug te brengen. Omdat *Cdx2* in de kiemcellen zelf niet tot expressie komt concluderen wij dat de Cdx eiwitten zorgen voor de wenselijke omgeving van die voorlopers.

De defecten in het laatste deel van de wervelkolom van Cdx mutanten kunnen gecorrigeerd worden door een overactivatie van de Wnt signaleringsroute in de embryonale groeizone. Het vervroegen van de expressie van Hox13 genen veroorzaakt ook een voortijdig eind aan axiale extensie. We tonen aan in **Hoofdstuk 4** dat Cdx mutaties, afname in de Wnt signaleringsroute en vervroegde expressie van Hox13 genen ook de morfogenese van de neurale buis en van het verteringssysteem aantasten. In het bijzonder ontstaan in alle drie gevallen extra neurale buizen buiten de normale neurale buis. De op elkaar lijkende fenotypes van deze genetische veranderingen bewijzen dat Cdx- en Hox-genen, en Wnt signalering de morfogenese van embryonale structuren in de drie kiemlagen, mesoderm, neurectoderm en endoderm controleren. Intrigerend is het feit dat deze genen hun patroonvormende rol in de voorloper zone van het embryo spelen, veel vroeger dan het stadium waarop de defecten zich openbaren.

Cdx genen worden inactief op het einde van embryogenese, behalve in het darmepitheel waar *Cdx1* en *Cdx2* tot expressie blijven komen. *Cdx2* in het bijzonder blijft actief in de dunne en dikke darm. In **Hoofdstuk 5** beschrijven we de gevolgen van *Cdx2* inactivatie in darmepitheel en specifiek in de stam cellen van de crypten van de dunne darm. In de afwezigheid van *Cdx2* treden veranderingen op in de eigenschappen van het darmepitheel, dat op maagepitheel gaat lijken en moleculaire markers specifiek voor de maag tot expressie gaat brengen. De crypt-stamcellen verliezen hun groeicapaciteit, *in vivo* in muis *Cdx2* mutanten, en in vitro wanneer de crypten in kweek gebracht worden in geschikt medium. Wel slaagt het kweken van deze crypten in maag- medium, en groeien ze als maagachtige structuren volgens meeste, zij het niet alle, criteria. We concluderen dat de afwezigheid van *Cdx2* het dunnedarmepitheel gedeeltelijk transformeert in maagepitheel.

De implicaties van dit werk worden in **Hoofdstuk 6** besproken.

STRESZCZENIE W JĘZYKU POLSKIM

Genom myszy zawiera trzy geny Cdx (*Cdx1, Cdx2, Cdx4*), które pełnią rozmaite funkcje podczas rozwoju zarodkowego. Ekspresja *Cdx2* wykrywalna jest już w stadium blastocysty w trofektodermie, gdzie jest gen ten odpowiedzialny jest za prawidłową implantację zarodka w ścianie macicy. W okresie rozwoju postimplantacyjnego, ekspresja genów Cdx wykrywalna jest w trzech listkach zarodkowych (ektodermie, mezodermie oraz enodermie) od rozpoczęcia gastrulacji, 6 dni po zapłodnieniu, i utworzenia cylindra zarodkowego, przez okres trawania osiowego rozwoju zrodka aż do jego zakończenia,13 dni zapłodnieniu. Konsekwencją mutacji w genach Cdx powodują skrócenie osi zarodka o różym nasileniu w zależności od serii zmutowanych alleli. **Rozdział pierwszy** opisuje przegląd rozwoju zarodkowego myszy i roli genów Cdx w tym procesie.

Osiowy rozwój zarodka jest to process, w którym ciało zarodka poniżej głowy czyli składające się z tułowia oraz ogona budowane jest poprez podziały i róznicowanie się komórek progenitorowych usytułowanych w zawiązku ogona (nazwanym również tylną strefą wzrostu). Konsekwencją tego procesu jest powstanie zawiązków wszystkich tkanek i organów stanowiących tułów oraz ogon. W **rozdziale drugim** zadaliśmy pytanie czy komórki progenitorowe odpowiedzialne za proces osiowego rozwoju zarodka, w szególności te, które posiadają jednocześnie właściwości komórek macierzystych, wykazują automoniczne wady w mysich zarodkach z mutacjami w genach Cdx. Aby odpowiedzieć na to pytanie wykorzystaliśmy technikę transplantacji populacji komórek z zarodków z mutacjami w genach Cdx do dzikich zarodków. Nasze dane wskazują iż, pomimo mutacji genetycznych komórki progenitorowe oraz ich komórki potomne mają te same zdolnosci tworzenia zawiązków tkanek jak populacja dzikich komórek. Nasze wyniki sugerują iż geny Cdx stymulują ścieżki sygnalizacyjne aktywne w obrębie tylnej strefy wzrostu, która pozwala komórkom progenitorowym tworzyć zawiązki tkanek oraz organów.

We wczesnym zarodku postimplantacjnym ekspresja *Cdx2* rozpoczyna się w stadium strugi pierwotnej w tylnej części zarodka oraz u podstawy przyszłej omoczni. Jest to miejsce gdzie pojawiają się pierwotne komórki płuciowe, czyli komórki prekursorowe oocytów i plemników. W **rozdziale trzecim** opisaliśmy populację pierwotnych komórek płuciowych w zarodkach z mutacjami w genach *Cdx2* oraz *Wnt3a*. Odkryliśmy, iż obie mutacje powodują zmniejszenie populacji tych komórek ale nie wpływają na ich pojawianie się u podstawy omoczni. Dodatkowo mutanty *Cdx2* mają poważnie upośledzony rozwój omoczni co związane jest z niewystarczającym poziomem sygnalizacji, która normalnie podtrzymuje komórki progenitorowe pierwotnych komórek płuciowych oraz komórek progenitorowych omoczni. W naszych doświadczeniach wykazaliśmy iż, dodanie do hodowli tylnych części zarodków zwierających pierwotne komórki płuciowe białka Wnt3a lub Bmp4, czyli białek sygnalizacyjnych normalnie obecnych w tym rejonie zarodka, przywraca normalny poziom populacji pierwotnych komórek płuciowych. Proponujemy iż, *Cdx2* stymuluje populację pierwotnych komórek płuciowych poprzez stymulację scieżek sygnalizacyjnych Wnt oraz Bmp.

W **rozdziale czwartym** opisane zostały wady rozwojowe szeregu mutantów (Cdx, *Wnt3a*, Hox13) których konsekwencją jest niedorozwój końcowego odcinka układu pokarmowego oraz moczowego. Konsekwencją mutacji wymienionych powyżej genów jest m. in. brak odbytu oraz połączenie ujścia jelita grubego oraz ujścia cewki moczowej w kloakę, bez otwarcia na zewnątrz, co umiemożliwia defekację oraz oddawanie moczu. Tym nieprawdłowościom towarzyszą także zniekształcenia dolnego odcinka kręgosłupa oraz cewki nerwowej. Opisany zespół wad jest charakterystyczny dla ludzkiego zespołu regresji kaudalnej i charakteryzuje się wysoką śmiertelnością noworodków. Komórki progenitorowe tworzące zawiązki układów pokarmowego oraz moczowego, jak również cewki nerwowej oraz kręgosłupa zlokalizowane są we wspomnianej wcześniej tylnej strefie wrostu oraz podlegają regulacji poprzez geny Cdx i Hox oraz ścieżki sygnalizacyjne m. in ścieżkę Wnt. Nasze wyniki po raz pierwszy wskazują na kluczowe znaczenie genów Cdx i Hox w prawidłowym procesie formowania tkanek i układów dolnego odcinka ciała, w szczególności układu poarmowego oraz moczowego.

W dorosłym życiu myszy ekspresja *Cdx1* i *Cdx2* podtrzymana jest w nabłonku układu pokarmowego, w jelicie cienkim oraz jelicie grubym. W **rozdziale piątym** opisany został rezultat inaktywacji genu *Cdx2* w komórkach macierzystych jelita cienkiego. Komórki macierzyste układu pokarmowego charakteryzują się ekspresją białka Lgr5 i są odpowiedzialne za odnowę kompletnego nabłonka jelita w warunkach równowagi homeostatycznej. Lgr5 pozytywne komórki macierzyste pozbawione białka Cdx2 w warunkach *in vivo* nie są w stanie pop-

rawnie proliferować oraz odtworzyć nabłonka jelita. W zamian, tworzą one podpowierzchniowe cysty które charakteryzują się ekspresją niektórych markerów chrakterystycznych dla nabłonka żołądka. Lgr5 pozytywne komórki mogą zostać wyizolowane z nabłonka jelita oraz hodowane *in vitro* w hodowlach z substytutem macierzy pozakomórkowej. W wyniku inaktywacji genu *Cdx2* w warunkach in vitro Lgr5 pozytywne komórki nie są w stanie stworzyć struktur chrakterystycznych dla dzikich hodowli. Jednakże hodowane w pożywce używanej dla hodowli żołądkowych tworzą struktrury morfologicznie charkterystyczne dla tychże hodowli. Podobnie do sytuacji *in vivo*, część markerów charakterystycznych dla żołądka była obecna w hodowlach.

Rozdział szósty zawiera ogólna dyskusję podsumowującą poszczególne rozdziały.

DANKWOORD

It's time for the most pleasant part of the writing process, not the easiest one though. When I was a teenager, I had a dream, a dream to become a scientist. As stubborn as I am, I tried very hard to make it come true. Five years ago I came to Utrecht to begin my scientific career. With a lot of hope, and fear, I had begun confrontation with a new environment and culture, far from home. Coming here was the most difficult decision of my life, as I had to leave behind everything and everybody I love. So far, that was the best decision I've ever made. As we say: what doesn't kill you makes you stronger, this experience did make me stronger.

Jacqueline, thank you very much for giving me the opportunity to work in your group. I didn't really believe that I could do it, but I it happened. I did my PhD! You made my dream come true. Thank you for your patience in correcting all the texts that I wrote (with a random use of articles). I think I will never learn how to do that correctly. Thank you for the guidance these last more than 4 years. I learnt a lot from you. I wish you all the best for future. Since a lot of people bugged me with learning Dutch...well I did my best so don't laugh...

Cesca… well, where to start?…You perfectly know what these dots mean ☺. (haha, I see 10.000 thoughts traveling with a speed of light through your brain now ☺). Jij was er altijd voor mij, in goede tijden en in slechte tijden. Jij en je familie hebben een dom Pools meisje geadopteerd. Hiervoor ben ik jullie eindeloos dankbaar. Jij zal altijd een plekje in mijn hart hebben.... We hadden een geweldige tijd, in het lab èn buiten het lab. Jij ben niet altijd de makkelijkste persoon om mee om te gaan, maar dat ben ik ook niet, dat is een van de dingen die ervoor zorgde dat er een mooie vriendenschap tussen ons heeft kunnen ontstaan (en ons onhandig gevoel voor humor, belachelijke discussies en vele andere dingen die moeten hier niet worden vermeld ☺). Soms heb ik het gevoel dat je mij beter kent, dan ikzelf. Bedankt voor alles.

Sylwia, you're guilty on all charges, you made me come here so if not for you, this definitely wouldn't have happenned. And that's a good thing ☺. You're also the only person who made me cry!!! And that was even before I really started!!! But again you're also the one who showed me that nothing is impossible and that I could achieve whatever I want if only I try hard enough. Splot dziwnych wydarzeń sprawił, że tu jestem i Ty wiesz najlepiej ze wszystkich że było cholernie ciężko. Sylwia, nie ma słów, które oddadzą moja wdzięczność za wszystko, za to że jesteś, że zawsze mnie wysłuchiwałaś i pocieszałaś, ale też myślę że słowa nie są potrzebne. Wiesz, że bez Ciebie bym sobie nie poradziła (nawet jesli mnie do płaczu potrafisz doprowadzić, haha). Mam nadzieję, że będziemy się wzajemnie wpierać jeszcze przez długie lata.

Moja polska bando: Sylwia, Ania, Karolina, Majka…jak śpiewa Johnny Cash: you are my sunshine, my only sunchine, you make me happy when skies are grey…wiecie jak leci dalej ☺. Jesteście moja rodziną tutaj i kocham was wszystkie. Sylwia, Ania i ja, trójca z zakładu embriologii UW. Kto by pomyślał że nasze drogi tak się splotą po zakończeniu licencjatu. Nadal się nie mogę wyjść z zadziwienia jak nasze życie się potoczyło. Ania, mam nadzieję, że znajdziesz wymarzoną pracę i szczęście czy to w Chinach czy w Holandii czy gdzieś indziej na końcu świata. Wszystkie wiemy że, nie ma granic których nie można przekroczyć, (Anka, wiem że się sentymentalna robię ale o to tu chodzi). Karolina, muszę przyznać że, jak spotkałam Cię po raz pierwszy w Warszawie to pomyślałam sobie że, tej to lepiej nie nastąpić na odcisk…haha. Nie pomyliłam się. Jesteś niesamowitą babką, rany jak ja bym chciała miec twoja charyzmę i pewność siebie. Strasznie się cieszę że, tu jesteś. Majka, z Tobą było zupełnie odwrotnie. Jak spotkałyśmy się po raz pierwszy to sobie pomyślałam: rany takie dziecko, co ona tu robi?…haha w małym ciele wielki duch ☺. Niezaradną istotką

to Ty zdecydowanie nie jesteś, haha. Bardzo się że tu się zjawiłaś i na pewno nie zapomnę naszych rozmów jeszcze na Lombokstraat. Wiecie że, u mnie raczej cieżko z wyrażaniem uczuć więc powiem Wam tylko tyle. Kocham Was wszystkie.

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LIST OF PUBLICATIONS

Cdx2 determines the fate of post natal intestinal endoderm
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Genesis. 2008 Jul;46(7):335-46.

* equal contribution

CURRICULUM VITAE

Monika Białecka was born on 3rd of May 1980 in Warsaw, Poland. In June 1999 she finished the pre-university education at the XXII Liceum Ogólnokształcace im. Jose Marti in Warsaw. Then she enrolled as a student at the faculty of Biology at the Warsaw University. She received her Bachelor diploma in 2002. In the same year she did her first student internship at the Institute of Genetics and Animal Breeding, Polish Academy of Sciences in the group of prof. Jacek Modlinski where she learnt the isolation and maintenance of mouse embryonic stem cells and mouse embryonic fibroblast. This first contact with mouse embryos determined the future direction of her interests. In 2004 she obtained her Master diploma at the Warsaw University. She conducted research for her master project at the Department of Embryology under supervision of prof Marek Maleszewski. During this project she participated in the research on the role of CD9 protein on the oocyte surface and the ability of sperm and oocyte fusion during fertilization in the mouse.

In 2006 she came to the beautiful city of Utrecht to start an internship in the group of prof. Christine Mummery at Hubrecht Institute within the frame od Cancer Genomics and Developmental Biology Master program. During that internship, under direct supervision of dr. Susana Chuva de Sousa Lopes, she worked on optimizing the method of generation of germ cells (with a focus on oocyte derivation) from the human embryonic stem cells. A year later she had begun her PhD studies in the group of dr. Jacqueline Deschamps on the role of Cdx genes in mouse development. The result of this study is described in this book. In the future she is planning to continue her research in science as a post-doctoral fellow.