Cdx1 and Cdx2 Exhibit Transcriptional Specificity in the Intestine

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Abstract

The caudal-related homeodomain transcription factors Cdx1 and Cdx2 are expressed in the developing endoderm with expression persisting into adulthood. Cdx1<sup>−/−</sup> mutants are viable and fertile and display no overt intestinal phenotype. Cdx2 null mutants are peri-implantation lethal; however, conditional mutation approaches have revealed that Cdx2 is required for patterning the intestinal epithelium and specification of the colon. Cdx2 is also necessary for homeostasis of the intestinal tract in the adult, where Cdx1 and Cdx2 appear to functionally overlap in the distal colon, but not during intestinal development. Cdx1 and Cdx2 exhibit complete overlap of expression in the intestine, although they differ in their relative levels, with Cdx1 maximal in the distal colon and Cdx2 peaking in the proximal cecum. Moreover, Cdx1 protein is graded along the crypt-villus axis, being abundant in the crypts and diminishing towards the villi. Cdx2 is expressed uniformly along this axis, but is differentially phosphorylated; the functional relevance of these expression domains and phosphorylation is currently unknown. Cdx1 and Cdx2 have been suggested to exhibit functional specificity in the intestinal tract. In the present study, using cell-based models, we found that relative to Cdx1, Cdx2 was significantly less potent at effecting a transcriptional response from the Cdx1 promoter, a known Cdx target gene. We subsequently assessed this relationship in vivo using a “gene swap” approach and found that Cdx2 cannot substitute for Cdx1 in this autoregulatory loop. This is in marked contrast with the ability of Cdx2 to support Cdx1 expression and function in paraxial mesoderm and vertebral patterning, thus providing novel in vivo evidence of context-dependent transcriptional specificity between these transcription factors.

Introduction

The intestinal tract is derived primarily from definitive endoderm, formed as epiblast cells ingress through the primitive streak, with some contribution from visceral endoderm [3]. The gut is subsequently patterned in an anterior to posterior order, which is reflected by the distinct functionalities of the esophagus, stomach, small and large intestines and associated accessory organs [4]. The small intestine is a highly specialized structure characterized by the finger-like villus projections and invaginating crypts which together comprise the crypt-villus axis. A pool of stem cells is housed in the base of the crypt region [5,6] which divide to produce highly proliferative transit-amplifying (TA) cells. These subsequently differentiate into enterocytes, Goblet cells, and enteroendocrine cells which migrate towards the tip of the villus and are shed 5–7 days later in the mouse. A fourth TA cell derivative, the Paneth cell, migrates to the base of the crypt and reside there with a lifespan of approximately 28 days. The colon lacks villi, which are replaced with a flattened epithelium which harbors mostly colonocytes and Goblet cells [4].

While the molecular mechanisms governing intestinal patterning are incompletely understood, the Cdx gene products are known to play an important role in this process [7,8,9,10]. Cdx1, Cdx2 and Cdx4 are homeodomain transcription factors related to caudal in Drosophila. Cdx1 and Cdx2 are expressed in the developing endoderm, where their expression persists in the intestine throughout life [1,2,11,12]. Cdx1<sup>−/−</sup> mutants are viable and fertile and exhibit vertebral homeotic transformations, but no overt intestinal phenotype [13]. Cdx2<sup>−/−</sup> mutants are peri-implantation lethal [11,14], however, conditional deletion strategies have revealed key roles for Cdx2 in diverse processes, including axial elongation and mesoderm patterning [15,16,17] and in the definitive endoderm and intestinal epithelium [7,8,9]. Although poorly conserved outside of the homeodomain, considerable evidence suggests that the Cdx proteins functionally overlap in several developmental processes including neural tube closure, axial elongation and mesodermal patterning [15,18,19,20]. This is consistent with gene substitution approaches that have shown that Cdx2 can replace Cdx1 in vertebral patterning [21]. However, the functional relatedness between Cdx1 and Cdx2 in the intestine has not been thoroughly investigated in vivo.

Cdx1 and Cdx2 are differentially expressed in the intestinal epithelium, with Cdx1 highest in the distal colon and Cdx2 maximal in the cecum. Furthermore, Cdx1 expression is graded along the crypt-villus axis, with more abundant levels in the crypts relative to the villi, while Cdx2 is expressed uniformly along this axis, but is differentially phosphorylated [1,2]. Although Cdx1 appears dispensable for development of the developing small intestine [7,13,22], Cdx1, together with Cdx2, may play a role in...
specification of the colon [9]. Furthermore, in the adult, Cdx1 functionally overlaps with Cdx2 in regulating intestinal homeostasis and colon patterning [8,23]. In contrast to these observations, a number of studies in tissue culture models suggest that Cdx1 and Cdx2 exhibit specificity in the intestine. For example, the Cdx target apical sodium-dependent bile acid transporter (ASBT) is preferentially regulated by Cdx2 [24]. Furthermore, the calcium channel MS4AI2 is responsive to Cdx2, but not Cdx1 [25], while the intestinal alkaline phosphatase gene is activated by Cdx1 and inhibited by Cdx2 [26]. Conversely, a number of intestinal genes have been reported to respond similarly to Cdx members in tissue culture models, such as Slc5a8 [27]. Functional equivalence between Cdx members is further exemplified by loss of expression of many intestine-specific genes such as Treh, Lct and Heph in Cdx2−/− and Cdx1−/−/Cdx2−/− mutants, while other genes, such as Slc7a8 and Alpi, appear to exhibit Cdx-type specific response [8].

The above observations suggest that Cdx1 and Cdx2 may be functionally distinct in certain contexts. To examine this further, we assessed regulation of the Cdx1 promoter, which is a Cdx1 target gene involved in an autoregulatory loop [28]. Using tissue culture models, we found that Cdx2 is significantly less potent compared to Cdx1 on this promoter, and that this difference can be mapped to differences in N-terminal transactivation sequences. To test this interaction in vivo, we examined mice in which Cdx2 had been substituted for Cdx1 (termed Cdx2ki2ki hereafter) [21] and lacking endogenous Cdx2. Using this model, we find that Cdx2 cannot support the Cdx1 autoregulatory loop, phenocopying loss of Cdx function and leading to intestinal failure. In contrast, prior work has shown that Cdx2 can support expression from the Cdx1 locus in paraxial mesoderm [21]. These observations provide novel evidence that Cdx members exhibit context-dependent functional specificity in regulating the Cdx1 promoter.

Results

Knockout studies have revealed roles for Cdx1 and Cdx2 in anterior-posterior patterning of the endoderm and mesoderm, and in some cases have suggested functional similarity in certain of these programs [17,18,21,22,29,30,31]; however, the specificity of Cdx members on different target genes remains unclear. An autoregulatory loop comprised of Cdx1 and LEF1 functioning through a LEF/TCF response element in the proximal Cdx1 promoter has been shown to be critical for Cdx1 expression [28]. To assess if Cdx2 could function in a comparable manner, we used transfection assays to compare the ability of Cdx1 or Cdx2 to elicit expression from the Cdx1 promoter alone or with LEF/β-catenin as previously described [29]. We found that, compared to Cdx1, Cdx2 was compromised in its ability to transactivate from the proximal Cdx1 promoter (Fig. 1A), an outcome that was not due to differences in protein levels (Fig. 1B, 1C). This finding is in contrast to regulation of a promoter derived from the Cdx target locus in paraxial mesoderm [21]. These observations provide novel evidence that Cdx members exhibit context-dependent functional specificity in regulating the Cdx1 promoter.

Cdx1 N-terminal Sequences Confer Specific Transcriptional Activity

The Cdx1 autoregulatory loop is thought to be governed by a Cdx1-LEF/TCF complex, with only the latter directly associated with DNA regulatory motifs [28,33]. To determine if Cdx1 and Cdx2 differentially interact with LEF/TCF members, we compared their association with LEF1 or TCF4 (also known as TCF72), the predominant LEF/TCF family member in the intestine [34,35]. We found that both Cdx1 and Cdx2 interacted comparably with either LEF1 or TCF4 (Fig. S1 and data not shown). These results suggested that differential affinity between Cdx and LEF/TCF proteins does not underlie the disparate transactivation competency observed between Cdx1 and Cdx2 on the Cdx1 promoter.

To begin to assess the basis for the differential transcriptional potency between Cdx1 and Cdx2, chimeric proteins in which the N-terminal sequences of each family member were exchanged were assessed for regulation of the Cdx1 promoter. This analysis revealed that Cdx1, but not Cdx2, N-terminal sequences were more potent at inducing transcription from this promoter (Fig. 2A). In addition, chimeric proteins harboring the N-terminal of Cdx1 fused to the DNA binding HMG domain of LEF1 or TCF4 were also more effective at eliciting expression from the Cdx1 promoter than the comparable Cdx2 derivatives (Fig. 3A). These differences were not due to variance in protein levels (Fig. 2B, 2C, 3B, 3C), but rather suggest that the basis for the observed functional differences between Cdx1 and Cdx2 lies in properties inherent to their N-terminal transactivation sequences.

Cdx2 Cannot Support Cdx1 Autoregulation in vivo

As described above, and by others, Cdx members exhibit different transcriptional potential on a number of target genes in tissue culture models [24,25,26,27]. It is, however, unclear if these differences hold in vivo. To investigate this, we used a previously described knock-in allele in which the Cdx2 open reading frame was inserted into the Cdx1 locus (hereafter designated Cdx12ki2ki), resulting in replacement of Cdx1 with Cdx2 [21]. We then crossed this line with the conditional Cdx2fl/fl villin Cre ER3 line, and mated Cdx2fl/2ki/Cdx2fl/2ki villin Cre ER3 females, deleting Cdx2 in the intestinal epithelium either at E13.5 or in the adult as previously described [7,23]. Cre-positive offspring (referred to as Cdx12ki2ki/Cdx2fl/−) were anticipated to lack expression of Cdx2 from the endogenous allele [7] and to express Cdx2 under the regulation of the Cdx1 promoter. Littermate controls (Cdx12ki2ki) were anticipated to express Cdx2 from both the endogenous and knock-in alleles.

As expected, neither Cdx12ki2ki/Cdx2fl/− nor Cdx12ki2ki/Cdx2fl/− mice exhibited Cdx1 expression (Fig. 4A i-iii), consistent with prior work [21]. Cdx12ki2ki/Cdx2fl/− mice displayed robust Cdx2 expression along the entire crypt-villus axis, comparable to wild type controls (Fig. 4A; panels iv and v). In marked contrast, Cdx12ki2ki/Cdx2fl/− offspring had very low levels of Cdx2, with more robust staining at the base of the crypts and tapering off towards the villus tip (Fig. 4A vi). This is similar to (but much weaker than) the normal pattern of expression of Cdx1 (Fig. 4A, panel 1) [12,36]. Consistent with these observations, western blot analysis showed markedly decreased expression of Cdx2 in Cdx12ki2ki/Cdx2fl/− animals compared to controls (Fig. 4C).

The above data suggest that Cdx2 is not efficiently expressed from the Cdx1 promoter in the small intestine. To investigate this, we examined transcripts produced from the Cdx1 promoter by qPCR. The small intestine of Cdx12ki2ki mice exhibited a strong reduction in transcripts derived from the Cdx1 promoter (Fig. 4D), suggesting a failure in transcription. This was not due to unforeseen effects mediated by the knock-in per se, as Cdx12ki2ki mice produced comparable levels of both wild-type and knock-in messages (Fig. 4E). It is also notable that loss of Cdx2 in Cdx12ki2ki/Cdx2fl/− mice had no impact on expression from the Cdx1 promoter (Fig. 4D), consistent with the finding that loss of Cdx2 did not affect Cdx1 levels (Fig. 4B). A similar relationship was seen...
in the adult colon, as assessed by both immunohistochemistry (Fig. 5A) and western blot analysis (Fig. 5B), although there appeared to be a modest increase in Cdx2 levels in Cdx12ki/2ki mice compared to wild-type controls, suggestive of compensatory mechanisms. Taken together, these findings indicate that Cdx1, but not Cdx2, is necessary for transcription from the Cdx1 promoter in the intestine.

Expression of Cdx2 from the Cdx1 Locus does not Support Intestinal Development or Homeostasis

Previously, we demonstrated that loss of Cdx2 in the small intestine at E13.5 leads to transformation of the intestinal epithelium to a partial glandular stomach identity. Cdx1 has no discernable role in this process [7,13]. Immunohistochemistry and qPCR data indicated residual Cdx2 transcripts were produced from the Cdx1 promoter in Cdx12ki/2ki/Cdx2−/− mice. We therefore assessed the small intestine of Cdx12ki/2ki/Cdx2−/− mice to determine if this residual Cdx2 protein could support intestinal patterning.

Cdx12ki/2ki mice appeared normal (data not shown) and histological analysis revealed no apparent abnormalities in these animals (Fig. 6; panels i and ii). In contrast, the duodenum of Cdx12ki/2ki Cdx2−/− mice exhibited a disordered epithelium, with shortened villi and vacuolated cells (Fig. 6; panel iii), reminiscent of the outcome of Cdx2 loss at E13.5 [7] (Fig. 6, panel iv). As previously described [7], excision mediated by the villin Cre ERα transgene during development of the large intestine was not sufficient to warrant further study [7].

Periodic Acid-Schiff (PAS) stains mucins in the apical edge of glandular stomach, as well as in Goblet cells of the intestine, while Alcian Blue stains mucins only in Goblet cells of the intestinal

Figure 1. The Cdx1 promoter is differentially regulated. (A) Luciferase reporter assay in P19 embryonal carcinoma cells with reporters derived from Cdx1 orDll1 promoters or a synthetic Cdx response element (CaudalRE), as noted. Red diamond represent a Cdx response element (CDRE) and black diamond represents a LEF/TCF response element (LRE). Fold induction is shown relative to reporter vector in response to Cdx1 or Cdx2 alone or in combination with LEF1 and β-catenin. Western blot (B) and quantification (C) of Cdx1 and Cdx2 protein using β-galactosidase as a loading control. *P<0.05 by student’s t-test.
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epithelium and not the stomach [37]. Cdx2<sup>−/−</sup> mice exhibit ectopic PAS staining along the apical edge of the aberrant villi, indicative of a transformation to pyloric stomach (Fig. 6 panel xii), as well as supernumerary intestinal goblet cells due to aberrant Notch signaling [7,22]. In contrast, neither Cdx1<sup>−/−</sup> nor Cdx2<sup>−/−</sup> mice exhibited ectopic PAS or Alcian Blue staining, although Cdx1<sup>−/−</sup>Cdx2<sup>−/−</sup> mice appeared to have more Goblet cells (Fig. 6 panels vi, vii, x, xi and data not shown). These results suggest that expression of Cdx2 under Cdx1 regulatory elements mice partially supports Cdx2-dependent function in the small intestine.

In wild type mice, Cdx1 protein levels are maximal in the distal colon, while Cdx2 peaks in the proximal cecum and diminishes in either direction [2,12,36,38,39]. Cdx members are critical for differentiation and homeostasis of the entire adult intestinal tract, including the large intestine [8,23,40]. In this regard, combined loss of Cdx1 and Cdx2 results in anteriorization of the distal colon to a cecal character [23], revealing a role for Cdx1 in the colon that is seen only with concomitant loss of Cdx2. To test if Cdx2

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**Figure 2. Differential regulation by N-terminal Cdx transcriptional activation sequences.** (A) Regulation of a Cdx1 promoter reporter vector in P19 embryonal carcinoma cells. Fold induction is shown relative to reporter vector alone in response to fusion proteins harboring the Cdx1 N terminal and Cdx2 homeodomain (Cdx1 N terminal) or the converse construct (Cdx2 N terminal). Transfections were conducted in combination with either LEF1 and β-catenin or TCF4 and β-catenin. Western blot (B) and quantification (C) of Cdx1 N-terminal and Cdx2 N-terminal proteins using β-galactosidase as a loading control. *P<0.05 by student’s t-test.

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could drive sufficient expression from the Cdx1 locus to fulfill Cdx function in the distal intestine, we examined the colon in Cdx1<sup>2ki/2ki</sup>Cdx2<sup>2-/-</sup> offspring. While Cdx1<sup>2ki/2ki</sup> colons appeared normal, Cdx1<sup>2ki/2ki</sup>Cdx2<sup>2-/-</sup> colons exhibited scalloped glands in the place of the typical flattened colonic epithelium, similar to those seen in Cdx1<sup>-/-</sup>Cdx2<sup>-/-</sup> offspring [23] (Fig. 7A), and suggestive of a conversion to a cecum-like phenotype. This is consistent with gain of expression of the cecal-enriched genes Defensin5 and TFF1 [41] (Fig. 7B). These data suggest a marked loss of Cdx function in the distal intestine [23], consistent with an inability of Cdx2 to drive expression from the Cdx1 locus in the intestinal tract in vivo.

**Discussion**

Recent studies have revealed critical functions for Cdx2 in diverse processes, including patterning the endoderm [7,8,9,23], intestinal differentiation [9,22,40] and axial elongation [17,18]. Despite divergence outside of their homeodomains, Cdx members exhibit significant functional overlap, as exemplified by the ability of Cdx2 to complement Cdx1 mutants in vertebral patterning [21]. However, the functional relatedness of Cdx members is less clear as regards the intestinal tract. To this end, we examined regulation of Cdx1, a known direct Cdx target gene expressed in the intestine [12,28]. We found that Cdx2 was unable to drive expression from the Cdx1 promoter in vitro, or in vivo. Furthermore, we found that Cdx2 could not be expressed from the Cdx1 locus at levels that suffice to support Cdx-dependent roles in the gastrointestinal tract. This represents the first in vivo demonstration of functional specificity between these family members.

**Specific Regulation of the Cdx1 Promoter**

We showed that Cdx2 is less efficient at transactivation from the Cdx1 promoter relative to Cdx1 using tissue culture based models. This finding was recapitulated in Cdx1<sup>2ki/2ki</sup>Cdx2<sup>-/-</sup> mice, which...
Functional Specificity of Cdx1 and Cdx2

A

i. WT

ii. Cdx1^{2ki/2ki}

iii. Cdx1^{2ki/2ki} Cdx2^{−/−}

iv. WT

v. Cdx1^{2ki/2ki}

vi. Cdx1^{2ki/2ki} Cdx2^{−/−}

αCdx1

αCdx2

B

WT

Cdx2^{−/−}

α-Cdx1

α-Actin

C

WT

Cdx1^{−/−}

Cdx2^{−/−}

Cdx1^{2ki/2ki} Cdx2^{−/−}

Cdx1^{2ki/2ki} Cdx2^{−/−}

α-Cdx2

α-Actin

D

Relative mRNA from Cdx1 locus

WT

Cdx1^{+/−}

Cdx1^{−/−} Cdx2^{−/−}

* *

E

Relative mRNA from Cdx1 locus

WT

knock-in

F

WT locus

Cdx1 E1

Knock in targeted locus

Cdx2 cDNA

Cdx1 E1

* *
have greatly diminished levels of Cdx2 and phenocopy intestinal Cdx loss of function models [22,23,31,40]. The loss of Cdx2 arises due to a failure of transcription at the Cdx1 locus, evident from transcriptional readout at the Cdx1 locus by qPCR. These findings illustrate that Cdx2 is less potent than Cdx1 in regulating the Cdx1 promoter throughout the extent of the intestinal tract and during development and in the adult.

N-terminal Sequences Convey Differential Cdx Function

Cdx1 and Cdx2 have divergent N terminal sequences which harbor poorly defined transactivation functions [2,42,43]. Consistent with previous work [28,33], we found that Cdx1 could direct transcription from its own promoter in concert with LEF/TCF in tissue culture models, but that this effect was not efficiently recapitulated by Cdx2. Although this auto-regulation relies on association with a LEF/TCF member bound to the proximal Cdx1 promoter [28], differential association between LEF/TCF and Cdx1 or Cdx2 did not appear to underline the specificity of Cdx1 regulation. Rather, this specificity appears to reside in the divergent N-terminal sequences, suggesting that the transactivation domain of Cdx1 differentially interacts with a transcriptional co-regulator(s) needed for transcription from the Cdx1 locus. Because Cdx members interact physically with LEF/TCF members through conserved homeodomain sequences, it is also unlikely that differential expression of LEF/TCF members underlies this effect. In this regard, recent work has suggested that Cdx2 is capable of association with a number of intestinal transcription factors in a manner that reflects the differentiation state of the cell [40], and it is possible that such a co-regulator may underlie Cdx-specific transcriptional regulation.

Figure 4. Cdx2 does not support expression from the Cdx1 promoter in the small intestine. (A) Immunohistochemistry for Cdx1 (i-iii) or Cdx2 (iv-vi) in the small intestine of E18.5 WT (i, iv), Cdx1^{+/2ki} (ii, v) or Cdx1^{2ki/2ki}Cdx2^{+/2ki} (iii, vi) animals. Western blot analysis for Cdx1 (B) or Cdx2 (C) and actin loading controls from small intestine. (D) qPCR analysis for transcripts from the Cdx1 locus in knock-in animals (red arrows in F) relative to WT (blue arrows in F). (E) qPCR analysis for WT (blue arrows in F) and knock-in (red arrows in F) transcripts from the Cdx1 locus heterozygous animals. *P<0.05 by student’s t-test. (F) Schematic representation of the wild type and targeted Cdx1 allele with primer sets for measuring WT (blue) and knock in (red) transcripts.

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Figure 5. Cdx2 cannot drive expression from the Cdx1 promoter in the adult colon. (A) Cdx2 immunohistochemistry and (B) Western blot analysis of Cdx2 in proximal and distal colon from WT (i), Cdx1^{+/-2ki} (ii) and Cdx1^{2ki/2ki}Cdx2^{+/2ki} (iii) mice.

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The paradigm of Cdx specificity may extend to other target genes. For example, the Cdx target *Dll1* is regulated by Cdx1 and Cdx2 in the presomitic mesoderm, but is not impacted by Cdx1 loss in the intestine [22]. Cdx1 and Cdx2 also exhibited different levels of transcriptional potency on the *Dll1* promoter in P19 embryocarcinoma cells in the current study. However, *Dll1* has also been shown to be a Wnt target [44,45] and similar levels of induction were seen with Cdx1 and Cdx2 in conjunction with LEF/β-catenin (Fig. 1A). This is again consistent with modulation of Cdx activity by collaborative partners [9,17].

![Figure 6. The Cdx2 knock in allele cannot complement loss of endogenous Cdx2.](image-url)

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Functional Specificity of Cdx Members

Substantial data suggests functional overlap between Cdx members in diverse ontogenic programs. For example, the phenotype of single versus compound Cdx loss suggests overlap as regards vertebral patterning, axial elongation and neural tube closure [13,14,15,17,19]. This is also consistent with gene substitution approaches which have shown that Cdx2 can fully complement Cdx1 loss in vertebral patterning [21]. Indeed, this latter observation used the same *Cdx1^2ki/2ki* line employed in the current study, clearly illustrating that Cdx2 can fulfill Cdx1 autoregulation in the mesoderm, but not in the intestine, and thus impacts on this target gene in a context specific manner.
Figure 7. The Cdx2 knock in allele cannot support colon homeostasis. (A) Hematoxylin and eosin staining of control adult cecum (i), Cdx1^{2ki/2ki} colon (iv) and proximal and distal colons from Cdx1^{2ki/2ki}Cdx2^{-/-} (ii, iii) and Cdx1^{-/-}Cdx2^{-/-} (v, vi) mice. Note the semblance of the proximal and distal colon in Cdx1^{2ki/2ki} and Cdx1^{-/-}Cdx2^{-/-} offspring to wild-type cecum. (B) qPCR analysis for the cecum-enriched transcripts TFF1 and Defensin5. *P<0.05 by student’s t-test compared to wild-type controls.

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In contrast to the above observations, work from tissue culture models presents conflicting evidence regarding specificity of Cdx1 and Cdx2 in regulation of intestinal genes [24,25,26,27,46,47]. Additional data from mouse models [7,9,10,23,40] show that loss of Cdx1 does not appear to play any role in patterning [7,13,40] or differentiation [22] of the small intestine, even in the absence of Cdx2. In contrast, functional overlap between Cdx1 and Cdx2 has been suggested in the adult GI tract, where deletion of Cdx2 results in an exacerbation of intestinal failure associated with Cdx2 loss and the appearance of a novel colon phenotype [23,40]. Finally, gene profiling of Cdx2−/− and Cdx1−/−Cdx2−/− mutants from villin-Cre ER1 conditional mice suggest that there are both common and specific intestinal Cdx target genes [8].

Transgenic models also suggest differential function between Cdx1 and Cdx2. For example, overexpression of Cdx2 in the gut epithelium is lethal [49] while overexpression of Cdx1 does not cause any overt phenotype [48,50]. However, these models resulted in abnormal Cdx expression, which may result in non-physiological impact. Similarly, misexpression of Cdx1 or Cdx2 in the stomach, which is normally devoid of Cdx, evokes slightly different phenotypes [51]. However, it is unknown if these outcomes reflect specific function or are due to differences in expression levels. Further characterization of Cdx1 and Cdx2 target genes and binding partners will be needed to better understand their molecular mechanisms of action.

**Experimental Procedures**

**Mice**

Cdx1−/−, Cdx2−/−, Cdx12−/−, and villin-Cre ER1 mice have been previously described [13,17,21,52,53]. Villin-Cre ER1-mediated deletion of Cdx2 was initiated by Tamoxifen administration at embryonic (E)13.5 or at 6 weeks of age as previously described [7,23]. At appropriate times, animals were sacrificed by cervical dislocation and gastrointestinal tracts were harvested at E18.5 or 6 days post-treatment in adults. Tamoxifen-treated non-transgenic littermates were used as controls in all instances. In all cases, experiments were repeated with a minimum of 3 different animals. The work described in this study was approved by the Animal Care and Veterinary Service of the University of Ottawa in accordance with the guidelines of the Canadian Council for Animal Care.

**Histology and Western Blot Analysis**

E18.5 intestinal tracts were processed for histological and immunohistochemical staining as previously described [7]. Slides were mounted using Permunt (Fisher) and images captured using a Zeiss Mirax Midi Scanner (Zeiss). Protein was harvested using lysis buffer (8 M urea, 4% CHAPS, 2 mM DTT, containing 1% Triton X-100, 1 mM DTT and protease inhibitors (1 μg/mL aprotinin, 1 μg/mL leupeptin, 1 μg/mL pepstatin A, 1 mM PMSF; Sigma) and lysed by sonication using a Branson Sonifier 450. Cell debris was cleared by centrifugation at 10,000 g.

**Plasmid Constructs**

The glutathione S-transferase (GST)-Cdx1 and GST-Cdx2 fusion proteins have been described previously [28]. GST-LEF1 and GST-TCF712 constructs were derived by subcloning appropriate open reading frames into pGEX4T-1. A FLAG-tagged TCF712 expression vector was generated using plasmid number 11031 [pG034 mTCF-4B from Addgene [55]. The LEF1-HA, Cdx1 and Cdx2 expression vectors and Cdx1-luciferase reporter vectors have been previously described [28].

**GST Fusion Proteins**

BL-21 bacteria were transformed with either empty GST expression plasmid, GST-Cdx1, GST-Cdx2, GST-LEF1 or GST-TCF712 fusion constructs. Cultures were grown to an OD500 of 0.5, treated with 0.5 mM IPTG (Bioshop), and cultured for a further 3 hours. Cells were then pelleted, resuspended in PBS containing 1% Triton X-100, 1 mM DTT and protease inhibitors (1 μg/mL aprotinin, 1 μg/mL leupeptin, 1 μg/mL pepstatin A, 1 mM PMSF; Sigma) and lysed by sonication using a Branson Sonifier 450. Cell debris was cleared by centrifugation at 10,000 g. Protein was harvested using the Bradford method [54]. Western blots were performed as previously described [7].

**Protein-protein Interaction Assays**

**Supporting Information**

Figure S1 Cdx1 and Cdx2 are comparable in binding TCF712 and LEF1 in vitro. Cdx1 and Cdx2 (A) or TCF712 and LEF1 (B) were transcribed and translated in vitro using the chlorophenolred-β-D-galactopyranoside (CPRG) assay system (Calbiochem) and used to correct for translation efficiency.
the cells Cre-ER^T2 line; P. Gruss and B. Meyer for the Cdx1^-/- mouse line and M. Mansfield and B. Hes for mouse husbandry.

Author Contributions
Conceived and designed the experiments: SG DL. Performed the experiments: SG AH. Analyzed the data: SG AH DL. Contributed reagents/materials/analysis tools: SG AH. Wrote the paper: SG DL.

References
1. Beck F, Erler T, Russell A, James R (1995) Expression of Cdx-2 in the mouse embryo and placenta: possible role in patterning of the extra-embryonic membranes. Dev Dyn 204: 219–227.
2. Guo RJ, Suh ER, Lynch JP (2004) The role of Cdx proteins in intestinal development and cancer. Cancer Biol Ther 3: 593–601.
3. Kwon GS, Voit M, Hadjantonakis AK (2008) The endoderm of the mouse embryo arises by dynamic widespread intercalation of embryonic and extraembryonic lineages. Dev Cell 15: 509–520.
4. Wells JM, Melton DA (1999) Vertebrate endoderm development. Annu Rev Cell Dev Biol 15: 393–410.
5. Munoz J, Stange DE, Schepers AG, van de Wetering M, Koo BK, et al. (2012) Cdx4 regulates mesenchymal differentiation and patterning of the foregut. Proc Natl Acad Sci U S A 109: 10053–10058.
6. Grainger S, Savory JG, Lohnes D (2010) Cdx2 regulates patterning of the intestinal epithelium. Dev Biol 339: 155–165.
7. Verzi MP, Shin H, Ho LL, Liu XS, Shivdasani RA (2011) Essential and redundant function of Caudal family proteins in activating adult intestinal progenitors. Mol Cell Biol 31: 6461–6473.
8. van Nes J, de Graaff W, Lebrin F, Gerhard M, Beck F, et al. (2006) The Cdx4 mutation affects axial development and reveals an essential role of Cdx genes in mouse development. EMBO J 25: 1674–1684.
9. Subramanian V, Meyer BI, Gruss P (1995) Disruption of the murine homeobox gene Cdx1 affects axial skeletal identities by altering the mesodermal expression domains of Hox genes. Cell 83: 641–653.
10. Verzi MP, Shin H, Ho LL, Liu XS, Shivdasani RA (2011) Essential and redundant function of Caudal family proteins in activating adult intestinal progenitors. Mol Cell Biol 31: 6461–6473.
11. Cdx2 regulates mesenchymal differentiation and patterning of the foregut. Proc Natl Acad Sci U S A 109: 10053–10058.
12. Grainger S, Savory JG, Lohnes D (2010) Cdx2 regulates patterning of the intestinal epithelium. Dev Biol 339: 155–165.
13. Savory JG, Bouchard N, Pierre V, Rijli FM, De Repentigny Y, et al. (2009) Cdx2 regulation of posterior development through non-Hox targets. Development 136: 4996–4110.
14. van den Akker E, Forlani S, Chawengsaksophak K, de Graaff W, Beck F, et al. (2002) Cdx1 and Cdx2 have overlapping functions in anteriorposterior patterning and posterior axis elongation. Development 129: 2181–2193.
15. van Nes J, de Graaff W, Lebrin F, Gerhard M, Beck F, et al. (2006) The Cdx4 mutation affects axial development and reveals an essential role of Cdx genes in the ontogenesis of the placental labyrinth in mice. Development 133: 419–428.
16. Gaunt SJ, Drage D, Troshak RW (2008) Increased Cdx protein dose effects intestinal axial patterning in transgenic lines of mice. Development 135: 2511–2520.
17. Grainger S, Lohnes D (2011) Cdx mediates neural tube closure through transcriptional regulation of the planar cell polarity gene Ptk7. Development 138: 1361–1370.
18. Young T, Rowland JE, van de Ven C, Bialecka M, Novoa A, et al. (2009) Cdx2 regulates axial development and reveals an essential role of Cdx genes in mouse development. Development 136: 4099–4110.
19. Galceran J, Sustmann C, Hsu SC, Folberth S, Grosschedl R (2004) LEF1-activated regulatory sequences mediate the transactivation capacity of the intestine-specific homeobox gene Cdx2. Dev Cell 19: 713–726.
20. Hofmann M, Schuster-Gossler K, Watabe-Rudolph M, Herrmann A, Herrmann AM, et al. (2002) Cdx2 regulation of posterior development through non-Hox targets. Development 129: 3805–3813.
21. Crissey MA, Guo RJ, Funakoshi S, Kongsong J, Liu J, et al. (2012) Cdx2 levels modulate intestinal epithelial-muscle intercalation and Paneth cell development. Gastroenterology 140: 517–528 e518.
22. Choi AM, Yao X, Gruss P, Jost JG (1995) Cdx-2 is essential for axial elongation in mouse development. Proc Natl Acad Sci U S A 96: 7318–7323.
23. Hryniuk A, Grainger S, Savory JG, Lohnes D, Rijli FM, et al. (2012) Cdx regulates mesenchymal differentiation and patterning of the foregut. Proc Natl Acad Sci U S A 109: 10053–10058.
24. Galceran J, Sustmann C, Hsu SC, Folberth S, Grosschedl R (2004) LEF1-activated regulatory sequences mediate the transactivation capacity of the intestine-specific homeobox gene Cdx2. Dev Cell 19: 713–726.
25. Grossmann J, Walther K, Artinger M, Rummele P, Woenckhaus M, et al. (2002) Cdx2 is essential for axial elongation in mouse development. Development 129: 2181–2193.
26. Grainger S, Savory JG, Lohnes D (2011) Cdx mediates neural tube closure through transcriptional regulation of the planar cell polarity gene Ptk7. Development 138: 1361–1370.
27. Alkhoury F, Malo MS, Mozumder M, Mostafa G, Hodin RA (2005) Differential regulation of intestinal alkaline phosphatase gene expression by Cdx1 and Cdx2. Am J Physiol Gastrointest Liver Physiol 289: G263–290.
50. Crissey MA, Guo RJ, Fort F, Li H, Katz JP, et al. (2008) The homeodomain transcription factor Cdx1 does not behave as an oncogene in normal mouse intestine. Neoplasia 10: 8–19.

51. Mutoh H, Sakurai S, Satoh K, Osawa H, Hakamata Y, et al. (2004) Cdx1 induced intestinal metaplasia in the transgenic mouse stomach: comparative study with Cdx2 transgenic mice. Gut 53: 1416–1423.

52. el Marjou F, Janssen KP, Chang BH, Li M, Hindie Y, et al. (2004) Tissue-specific and inducible Cre-mediated recombination in the gut epithelium. Genesis 39: 189–193.

53. Santagati F, Minoux M, Ren SY, Rijli FM (2005) Temporal requirement of Hoxa2 in cranial neural crest skeletal morphogenesis. Development 132: 4927–4936.

54. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254.

55. Lee YJ, Swencki B, Shoichet S, Shivdasani RA (1999) A possible role for the high mobility group box transcription factor Tcf-4 in vertebrate gut epithelial cell differentiation. J Biol Chem 274: 1566–1572.

56. Schef H, Lehmann KE, Buschmann IR, Unger T, Funke-Kaiser H (2006) Quantitative real-time RT-PCR data analysis: current concepts and the novel “gene expression’s CT difference” formula. J Mol Med (Berl) 84: 901–910.