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Establishment of Intestinal Identity and Epithelial-Mesenchymal Signaling by *Cdx2*

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SUMMARY

We demonstrate that conditional ablation of the homeobox transcription factor Cdx2 from early endoderm results in the replacement of the posterior intestinal epithelium with keratinocytes, a dramatic cell fate conversion caused by ectopic activation of the foregut/esophageal differentiation program. This anterior homeotic transformation is first evident in the early embryonic Cdx2-deficient gut as expression of several key foregut endoderm regulators was shifted caudally. While the intestinal transcriptome was severely affected, Cdx2-deficiency only transiently modified selected posterior Hox genes and the primary enteric Hox code was maintained. Further, we demonstrate that Cdx2-directed intestinal cell fate adoption plays an important role in the establishment of normal epithelial-mesenchymal interactions, as multiple signaling pathways involved in this process were severely affected. We conclude that Cdx2 controls important aspects of intestinal identity and development, and that this function is largely independent of the enteric Hox code.

Keywords

Cdx2; intestine; esophagus; endoderm; conditional gene ablation; *Cdx1*; *HNF1α* ; *HNF4α* ; *Foxa3*; *Wnt*; keratinocyte

INTRODUCTION

The mouse endoderm transforms from a two-dimensional epithelial sheet into the primitive gut tube at embryonic day 8.5–9.0 (E8.5–9.0). Subsequent morphological differentiation converts the pseudostratified endoderm layer into a tall columnar epithelium which lines the respiratory and gastrointestinal tracts (Wells and Melton, 1999). The primitive gut appears homogeneous from end to end, with distinct anterior-posterior (AP) regions adopting different fates in subsequent organogenesis. In the gastrointestinal tract, the foregut gives rise to the epithelia of esophagus, stomach, and duodenum, while midgut and hindgut become the small intestine, and the cecum and colon, respectively. Cross-talk between gut mesoderm and endoderm progressively commits the primary endoderm to specific fates (Grapin-Botton and Melton, 2000). Mutations in a number of *Hox* genes result in malformations in certain gut regions, but do not cause whole-sale AP transformation of the gut (Aubin et al., 1997; Boulet and Capecchi, 1996; Manley and Capecchi, 1995; Warot et al., 1997; Zacchetti et al., 2007),

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even though these Hox factors play important roles in patterning the mesoderm and neural tube (Deschamps et al., 1999; Krumlauf, 1994; McGinnis and Krumlauf, 1992).

AP asymmetry of the gut endoderm is evident with the onset of *Cdx2* expression in the hindgut at its inception (Beck et al., 1995). *Cdx2* is the mouse homologue of *AmphiCdx* in *Amphioxus* and *caudal* in *Drosophila*. It resides in the "*ParaHox*" gene cluster believed to have evolved from a "*ProtoHox*" cluster that gave rise to the definitive *Hox* gene clusters (Brooke et al., 1998). In *Drosophila*, *caudal* specifies posterior body segments (Macdonald and Struhl, 1986; Mlodzik et al., 1985; Moreno and Morata, 1999). Morpholino knockdown and overexpression studies in Zebrafish indicated essential roles of *caudal* orthologues in neural tube and intestinal development (Cheng et al., 2008; Flores et al., 2008; Shimizu et al., 2006; Skromne et al., 2007). Three mouse homologues, *Cdx1*, *Cdx2* and *Cdx4* (Duprey et al., 1988; Gamer and Wright, 1993; James and Kazenwadel, 1991), participate in the patterning of the vertebral column (Chawengsaksophak et al., 1997; Subramanian et al., 1995; van Nes et al., 2006) and in embryonic hematopoiesis (Wang et al., 2008), however, their role in endoderm development is less clear. Homozygous *Cdx1* or *Cdx4* mutants do not display gut defects (Subramanian et al., 1995; van Nes et al., 2006), while *Cdx2* null mutants die in utero before the onset of endoderm development (Chawengsaksophak et al., 1997; Tamai et al., 1999).

In the mouse embryo, *Cdx2* is expressed in nuclei of cells derived from the late-dividing blastomere, a precursor of trophectoderm (Deb et al., 2006). From E8.5 onward, *Cdx2* is activated in the embryo proper, predominantly the posterior gut (Beck et al., 1995). *Cdx2* expression subsequently becomes restricted to the intestinal epithelium, with a sharp anterior boundary marking the transition from stomach to duodenum (James et al., 1994; Silberg et al., 2000). Genetic analysis of *Cdx2* function in mammalian intestinal development has been limited to *Cdx2* heterozygous mice that form multiple colonic polyps (Chawengsaksophak et al., 1997). These polyps contain areas of squamous metaplasia in which the expression of the remaining wild type Cdx2 allele is extinguished through an unknown mechanism (Beck et al., 1999). However, it is still unclear as to: ***a.*** what transcriptional programs were altered in these epimorphic lesions; ***b.*** how comprehensive the impact of Cdx2-disruption is for cell fate determination; ***c.*** through which mechanisms loss of Cdx2 induces squamous metaplasia; ***d.*** through which mechanisms Cdx2 promotes intestinal differentiation; and ***e.*** what role Cdx2 plays in intestinal epithelial-mesenchymal interactions.

We have previously shown that ectopic expression of *Cdx2* in the gastric epithelium induces intestinal metaplasia (Silberg et al., 2002), an example of a posterior homeotic transformation. Here, we demonstrate that *Cdx2* is essential for the initial expression and/or subsequent maintenance of a group of pro-intestinal transcription factors, including *Cdx1*, *Isx*, *HNF1α* and *HNF4α* , which together activate the intestinal transcriptome. The expression of Cdx2 in the posterior gut epithelium antagonizes the foregut differentiation program, which becomes ectopically activated upon Cdx2-disruption, resulting in dramatic cell fate conversion. We further demonstrate that intestinal cell fate establishment by Cdx2 plays a critical role in instructing normal epithelial and mesenchymal interactions, in particular with respect to the integrity of Wnt and Hedgehog signaling.

RESULTS

Conditional ablation of *Cdx2* from the developing endoderm

Cdx2 null mice die before gastrulation (Chawengsaksophak et al., 1997; Tamai et al., 1999). Therefore, we derived a conditional *Cdx2* allele to study its role in the gut endoderm(**Suppl. Fig. 1A**). Correctly targeted embryonic stem cell clones were identified by Southern blot analysis (**Suppl. Fig. 1B**). After germ line transmission of the targeted allele, the FRT-flanked

neomycin resistance gene was removed by crossing to *Flp1* deleter mice (Rodriguez et al., 2000). *Cdx2*$^{loxP/+}$ mice were then intercrossed, resulting in *Cdx2*$^{loxP/loxP}$ mice that were viable and fertile (**Suppl. Fig. 1C**), confirming that the *Cdx2*^{loxP} allele is functionally wild type. Subsequent Cre-mediated gene ablation results in a null allele that lacks the homeobox domain. To ablate *Cdx2* conditionally in the developing gut, we bred *Cdx2*$^{loxP/+}$ mice to *Foxa3*Cre mice (Lee et al., 2005), which delete loxP flanked targets in early endoderm. Using the *Rosa26R* reporter line, we verified Cre activity in the primitive gut of E9.5 embryos, prior to the onset of gross morphological defects (Fig. 1A, B). Efficient deletion of *Cdx2* from mutant (*Cdx2*$^{loxP/loxP}$,*Foxa3*Cre^{+}) gut epithelia was evident with immunohistochemistry using an anti-Cdx2 antibody (Fig. 1C, D). The expression of Foxa1, a pan-endoderm marker, was unaffected (Fig. 1E, F). Examination of mutant embryos at mid and late gestation revealed equal efficiency of *Cdx2* ablation throughout the intestinal domain (Fig. 1I–N and **Suppl. Fig. 2**).

Intestinal growth is severely affected in *Cdx2* mutant mice

Although the mutant pups were born alive, they did not survive beyond postnatal day one (P1). We examined the gastrointestinal tract of mutant embryos at various developmental stages. The gross abnormalities of the mutant posterior gut region first became evident at E12.5 (Fig. 1G). In contrast to the control intestinal tract that ends with colon and rectum, the mutant intestine developed an abnormal distal structure that terminates in a blind-ended sac (Fig. 1G, arrow). Progressive defects in elongation of the mutant intestine began to appear at E14.5 (**Suppl. Fig. 3A**) and the mutant gastrointestinal tract developed a malformed cecum at the distal end, with no colon (Fig. 1H, arrow). Cross sections of the E14.5 mutant distal intestine revealed a dilated gut lumen (Fig. 1M–N). All mutant animals examined from E13 to P0 (n=56) demonstrated an absence of the colon, a phenotype reminiscent of the most severe cases of colonic atresia in humans (Etensel et al., 2005;Lau and Caty, 2006). The mutant duodenum was progressively distended and became translucent, likely due to fluid retention caused by distal obstruction (Fig. 2A–B). By E18.5, the duodenum was further dilated with 5–7 fold increase in diameter compared to the control tissue (Fig. 2C–D, D′). Thus, *Cdx2*-deficiency prevents colon formation and leads to complete intestinal obstruction.

While the mutant proximal and medial intestinal epithelia appeared less organized than the control epithelia, the overall histology at E14.5 differed only subtly, as both mutant and control gut epithelia appeared pseudostratified (Fig. 1I–N). However, defects in differentiation became more apparent later in development. Since the mutant animals die at P1, before the development of Paneth cells, we examined the differentiation of enterocytes (Fig. 2E–F, I–J), goblet cells (Fig. 2G–H, K–N), and enteroendocrine cells (Fig. 2M–N) at different stages using specific markers and found terminal differentiation severely impaired. Instead, the mutant posterior intestinal epithelium expressed a basal epithelial cell marker p63 from E15.5 (Fig. 2H).

Villus hypoplasia was detected from E16.5 throughout the mutant intestinal domain as compared to controls (Fig. 3A–B). Position-matched longitudinal histological sections of E18.5 control and mutant intestines revealed dramatic reductions of intestinal villi (Fig. 3C–J), with severity increasing from anterior to posterior(**Suppl. Fig. 3B**): The mutant duodenum contained villus-like epithelial foldings (Fig. 3D) that were significantly stunted and broadened (Fig. 3F, and **Suppl. Fig. 3C**), while the cuboidal epithelia of mutant jejunum and ileum were completely replaced with a flattened epithelium (Fig. 3H, J), and the mutant ileum and cecum lacked villi entirely (Fig. 3J, and **Suppl. Fig. 2F** for cecum). Intestinal epithelia containing mosaic *Cdx2*-deletion were observed in a few mutant embryos. Segments of Cdx2-positive epithelium were contiguous to Cdx2-deficient regions (Fig. 3K). Interestingly, cells that retained *Cdx2* expression were capable to form villi and elaborate goblet cells normally (Fig. 3L), while adjacent *Cdx2*-deficient cells failed to do so (Fig. 3M). Thus, *Cdx2* is required for initiation of intestinal differentiation and morphogenesis in a cell-autonomous fashion.

Excess proliferation and keratinocyte character in the mutant intestine

Ki67 staining, which marks transit amplifying cells, revealed an expanded proliferative compartment in the mutant duodenal epithelium (Fig. 3N, O). The proliferative index of the mutant duodenal epithelial cells, assayed by BrdU incorporation, was significantly increased in the mutant epithelium following either 1-hr or 24-hr labeling (**Suppl. Fig. 4A–C**). Interestingly, even after a short labeling period (40–60 min), more than 20% of $BrdU^+$ mutant cells were located at or above position 9 relative to the bottom of the nascent crypts (Fig. 3Q, R, and **Suppl. Fig. 4D**), while $BrdU^+$ control cells were restricted to the inter-villus space even after 24-hour BrdU labeling (Fig. 3P, R and **Suppl. Fig. 4E**). BrdU incorporation revealed a continuous proliferative cell layer across the mutant epithelial sheet in the posterior intestine (**Suppl. Fig. 5B**). We did not, however, detect a significant increase in the apoptotic rate in *Cdx2* mutants by either cleaved caspase-3 or TUNEL staining (**Suppl. Fig. 5D**). Thus, the failure to specify the colon was not caused by a lack of proliferative capacity or enhanced cell death in *Cdx2* mutant mice. The loss of terminal differentiation discussed above, and our failure to observe gastric glandular epithelial cell types using specific antibodies (not shown), suggest that the proliferative pattern of the mutant epithelium resembles that of early embryonic stages prior to intestinal differentiation.

To gain insight into the identity of the *Cdx2* mutant epithelial cells, especially those in the posterior intestinal epithelium, we examined their ultrastructural features using transmission electron microscopy. *Cdx2*-deficient epithelial cells failed to develop the brush border typical of enterocytes (Fig. 4A–D). Examination of the posterior intestine revealed multiple layers of flattened epithelial cells, with the axes of the nuclei parallel to the luminal surface (Fig. 4F). Unexpectedly, the mutant posterior intestinal epithelial cells contained abundant tonofilaments (Fig. 4G). Tonofilaments are typical of squamous epithelial cells and are frequently seen in the desmosomal junctions of keratinocytes (Fig. 4H), which contribute to stratified esophageal epithelia but are extremely rare in the normal intestine (Fig. 4E).

Squamous differentiation has been reported in colorectal adenoma(Ouban et al., 2002). To verify whether the *Cdx2*-deficient posterior intestine has molecular features of squamous epithelia, we performed immunohistochemistry for keratin 13 and p63, markers of the suprabasal and basal squamous epithelial cells in mouse esophagus, respectively (Fig. 5A, B). In contrast to the control ileum where neither gene was expressed (Fig. 5C–D), the *Cdx2* mutant epithelium was positive for both markers (Fig. 5E–G). Neither *keratin 13* nor *p63* was expressed in E10.5 wild type midgut or hindgut endoderm (**Suppl. Fig. 6C, D**). At E12.5, p63 expression was detected in foregut endoderm cells fated to become forestomach and pharynx **(Suppl. Fig. 6E, F**). Likewise, a marker of the anterior foregut endoderm, Sox2 (Que et al., 2007), was detected in the *Cdx2*-deficient ileum at an expression level equivalent to that of normal esophagus (Fig. 5H), confirming that the *Cdx2*-deficient posterior intestine was indeed anteriorized. These data indicate that the expression of squamous markers in mutant prospective intestine was not due to a developmental delay, but rather due to an ectopically activated foregut differentiation program.

Global activation of esophageal genes in the *Cdx2*-deficient posterior gut

We next performed gene expression profiling using RNA samples extracted from total E18.5 control and mutant ileum as well as normal esophagus. The morphological abnormalities of the mutant ileum precluded separation of the epithelium from mesenchyme. Of the 11,738 significantly changed genes, 268 genes demonstrated fold-changes above 50-fold compared to control ileum. Hierarchical clustering showed that the transcriptome of the mutant ileum resembled that of esophagus far more than that of normal ileum (**Suppl. Fig. 7A**). The similarity between the mutant ileum and control esophagus is highlighted by a heat map assembled from differentially expressed genes (Fig. 5I). Consistent with the morphological transformation,

virtually all intestine-specific genes were downregulated in the mutant ileum (Fig. 5J, **Suppl. Table 1**).

Next, we compared our microarray results with several previous intestine gene profiling studies (Bates et al., 2002; Li et al., 2007; Schroder et al., 2006). Among genes that show significant enrichment in E18.5 intestinal epithelium over mesenchyme (Li et al., 2007), 35.3% were significantly altered in *Cdx2*-deficient intestine. Likewise, 39.8% of genes that show enrichment in intestine over stomach (Bates et al., 2002) were significantly affected in our model. Furthermore, genes with highly specific expression patterns in the differentiated intestinal epithelium (Schroder et al., 2006) were all significantly downregulated in *Cdx2*-deficient mice.

In contrast, many genes involved in keratinocyte differentiation were significantly upregulated in the *Cdx2* mutant ileum (Fig. 5K, **Suppl. Table 2**; **Suppl. Fig. 7B**). Notably, nine out of the ten most highly upregulated genes in the mutant ileum were enriched in normal esophageal epithelium (**Suppl. Table 2**). Most of these genes, such as high molecular weight *keratins, keratin 5 and 13, small proline-rich protein 3, calmodulin-like 3, cornifelin, plakophilin 1,* and *dermokine,* play important roles in keratinocyte cell envelope formation and desmosome assembly. Thus, gene profiling analysis confirmed the transformation of *Cdx2*-deficient prospective intestinal endoderm domain to an esophageal cell fate.

Cdx2-deficiency alters the AP distribution of transcriptional regulators

To gain a mechanistic insight to the cell fate switch in *Cdx2*-deficient posterior intestine, we analyzed transcriptional regulators known to be crucial in regulating intestinal differentiation. Along with the striking decrease of *Cdx2* mRNA itself, several intestine-enriched transcription factors, including *HNF1α* , *HNF4α* , *Isx* and *Cdx1*, were dramatically reduced in expression in the mutant intestine (**Suppl. Table 1**). Next, we performed quantitative reverse transcriptase PCR (Q-RTPCR) analysis on developing gastrointestinal tracts at earlier embryonic time points. A significant reduction in mRNA levels of *HNF1α* , *HNF4α* and *Isx* was already evident in the E12.5 mutant gut (Fig. 6B–D). At E14.5, expression of all these factors as well as *Cdx1* was significantly reduced in both proximal and distal intestine (Fig. 6A–D). These data indicate that the reduced expression of these transcription factors at E18.5 was not due to a secondary effect of abnormal development, but due to an impairment of the initial activation of these genes.

The expression of *Math1*, a basic helix-loop-helix transcription factor that plays a role in the differentiation of intestinal secretory cell types (Yang et al., 2001), was significantly reduced in mutant distal intestines from E12.5 onward (Fig. 6E). Conversely, qRT-PCR of *Sox2* and *Pax9* demonstrated that these foregut-enriched genes were ectopically activated in the mutant posterior intestine as early as E12.5, at a level equivalent to the stomach (Fig. 6G, H). Activation of *Sox2* was detected even in the anterior mutant intestines (Fig. 6G), strongly supporting an early anteriorization event that subsequently drives the ectopic activation of foregut transcriptional program in the mutant gut (Fig. 6J).

qRT-PCR confirmed the decrease of *Indian Hedgehog (Ihh)* expression and the dramatic activation of *Wnt10a* in E14.5 mutants (Fig. 6F, I). In the normal gastrointestinal tract, *Wnt10a* expression is excluded from the intestinal domain from E12.5 (Fig. 6I). These changes in the expression pattern of signaling molecules likely reflect a consequence of early cell fate transition, resulting from the altered transcriptional program in the Cdx2-deficient intestine. We also confirmed the changes of *Cdx1, HNF1α* , *HNF4α* , *Sox2* and *Wnt10* expression at the protein levels using E18.5 tissue lysates (Fig. 5H and Fig. 6K).

To investigate whether *Cdx2* directly regulates the expression of *HNF1α , Cdx1 and HNF4α* in the embryonic intestines, we first examined the regulatory sequences of *HNF1α , HNF4α* and *Cdx1*. Among multiple Cdx binding sites within the 5′ upstream region of *HNF1α* , those located near the transcription initiation site were most conserved from *Xenopus* to human (**Suppl. Fig. 8A**). Less conserved Cdx binding sites were identified in the *HNF4α* and *Cdx1* 5′ upstream sequence (**Suppl. Fig. 8B, C**). These Cdx sites are occupied by Cdx2 *in vivo*, as demonstrated by chromatin immunoprecipitation (ChIP) (Fig. 6L).

Cdx2-deficiency affects expression of selected enteric *Hox* genes

In a number of non-endoderm tissues, Cdx factors exert their developmental effect via regulating Hox transcription factors (Charite et al., 1998; Shimizu et al., 2006; Subramanian et al., 1995; Wang et al., 2008), which are key players in the primary AP patterning process of the vertebrate embryo (Krumlauf, 1994). Overexpression or inactivation of specific *Hox* genes has been shown to affect gastrointestinal development (Aubin et al., 1997; Boulet and Capecchi, 1996; Kondo et al., 1996; Pollock et al., 1992; Wolgemuth et al., 1989), while a cluster of *Hoxd* genes controls the formation of the ileo-cecal sphincter (Zakany and Duboule, 1999). Our microarray data indicated that a number of intestine-enriched *Hox* genes, including *Hoxa5, Hoxb5, Hoxb6, Hoxa7* and *Hoxb7*, continue to be expressed in the mutant ileum at a level similar to controls **(Suppl. Fig. 9)**. However, *Hoxc9*, a gene expressed in the posterior midgut and hindgut (Grapin-Botton and Melton, 2000; Roberts, 2000), was decreased 6.2-fold in the mutant ileum (**Suppl. Fig. 9B**).

Next we examined the AP distribution of representative *Hox* genes in the early gut where their expression patterns have well been documented (Choi et al., 2006; Grapin-Botton and Melton, 2000; Pitera et al., 1999; Roberts, 2000). Levels of *Hoxa3, Hoxb3, Hoxb4, Hoxc4, Hoxd4, Hoxb5, Hoxc5, Hoxa7* and *Hoxb7* mRNA were not significantly changed in the *Cdx2* mutant (Fig. 7A–H, **Suppl. Fig. 10A**). In contrast, at E12.5, *Hoxc8, Hoxb9, Hoxc9, Hoxa13* and *Hoxd13* mRNA levels were significantly lower in the mutant posterior intestine (Fig. 7I–L, **Suppl. Fig. 10B**). At E14.5, however, most of these posterior *Hox* genes had recovered to match the levels of the control intestine **(**Fig. 7I–L, **Suppl. Fig. 10B**).

Most *Hox* genes analyzed are expressed in the gut mesenchyme (Li et al., 2007), while some, such as *Hoxa3, Hoxb4, Hoxc5, Hoxb9, Hoxc9, Hoxa13* and *Hoxd13*, are also active in the epithelium (Grapin-Botton and Melton, 2000; Roberts, 2000). Our results demonstrate that *Cdx2*-deficiency in the early gut endoderm transiently modifies the expression of selected posterior *Hox* genes, but had no impact on anterior *Hox* genes. While the posterior *Cdx2*-deficient gut was anteriorized as early as E12.5 (Fig. 6G–J), the maintained expression of *Hoxc9*, *Hoxa13* and *Hoxd13* in this domain (Fig. 7J–L) indicates that the mutant gut had retained its primary enteric *Hox* code.

In addition, the *Cdx2*-deficient gut demonstrated normal AP expression of *Pdx1*, a second *“Parahox”* gene, which remained restricted to the duodenum even in the absence of Cdx2 (**Suppl Fig. 10E, F**). Furthermore, the expression of *Barx1*, a stomach specific mesenchymal transcription factor (Kim et al., 2005), also maintained its expression domain in Cdx2 mutant embryos (**Suppl. Fig. 10C**). These data further support the notion that the *Cdx2*-deficient gut retained certain AP values.

Cdx2-deficiency affects intestinal epithelial-mesenchymal interactions

We found dysregulation of Wnt ligand expression in *Cdx2*-deficient intestine. In addition to the ectopic activation of *Wnt10a* as a result of anteriorization of the mutant ileum (Fig. 6I, K), multiple other *Wnts*, as well as the Wnt target genes CD44, cyclin D1, Sox9 and the Tcf factors, were significantly upregulated in the *Cdx2*-deficient intestine (Fig. 8A, B; **Suppl. Fig. 11A–**

D). In contrast to Wnt, expression of *Ihh* and *Sonic hedgehog* (*Shh*) was significantly reduced in the *Cdx2*-deficient ileum (Fig. 8A), consistent with the decreased expression of *Hedgehog-interacting protein (Hhip)*, a primary hedgehog target expressed by the intestinal mesenchyme (Li et al., 2007). The severely expanded smooth muscle layer we observed in the mutant duodenum (Fig. 3F) and jejunum (Fig. 8C–F) may reflect the decreased hedgehog signaling activity, as inhibition of Hedgehog signaling in the intestine causes smooth muscle expansion (Madison et al., 2005).

The expression of *desmin*, a marker of smooth muscle progenitors but not by myofibroblasts (Adegboyega et al., 2002), was increased 6.2-fold in the *Cdx2*-deficient intestine and accompanied with a significant decrease of several *myosin* genes (**Suppl. Fig. 11E**), suggesting an altered myogenic process and terminal differentiation of smooth muscle cells in the *Cdx2* mutant gut. The *myosin* gene expression profile in the *Cdx2* mutant ileum highly resembled that of wild-type esophagus (**Suppl. Fig. 11E**), illustrating a potent epithelial-to-mesenchymal regulatory role that affected the smooth muscle differentiation.

DISCUSSION

Cdx2 and the AP patterning of the gut

The *Cdx2*-deficient gut displays severe hindgut abnormalities with a failure of colon development and a complete terminal blockage. Partial or complete colonic atresia has been reported as a human congenital disorder (Etensel et al., 2005). Mutations in *PDX1*, a neighboring "*Parahox*" gene, cause pancreatic agenesis in humans (Stoffers et al., 1997). Our findings support the notion that the *Parahox* genes specify regional identities to the vertebrate gut, and suggest further that mutations in *CDX2* or its targets could contribute to colonic atresia in human.

The expression domains of multiple important foregut regulators including Sox2, Pax9 (Grapin-Botton and Melton, 2000), p63 (Glickman et al., 2001), were dramatically extended towards the posterior of the *Cdx2* mutant gut tube (Fig. 8G). None of the previously reported mutant mice had such a dramatic impact on AP patterning of the gut (Aubin et al., 1997; Boulet and Capecchi, 1996; Manley and Capecchi, 1995; Warot et al., 1997; Zacchetti et al., 2007). When a cluster of *Hoxd* genes was deleted, no dramatic disruption of intestinal identity was observed, except that induction of the cecum was affected (Zacchetti et al., 2007). The cecum was correctly induced in *Cdx2* mutant embryos, consistent with the observation that the primary enteric *Hox* code was maintained.

Though Cdx factors have been proposed to function via regulation of *Hox* gene expression in several non-endoderm tissues (Charite et al., 1998; Subramanian et al., 1995; Wang et al., 2008), the expression of anteriorly localized intestinal *Hox* genes was independent of Cdx2. *Cdx2*-deficiency transiently delayed the expression of several posterior intestinal *Hox* genes at early embryonic stages; however, these genes maintained their relative AP position. The regulation of posterior *Hox* genes by Cdx factors has been reported in Zebrafish hindbrain (Shimizu et al., 2006), however, functional rescue by downstream Hox factors remains controversial (Skromne et al., 2007). Our findings indicate that *Cdx2*-deficiency does not profoundly influence the primary enteric *Hox* code.

Cdx2 regulates pro-intestinal transcription factors

Cdx1, whose gut expression pattern resembles that of *Cdx2* (Silberg et al., 2000), has the capability to drive intestinal differentiation in a gain-of-function setting (Mutoh et al., 2004). Redundancy between all three Cdx proteins has been reported in a number of non-endoderm tissues (van den Akker et al., 2002; van Nes et al., 2006; Wang et al., 2008). Therefore, it was

surprising to see the near-complete homeotic transformation of the *Cdx2*-deficient intestine, as some compensation was anticipated. We established that *Cdx1* activation is directly dependent on *Cdx2*. This transcriptional hierarchy between the two *Cdx* genes reflects their sequential expression pattern in the gut endoderm, where *Cdx2* precedes *Cdx1* by a few days (Hu et al., 1993; Meyer and Gruss, 1993; Silberg et al., 2000). In fact, the expression of *Cdx1* starts only when villus morphogenesis and epithelial maturation begin (Hu et al., 1993). Our data provide further evidence for the evolutionary significance of the *"Parahox"* cluster, where *Cdx2*, but not *Cdx1* or *Cdx4*, is located. Thus, *Cdx1* is controlled by the more ancient *caudal* orthologue *Cdx2* in gut endoderm to facilitate the developmental and anatomical complexity of the organ.

Similar to *Cdx1*, *Isx* is another intestine-specific transcription factor whose expression initiates during epithelial differentiation (Choi et al., 2006), consistent with its dependency on Cdx2. In addition, the maintenance of *HNF1α* and *HNF4α* expression in the embryonic intestine is directly controlled by Cdx2. Single-gene ablation of *Cdx1*, *Isx*, *Hnf1α* or *Hnf4α* in mice had no effect on the establishment of the intestinal epithelium (Choi et al., 2006; Garrison et al., 2006; Lee et al., 1998; Pontoglio et al., 1996; Shih et al., 2001; Subramanian et al., 1995). Nevertheless, Cdx1 (Mutoh et al., 2004), Isx (Choi et al., 2006), HNF1α (Martin et al., 2000) and HNF4α (Garrison et al., 2006) regulate the expression of numerous intestinal genes. Our data support the notion that Cdx2 functions upstream of a group of pro-intestinal transcription factors, with which it synergizes to promote intestinal cell fate (Fig. 8G).

Cdx2 antagonizes the foregut differentiation program

Our conditional Cdx2 mutants recapitulate the finding of squamous metaplasia in $Cdx2^{+/-}$ mouse colonic lesions, where Cdx2 expression was diminished (Beck et al., 1999). When *Cdx2* was removed in our model, anteriorization was first evident with the caudal extension the of Sox2 and Pax9 expression domains (Fig. 8G). This was followed by squamous differentiation around E14.5–15.5, leading to genome-wide activation of esophageal genes. Due to the lack of pro-intestinal regulators, the prospective intestinal epithelial domain was replaced by keratinocytes. These data provide molecular evidence that *Cdx2* normally represses a foregut differentiation program in the posterior gut, explaining the epimorphic changes observed previously (Beck et al., 1999).

We demonstrate that the normal gastrointestinal expression domain of *Wnt10a* is opposite to that of *Cdx2*, mimicking the expression pattern of *Sox2*. *Cdx2*-deficiency led to ectopic activation of *Wnt10a* expression in the caudal intestine, possibly as a consequence of the ectopically differentiated squamous cells. Recent findings suggest that ectodermal dysplasia in humans is associated with Wnt10a mutations (Adaimy et al., 2007), while mis-regulation of *Wnt10a* was found in gastrointestinal cancer (Kirikoshi et al., 2001). We speculate that this gene may involve in keratinocyte differentiation during upper gastrointestinal development. In summary, we have identified *Cdx2* as a master regulator in the posterior endoderm, demonstrating that this gene is essential for the establishment of intestinal identity.

Materials and Methods

Histology, immunohistochemistry and immunofluorescence

Hematoxylin, eosin, and alcian blue staining was performed in the Morphology Core of the Penn Center for Molecular Studies in Digestive and Liver Diseases. Alkaline phosphatase staining was performed using the Vector Red Alkaline Phosphatase Substrate Kit I (Vector Laboratory, SK-5100). The ABC detection system (Vector Laboratory, PK-6100) was used for immunohistochemistry. Cy2- and Cy3-conjugated fluorescent secondary antibodies were purchased from Jackson Laboratory.

For quantification of intestinal length as well as villi number, length and width, images of matched control and mutant intestines were analyzed using ImageJ software (NIH). For cell number quantification, $BrdU^+$ cells within 50 continuous villi were manually counted from three slides of control and mutant intestines. The percentage of $BrdU^+$ cells at each cell position was calculated with the cell located at the bottom of the inter-villus pocket designated as position 0.

Electron microscopy

Fresh intestinal tissues were washed with PBS and suspended in a fixative solution of 2.5% cacodylate-buffered glutaraldehyde and 4% paraformaldehyde (pH 7.4) for 6 hours. Tissues were rinsed in a cacodylate-buffered solution, post-fixed with 2% cacodylate-buffered OsO4 dehydrated with graded ethanol, clarified in propylene oxide and embedded in Epon. Seventy nm thin sections were obtained with a Leica UCT ultramicrotome using a Diatome diamond knife and placed on 200 mesh copper grids. Sections were stained with an alcoholic solution of uranyl acetate, followed by a solution of bismuth subnitrite. These sections were examined under a JEOL JEM1010 electron microscope and digital images were captured using AMT Advantage HR aided Hamamatsu CCD camera. All EM supplies were purchased from Electron Microscopy Sciences, Fort Washington, PA, and Ted Pella, Redding, CA.

Western blot analysis

Fresh intestinal tissue lysates were prepared in lysis buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 10 mM EDTA, 0.02% NaN_3, 50 mM NaF, 1 mM Na_3VO_4, 1% NP40, 1 mM PMSF, and protease inhibitors (Sigma), from E18.5 mouse intestines. 15 ug total lysates were heated at 70°C for 10 min in 4× LDS buffer (Invitrogen), and loaded on 4%–12% SDS-PAGE (Invitrogen). Proteins were transferred to PVDF membranes (Invitrogen). Membranes were stripped in Western stripping buffer (Pierce) and reprobed sequentially with corresponding antibodies.

Chromatin immunoprecipitation

E16.5 control and mutant intestinal tissues were finely minced into small pieces followed by 10 min cross-linking with 1% formaldehyde at 37°C and subjected to chromatin purification and immunoprecipitation as previously described (Rubins et al., 2005) using anti-Cdx2 antibodies (Funakoshi et al., 2008). Input chromatin and ChIP DNA were used as template in quantitative genomic PCR using a MX3000 PCR machine (Stratagene). The 28S ribosomal genes were used in as internal reference.

Microarray analysis

One centimeter of the ileum, immediately above the cecum, was dissected from three E18.5 mutant and three control embryos. 250 ng of total RNA was amplified and labeled with Cy3 using the Low Linear Amplification Kit (Agilent Technologies, CA). This labeling reaction produced 1.75 – 2.0 μg of Cy3-labeled cRNA (anti-sense), by first converting mRNA primed with an oligo (d)T-T7 primer into dsDNA with MMLV-RT and then amplifying the sample using T7 RNA Polymerase in the presence of Cy3-CTP. After purification, 1.65 μg of cRNA was fragmented and hybridized to the Whole Mouse Genome Oligo Microarray (G4122A; Agilent Technologies, CA) array for 17 hr. at 65°C.

Microarray slides were washed and scanned with an Agilent G2565BA Microarray Scanner. Images were analyzed with Feature Extraction 9.5 (Agilent Technologies, CA). Mean foreground intensities were obtained for each spot and imported into the mathematical software package “R”. The Cy3 (green) intensities were corrected for the scanner offset but not further background corrected. The dataset was filtered to remove positive control elements. Using the

negative controls on the arrays, the background threshold was determined and all values less than this value were set to the threshold value. Finally, the data were normalized using the Quantile Normalization package in "R" (Bolstad et al., 2003). Complete statistical analysis was then performed in "R" using both the LIMMA and SAM packages to identify statistically significant differential gene expression between the three groups. Microarray data have been deposited in ArrayExpress (www.ebi.ac.uk) under accession number XXX.

Quantitative RT-PCR analysis

Total RNA samples were extracted from E12.5–E18.5 gut tissues. For E12.5 tissues, one biological sample was pooled from 2–3 guts of like genotype. cDNA synthesis and quantitative RT-PCR analysis was performed as described previously (Gao et al., 2007). qRT-PCR primer sequences are available upon request.

Clustering analysis and generation of heat map

Hierarchical clustering was performed on the samples (arrays) using the "R" package "pvclust" (Suzuki and Shimodaira, 2006). Additional hierarchical clustering on differentially expressed genes and generation of heat maps was performed using the TM4 Multiple Experiment Viewer software package (Saeed et al., 2003).

Gene functional category and pathway analysis

Gene functional classification was performed on differentially expressed genes with at least a 4-fold change between control and Cdx2-deficient ileum. The Refseq_mRNA IDs of these genes were used for analysis by DAVID Bioinformatics resources, NIH (Dennis et al., 2003). Data were also analyzed through the use of Ingenuity Pathways Analysis (Ingenuity System, www.ingenuity.com) as described previously (Phuc Le et al., 2005).

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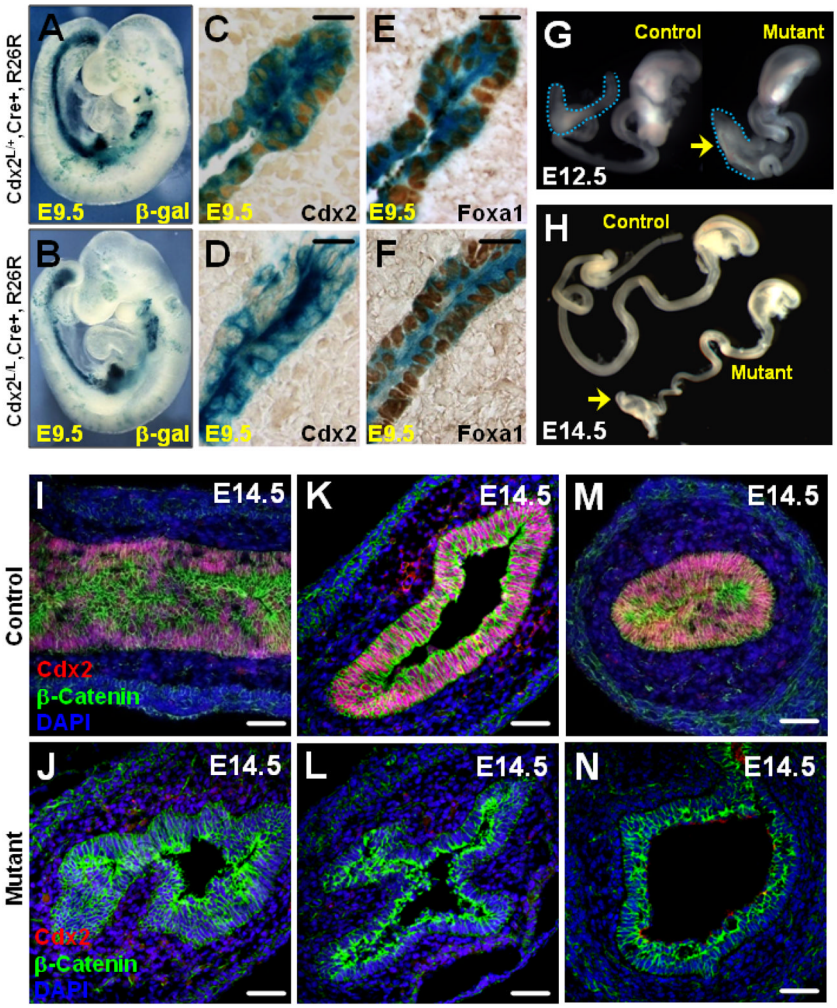

Figure 1. Conditional ablation of *Cdx2* in mouse endoderm leads to abnormal intestinal growth and colon dysgenesis

(A–B) Whole mount β-galactosidase staining of E9.5 *Cdx2*$^{loxP/+}$, *Foxa3*Cre, *R26R* and *Cdx2*$^{loxP/loxP}$, *Foxa3*Cre, *R26R* embryos.

(C–D) Immunohistochemistry for Cdx2 in E9.5 control and mutant embryos.

(E–F) A pan endoderm marker, Foxa1, continues to be expressed in the *Cdx2*-deficient endoderm.

(G) The distal intestine of the E12.5 mutant gut forms a blind-ended sac (arrow).

(H) At E14.5, abnormal intestinal growth and terminal blockage are evident in the mutant posterior gut, with no colon formation. Arrow points to malformed cecum.

(I–N) Confocal immunostaining for β-catenin (green) and Cdx2 (red) was performed on E14.5 anterior (I,J), medial (K,L) and posterior (M,N) intestinal sections. Nuclei were counterstained by DAPI in blue. Scale bars: 50 μm.

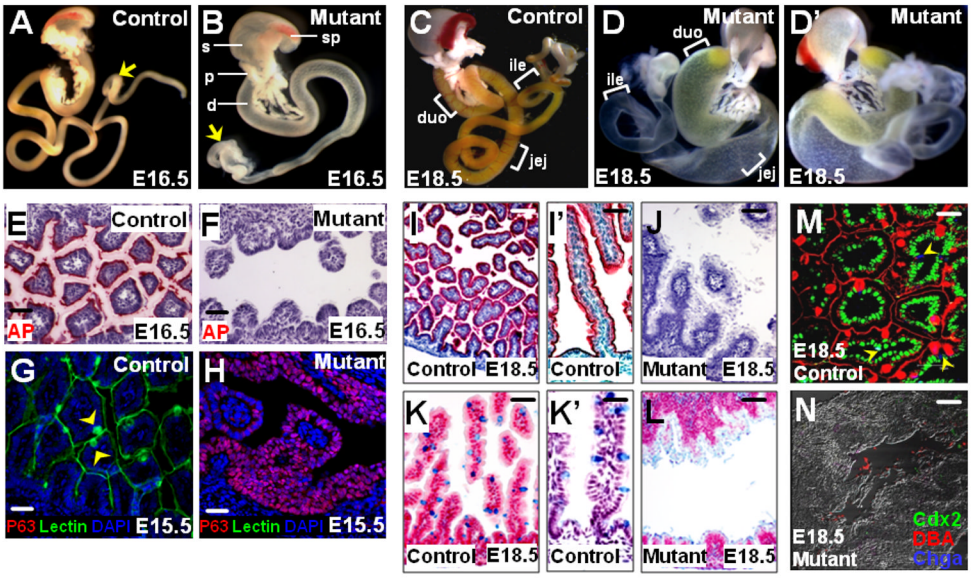

Figure 2. Initial intestinal differentiation is blocked in the Cdx2-deficient gut

(A–B) Severe intestinal shortening is evident in the E16.5 mutant duodenum. The colon fails to form in Cdx2 mutant embryos. Arrows point to cecum. *d*, duodenum; *p*, pancreas; *s*, stomach; *sp*, spleen.

(C–D′) E18.5 mutant intestine is dilated due to terminal blockage. duo, duodenum; jej, jejunum; ile, ileum.

(E–F) Alkaline phosphatase staining (red) of E16.5 control and mutant jejunum sections.

(G–H) Confocal immunostaining for p63 (red) and DBA lectin (green) of E15.5 control and mutant jejunum sections. Nuclei were counterstained by DAPI in blue.

(I–J) Alkaline phosphatase staining (red) of E18.5 control and Cdx2 mutant.

(K–L) Alcian blue staining (blue) of E18.5 control and Cdx2 mutant jejunum sections demonstrates absence of goblet cells in mutant epithelium.

(M–N) Confocal immunostaining of Cdx2 (green), lectin DBA (red) and chromogranin A (blue) demonstrates absence of lectin-positive intestinal mucosa as well as enteroendocrine cells in Cdx2 mutants. Arrowheads in M point to the rare enteroendocrine cells in the control intestine. Scale bars: 75 μm in E–F, I–L; 50 μm in G–H, I′, K′ and M–N.

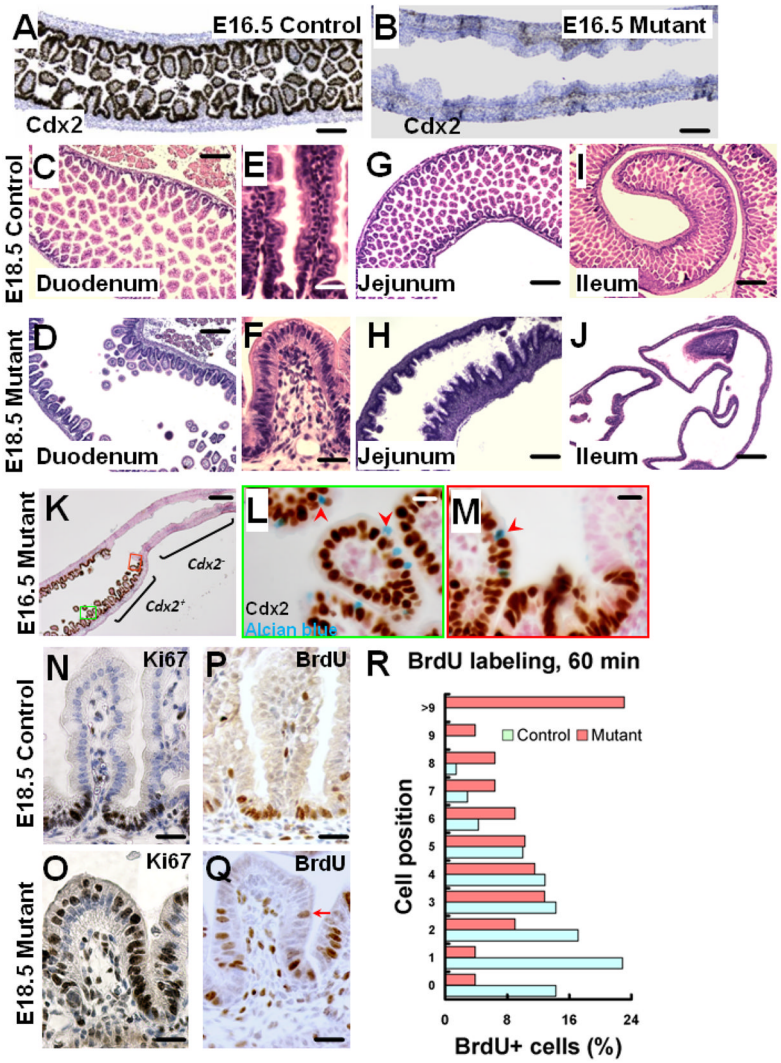

Figure 3. ***Cdx2*-deficiency affects villus morphogenesis and proliferation pattern**

(A–B) Immunohistochemistry for Cdx2 on E16.5 control and mutant ileal sections.

(C–J) H&E staining of position-matched E18.5 longitudinal sections of control and mutant duodenum, jejunum and ileum. The mutant duodenal epithelial folding is stunted (F).

(K–M) Mosaic *Cdx2* deletion in E16.5 mutant ileum is indicated by Cdx2 immunostaining (brown). $Cdx2^+$ epithelia are contiguous to regions of $Cdx2^-$ epithelia (K). Differentiated goblet cells (alcian blue) were observed only in the $Cdx2^+$ epithelial cells (arrowhead in L and M), but not in the $Cdx2^-$ epithelium.

(N–O) Immunohistochemistry for Ki67 on E18.5 duodenal sections.

(P–Q) BrdU incorporation followed by immunohistochemistry illustrates that proliferating cells are distributed throughout the stunted villi of Cdx2-deficient mice, in contrast to control $BrdU^+$ cells that are localized to the intervillus region.

(R) Quantification of $BrdU^+$ cells located along the crypt-villus axis after 60 minutes of in vivo labeling.

Scale bars: 75 μm in A–B; 100 μm in C–D, G–K; 50 μm in E–F, L–Q.

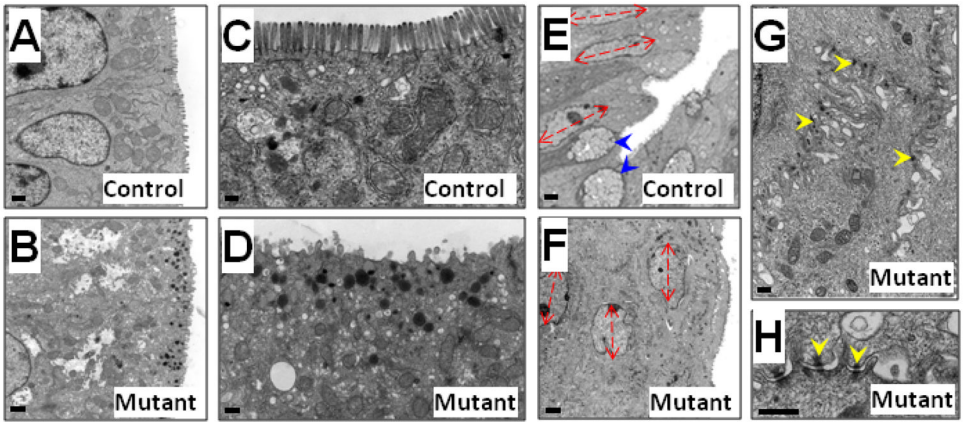

Figure 4. The *Cdx2*-deficient intestine lacks intestine-specific ultrastructural features

(A–D) Electron micrographs of E18.5 control and mutant anterior intestinal epithelium. Mutant cells lack brush border (D).

(E–F) Electron micrographs of E18.5 control and mutant posterior intestinal epithelium. Double-ended arrows indicate the orientations of the mutant nuclei which are parallel to the luminal surface (F), as opposed to the perpendicular orientation in control cells (E). Blue arrowheads point to goblet cells in control tissue (E).

(G–H) Mutant ileal cells contain abundant tonofilaments (yellow arrowheads in G) across intercellular junctions where desmosome-like structures (yellow arrowheads in H) are assembled. Scale bars: 2 microns in A–B, E–F; 500 nm in C–D, G–H.

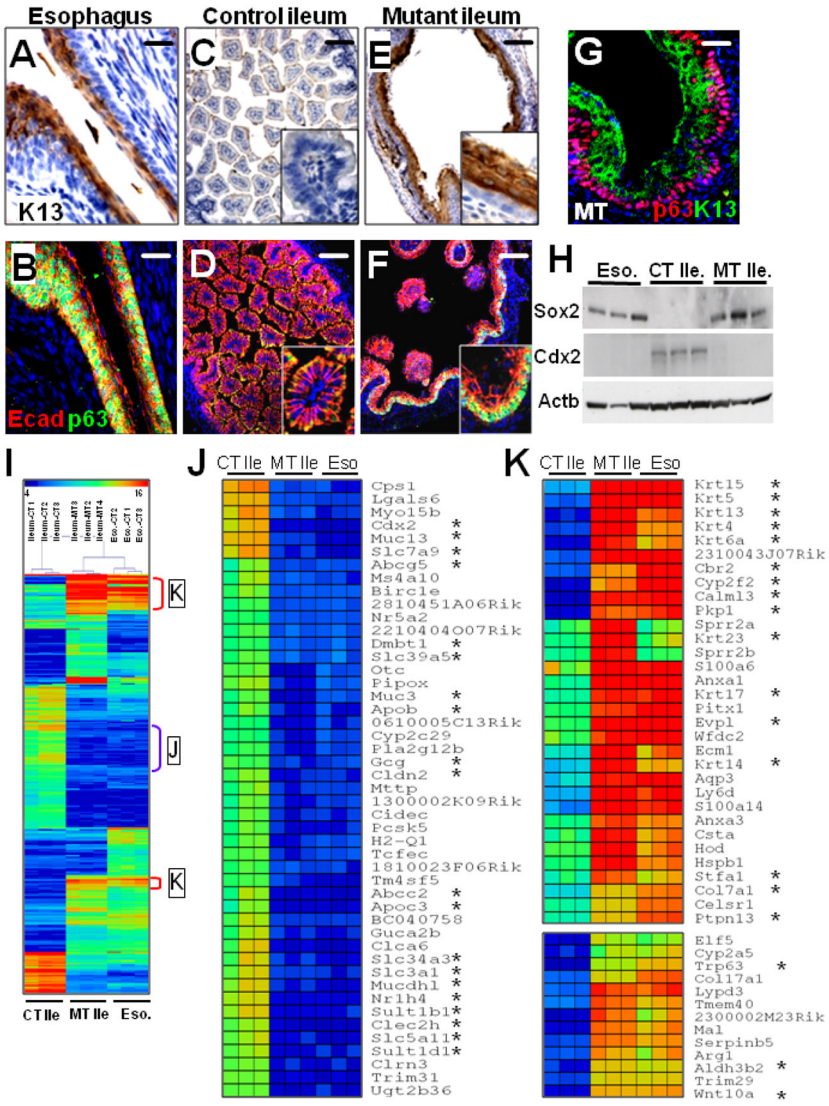

Figure 5. The *Cdx2*-deficient posterior intestine activates esophageal genes

(A–B) The E18.5 control esophageal epithelium expresses both keratin 13 and p63 (green). Sections were counterstained by E-cadherin (red) and DAPI (blue) in B.

(C–F) The E18.5 control ileal epithelium lacks keratin 13 and p63 staining, while the Cdx2 mutant ileal epithelium expresses both markers.

(G) Confocal immunostaining for keratin 13 (green) and p63 on Cdx2 mutant ileum.

(H) Western blot for Sox2, a foregut endoderm transcription factor.

(I) Heat maps, generated for genes with at least 20-fold change in mutant ileum compared to control ileum. Scales of the heat map are log based. The blue and red brackets indicate significantly down- and up-regulated genes listed in J and K.

(J) A partial list of intestinal genes (asterisks) that were extinguished in the mutant ileum.

(K) A partial list of esophageal genes (asterisks) that are significantly activated in the mutant ileum. Scale bars: 50 μm in A–B, G; 75 μm in C–F.

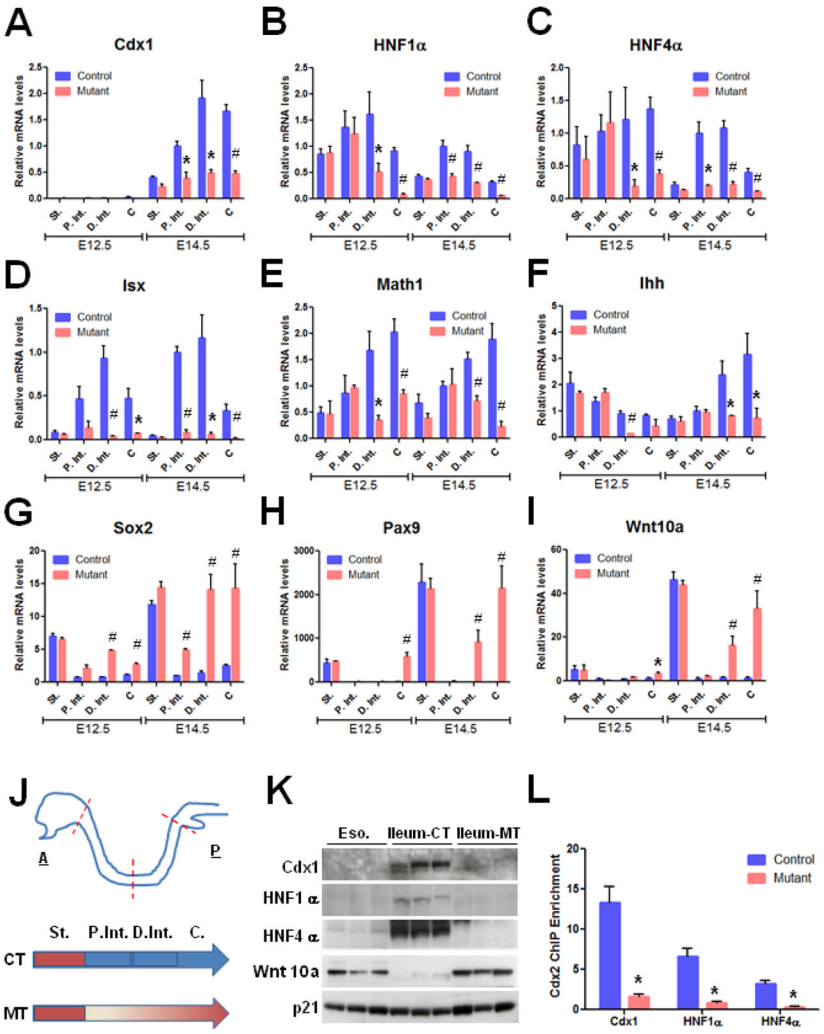

Figure 6. ***Cdx2*-deficiency replaces pro-intestinal factors with foregut regulators**

(A–I) Quantitative RT-PCR analysis was performed on E12.5 and E14.5 control and mutant stomach (St.), proximal small intestine (P. Int.), distal small intestine (D. Int.) and cecum (C), using gene specific primers. *, $p<0.05$; #, $p<0.01$. Error bar shows S.E.M.

(J) The top diagram illustrates tissue segments of the E14.5 gut, from anterior (A) to posterior (P) end, used in the qRT-PCR analysis. Diagram at the bottom summarizes the anteriorization event that occurred in the mutant intestinal domain. The foregut differentiation program is shown in red while intestinal differentiation is depicted in blue.

(K) Western blots for Cdx1, HNF1α , HNF4α Wnt10a.

(L) ChIP assay for Cdx2 occupancy of the *HNF1α* , *HNF4α* and *Cdx1* promoters. No enrichment is detected in ChIP samples derived from *Cdx2*-mutant intestines. *, $p<0.05$.

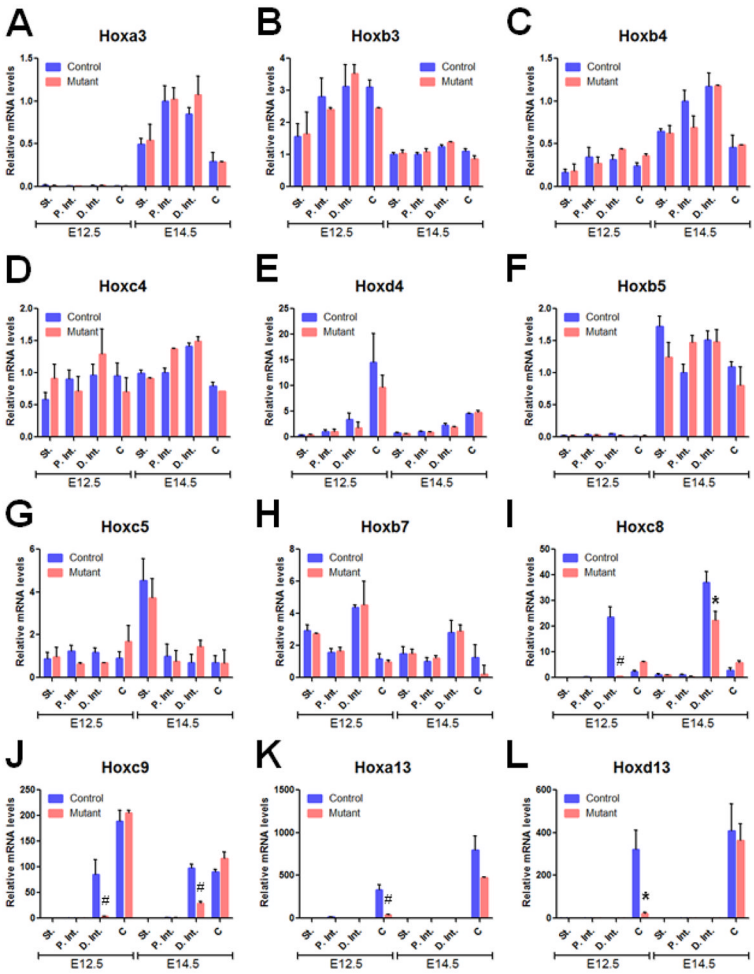

Figure 7. *Cdx2*-deficiency affects selected posterior enteric *Hox* genes

(A–L) Quantitative RT-PCR analysis of E12.5 and E14.5 control and mutant stomach (St.), proximal small intestine (P. Int.), distal small intestine (D. Int.) and cecum (C), using gene specific primers. *, $p<0.05$; #, $p<0.01$. Error bar shows the S.E.M.

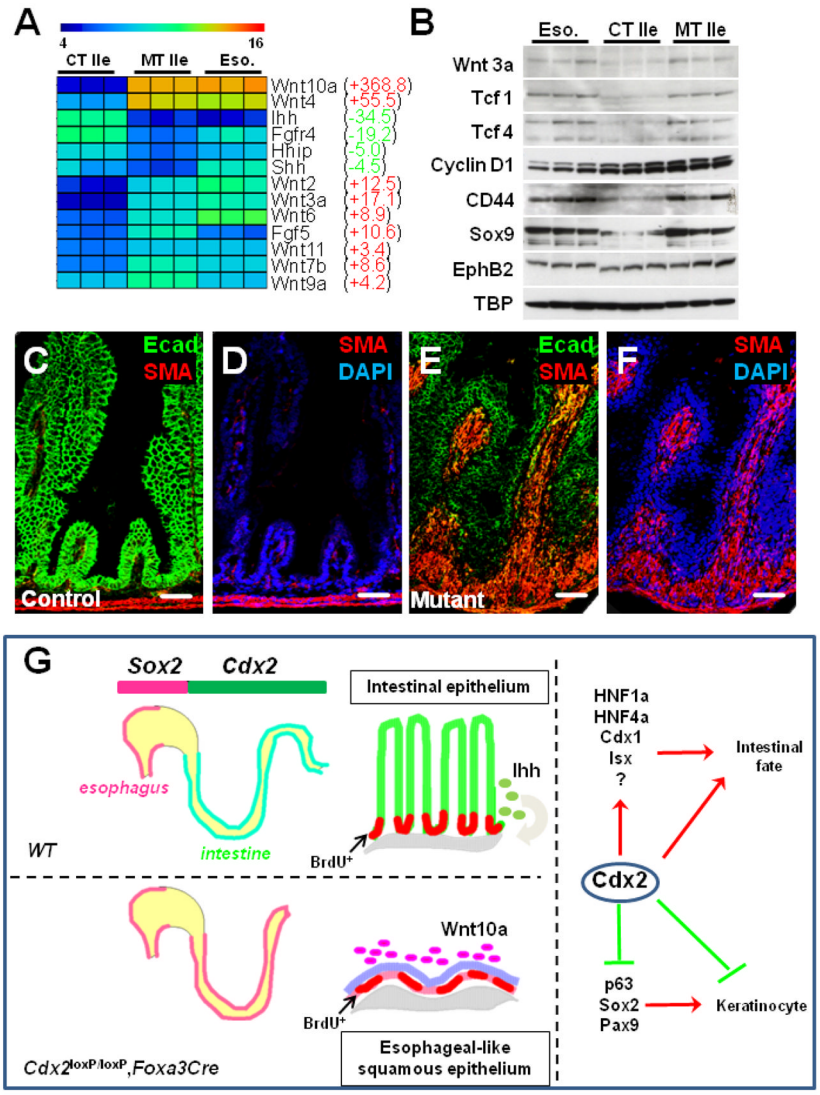

Figure 8. *Cdx2*-deficiency affects epithelial-mesenchymal signaling

(A) Heap map shows alterations in the expression of *Wnt* and *Hedgehog* genes in the Cdx2 mutant gut. Positive and negative fold changes are shown in red and green numbers, respectively. n=3 for each tissue type.

(B) Western blots demonstrate upregulation of several Wnt targets in the Cdx2 mutant intestine.

(C–F) Confocal immunostaining for smooth muscle actin (SMA). Sections were counterstained for E-cadherin (green) and DAPI (blue).

(G) Expression of *Cdx2* in midgut and hindgut endoderm directs these domains towards the intestinal cell fate. Cdx2 promotes intestinal identity via a feed-forward mechanism involving activation of pro-intestinal transcription factors, while a foregut/esophageal cell differentiation program is repressed by *Cdx2* in the posterior gut (right panel). Removing *Cdx2* from posterior endoderm (bottom left) leads to replacement of intestinal epithelial identity by ectopic foregut epithelial differentiation. Cdx2 is critical for the expression of signaling molecules, the epithelial-mesenchymal interaction, and the intestinal proliferation pattern.