The Role of Cdx in Intestinal Development

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Laser-assisted blastocyst dissection and subsequent cultivation of embryonic stem cells in a serum/cell free culture system: applications and preliminary results in a murine model

Noriko Tanaka[1], **Takumi Takeuchi**[1], **Queenie V Neri**[1], **Eric S Sills**[2] and **Gianpiero D Palermo**[1*]

Journal of Translational Medicine 2006, **4**:20 doi:10.1186/1479-5876-4-20

Self-Renewal and Cancer of the Gut: Two Sides of a Coin

Freddy Radtke and Hans Clevers

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ABSTRACT

The products of the Cdx genes, *Cdx1*, *Cdx2* and *Cdx4*, are known to play essential roles in many developmental processes including neural tube closure, axial elongation and patterning the anterior-posterior axis of the developing embryo. Cdx1 and Cdx2 are both expressed in the endoderm of the embryo and persist throughout adulthood in the intestinal epithelium, but their functions and mechanisms of action in this lineage are poorly understood, in part due to the peri-implantation lethality of *Cdx2*$^{-/-}$ mice. To circumvent this limitation, a conditional loss of function strategy was used to inactivate Cdx2 in the intestinal epithelium. These conditional mutants were also crossed to *Cdx1*$^{-/-}$ mice, which are viable and fertile, to examine potential functional compensation between these family members. The major findings of this study are that Cdx2 regulates patterning and differentiation of the small intestinal epithelium, while Cdx1 does not appear to make a contribution to either process. Furthermore, Cdx operates upstream of Notch ligand *Delta-like 1* (*Dll1*) in endoderm and mesoderm derivatives, demonstrating that Cdx function is similar in different lineages. Finally, Cdx2 cannot fulfill the requirement for Cdx1 in regulation of its own promoter in the intestine. This is the first *in vivo* evidence that these two family members have context-dependent functional specificity. Altogether, this study underscores critical roles and mechanisms of action for Cdx members in the developing intestine and mesoderm.

RÉSUMÉ

Les produits des gènes *Cdx, Cdx1, Cdx2* et *Cdx4,* sont connues pour jouer un rôle essentiel dans de nombreux processus de développement, notamment la fermeture du tube neural, l'allongement axial et la structuration de l'axe antéro-postérieur de l'embryon en développement. *Cdx1* et *Cdx2,* mais pas *Cdx4,* sont exprimés dans l'endoderme de l'embryon, continuent à l'âge adulte dans l'épithélium intestinal. Cependant, leurs fonctions et mécanismes d'action sont mal connus, en partie à cause de la létalité péri-implantatoire des souris *Cdx2*$^{-/-}$. Pour contourner cette limitation, une perte conditionnelle de la stratégie de la fonction a été utilisée pour inactiver conditionnellement *Cdx2* dans l'épithélium intestinal. On a combiné ces mutants conditionnels avec les souris *Cdx1*$^{-/-}$, qui sont viables et fertiles, afin d'examiner le potentiel de compensation fonctionnelle entre ces membres de famille. Les conclusions principales de cette étude sont que Cdx2 règle la structuration et la différentiation de l'épithélium intestinal, tandis que Cdx1 ne semble pas contribuer à ces processus. En plus, Cdx règle aussi l'expression endodermal et le mésodermal du ligand de Notch, *Delta-like 1* (*Dll1*), démontrant que la fonction de Cdx peut être similaire dans des lignages différentes. Finalement, dans l'intestin, Cdx2 ne peut pas satisfaire à l'exigence de Cdx1 dans la régulation de son propre promoteur. C'est la première preuve *in vivo* que ces deux membres de famille spécificité fonctionnelle dans certains contextes. Au total, cette étude met en évidence les rôles essentiels et les mécanismes de fonction des membres Cdx dans l'intestin et le développement du mésoderme.

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LIST OF ABBREVIATIONS

Abbreviation	Meaning
AB	Alcian Blue
ADAM	a disintegrin and metalloprotease
ADH	alcohol dehydrogenase
ANT-C	antennapedia complex
AP	anterior-posterior
APC	adenomatous polyposis coli
ATP	adenosine triphosphate
BarX	Bar class of homeobox
bHLH	basic helix-loop-helix
Bmi	B lymphoma Mo-MLV insertion region 1 homolog
BMP	Bone Morphogenic Protein
BrdU	bromodeoxyuridine
Brg	Brahma related gene
BX-C	bithorax complex
Caco	cancer of the colon
cad	caudal
CBC	crypt base columnar
CBP	Creb binding protein
CDRE	Cdx response element
Cdx	caudal related homeobox
CHD	chromodomain helicase DNA binding
ChIP	chromatin immunoprecipitation
ChIP-seq	ChIP-sequencing
CK	casein kinase
CSL	CBF-1-Su(H)-Lag-1
CtBP	C terminal binding protein
Cyp	cytochrome P450
DCLK1	doublecortin-like kinase
DEPC	diethyl-pyrocarbonate
Dhh	Desert Hedgehog
DIG	digoxigenin
Dll	Delta-like
DNA	deoxyribonucleic acid
DSL	Delta-Serrate-Lag-2
DTT	dithiothreitol
Dusp	dual specificity phosphatase
E	embryonic day

ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol tetraacetic acid
EMSA	electrophoretic mobility shift assay
en	engrailed
epCAM	epithelial cell adhesion marker
ER	estrogen receptor
ES	embryonic stem
Ets	E twenty-six
FACS	fluorescence activated cell sorting
FAP	familial adenomatous polyposis
FGF	fibroblast growth factor
flox	flanked by loxP
Fox	Forkhead box
ftz	fushi tarazu
Fz	frizzled
Gfi	growth factor independant
Ghrl	ghrelin
GI	gastrointestinal
Gli	gliobastoma
GSK	glycogen synthase kinase
H	histone
HAT	histone acetyl transferase
HD	homeodomain
HDAC	histone deacetylase
Hes	Hairy/enhanced/split
Hex	hematopoietically expressed homeobox
HOM-C	homeotic complex
Hox	homeobox
HRP	horseradish peroxidase
IAP	intestinal alkaline phosphatase
IEC	intestinal epithelial cell
IFABP	intestinal fatty acid binding protein
IgG	immunoglobulin Type G
Ihh	Indian Hedgehog
int	integration
ISWI	imitation SWI
Kdm	lysine-specific demethylase
Klf	kruppel like factor
L	lumbar
LacZ	Gene encoding β-galactosidase

LEF	lymphoid enhancer factor
Lfng	lunatic fringe
Lgr	Leucine-rich repeat-containing G-protein coupled receptor
LRE	LEF/TCF response element
LRP	LDL receptor-related protein
M	microfold
Mam	mastermind
Math	mouse atonal homologue
Min	multiple intestinal neoplasia
MINT	MSx2-interacting nuclear target protein
MMTV	mouse mammary tumour virus
mRNA	messenger RNA
Muc	mucin
NCoR	nuclear receptor co-repressor
NICD	Notch intracellular domain
NuRD	nucleosome remodeling
Oct	octamer-binding transcription factor
P	postnatal day
PAGE	polyacrylamide gel electrophoresis
PAS	periodic acid-Schiff
PBS	phosphate buffered saline
PcG	polycomb group
PCR	polymerase chain reaction
Pdx	pancreatic and duodenal homeobox
PFA	paraformaldehyde
PIC	pre-initiation complex
Pofut	protein O-fucosyltransferase
Pol II	RNA Polymerase II
Ptch	Patched
Ptf	proximal sequence element-binding transcription factor
Ptk	tyrosine-protein kinase-like
PVDF	polyvinylidene fluoride
qPCR	quantitative PCR
RA	retinoic acid
RALDH	retinaldehyde dehydrogenase
RAR	retinoic acid receptor
RARE	retinoic acid response element
Rbpj	recombining binding protein suppressor of hairless
RDH	retinal dehydrogenase
RNA	ribonucleic acid
RT-PCR	reverse transcriptase PCR

RXR	retinoid X receptor
SDS	sodium dodecyl sulfate
SHARP	split-and hairy-related protein
Shh	Sonic Hedgehog
SILAC	stable isotope labeling by amino acids in cell culture
SKIP	ShaI K+ channel interacting protein
SLC	solute carrier family
Smad	sma/mothers against decapentaplegic
Smo	Smoothened
SMRT	silencing mediator for retinoid or thyroid hormone receptors
SNF	sucrose non-fermenting
Sox	Sry-related HMG box
Spry	Sprouty
SWI	switch
T	Brachyury
TA	transit-amplifying
Tam	tamoxifen
TBP	TATA binding protein
TCF	T-cell factor
TF	transcription factor
TFF	trefoil factor
TGF	Transforming Growth Factor
TIC	tumour initiating cell
TLE	transducin-like enhancer of split
trxG	trithorax group
TSS	transcriptional start site
TUNEL	terminal deoxynucleotidyl transfer mediated dUTP nick end labeling
Wnt	wingless
WTX	Wilms tumour suppressor

DEDICATION

To aspiring individuals everywhere:

"*Today you are You, that is truer than true. There is no one alive who is Youer than You.*"

-Theodor "Seuss" Geisel (1904-1991)

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CHAPTER 1: INTRODUCTION

Intestinal pathologies

More than 20 million Canadians suffer from digestive health disorders, leading to 4% of the total annual deaths in Canada. For example, cancers of the digestive tract are second only to lung cancers in Canada, representing 20% of new cancer diagnoses and 10% of all cancer deaths. Another example is celiac disease, which results in the wasting of the intestinal epithelium in response to wheat sensitivity, and afflicts more than 300,000 Canadians.

As a part of disease processes such as cancer, trans-differentiation from one type of epithelium to another is common. For instance, in Barrett's esophagus, which is thought to lead to some esophageal cancers, the epithelium of the esophagus acquires an intestinal identity. Understanding the molecular regulation of intestinal development is therefore important for the treatment, diagnosis and prevention of such pathologies (CDHF, 2009).

EMBRYONIC DEVELOPMENT OF THE GUT

Endoderm formation

In mice, gestation typically lasts for 19 days. Fertilization of the oocyte leads to zygote formation and after several rounds of cell divisions and compaction, the mature blastocyst is formed around embryonic day (E)4.5. The blastocyst is composed of an inner cell mass and an outer trophectoderm lineage. The inner cell mass is further divided into the epiblast, which will later form embryonic derivatives, and the overlying primitive endoderm, which, along with the trophectoderm, forms the extraembryonic lineages

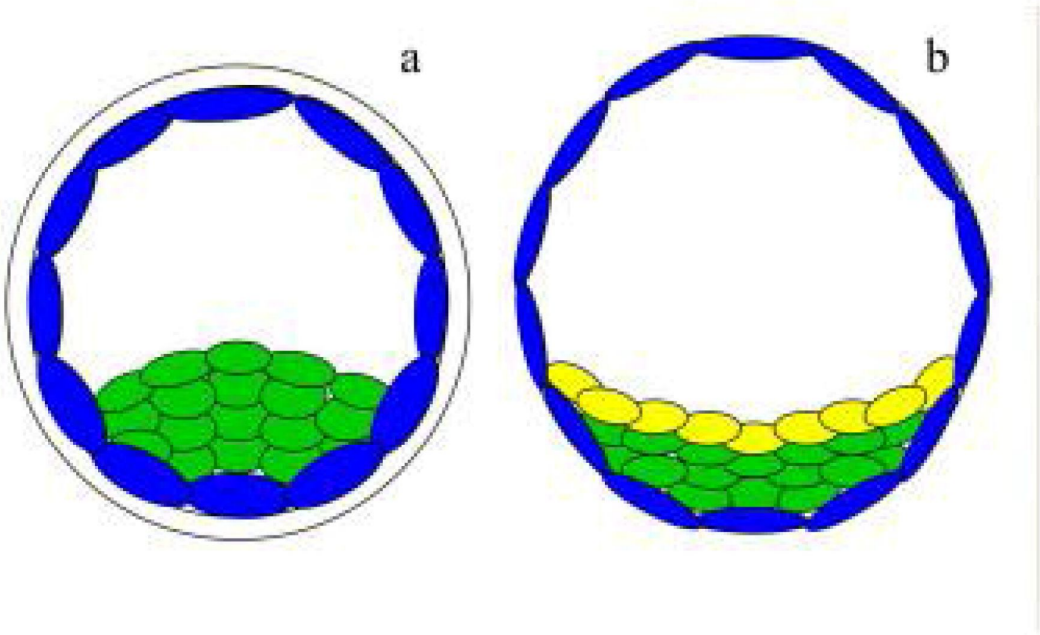


Figure 1: Organization of primitive endoderm. Schematic representation of expanded blastocyst with absence (a) and presence (b) of primitive endoderm (hypoblast) in a day 4 expanded blastocyst . In b, inner cell mass remnant is defined as the epiblast (green) and the primitive endoderm (yellow). Figure and legend adapted with permission from Takara, *et al,* 2006.

(Figure 1). The primitive endoderm will give rise to visceral endoderm, which underlies the epiblast, and parietal endoderm, which will underlie the yolk sac.

Around E6.5, the three embryonic germ layers are formed in a process termed gastrulation. Some of the epiblast cells undergo an epithelial to mesenchymal transition, delaminate and traverse the primitive streak, a transient structure through which epiblast cells will migrate. During this process, cells in the cup-shaped epiblast migrate through the primitive streak to form an inner, middle and outer layer of cells, termed ectoderm, mesoderm and endoderm, respectively (Figure 2). The ectoderm will give rise to the epidermis, neural tissues and many skeletal derivatives of the skull; the mesoderm will give rise to the muscular, urogenital, cardiovascular and elements of the axial and appendicular skeleton; and the endoderm will give rise to the digestive tract and its accessory organs (reviewed in Solnica-Krezel, 2005; Wolpert, 2004; Zorn and Wells, 2009).

Lineage tracing studies in many animal models have indicated how endoderm is formed. In zebrafish and *C. elegans*, it has been suggested that at least some endoderm and mesoderm cells arise from a common progenitor pool (reviewed in Rodaway and Patient, 2001). Early lineage tracing studies in mouse suggested that this mesendodermal pool may be evolutionarily conserved (Lawson and Pedersen, 1987). In this regard, mouse embryonic stem (ES) cells can be driven towards a cell type expressing markers of both endoderm and mesoderm *in vitro* (Kubo et al., 2004; Tada et al., 2005), but this population has yet to be definitively identified *in vivo*. In contrast, recent evidence suggests that if there is a common mesendodermal progenitor pool, it contributes to a relatively minor portion of the definitive endoderm (Tzouanacou et al., 2009).

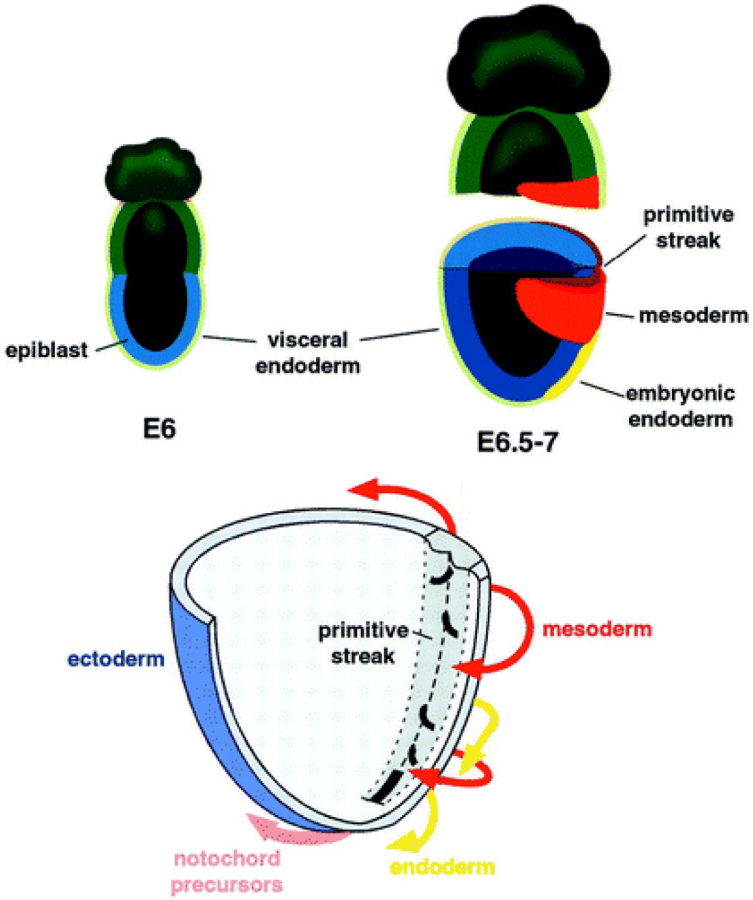


Figure 2: Gastrulation and endoderm formation. The *top panel* shows relative positions of the embryonic ectoderm (blue), mesoderm (red), and endoderm (yellow) during gastrulation of the mouse embryo in a partially cut-away view . All embryonic tissues derive from the blue region of the E6 embryo, which is called the primitive ectoderm or epiblast at this stage. Embryonic endoderm (yellow), which is first detected over the anterior primitive streak, migrates along the mid-line in an anterior direction. Migration of endoderm and mesoderm continues throughout gastrulation (E7-7.5) and is shown in more detail in the *bottom panel*. The arrows show the relative paths of migration of cells during mouse gastrulation. Figure and legend adapted with permission from Wells and Melton, 1999. ANNUAL REVIEW OF CELL AND DEVELOPMENTAL BIOLOGY by ANNUAL REVIEWS, INC Reproduced with permission of ANNUAL REVIEWS in the format Republish in a thesis/dissertation via Copyright Clearance Center.

In the mouse, the formation of the definitive endoderm begins with recruitment of epiblast cells through the anterior portion of the primitive streak during the early streak stage. Definitive endodermal cells are laid down in anterior to posterior order, such that the cells exiting the streak first will form the most anterior regions of the foregut, and those exiting last will form the most posterior regions of the hindgut (Lawson et al., 1986; Lawson and Pedersen, 1987). It was first thought that epiblast cells replace all of the overlying visceral endoderm; however, it has since been shown that these cells initially intermingle with visceral endoderm and expand to eventually displace most, but not all, visceral endoderm (Kwon et al., 2008; Lawson et al., 1986; Lawson and Pedersen, 1987). By the time gastrulation is complete, the endoderm has formed a simple pseudostratified epithelium which overlays the mesoderm (Grosse et al., 2011).

Intestinal patterning

The developmental process by which a tissue is separated into distinct anatomical or functional domains is referred to as patterning. The mechanisms of patterning in the intestinal epithelium remain poorly understood. Initiation of signaling cascades eventually leads to restricted expression of transcription factors along the primitive gut, and segregation of early endoderm into anterior and posterior domains is evident from gene expression studies beginning with late gastrula stage embryos. In the more anterior portion of the primitive streak, where anterior foregut endoderm will arise, transcription factors such as *Hex, Sox2*, and *Foxa2* are expressed (Ishii et al., 1998; Martinez Barbera et al., 2000; Monaghan et al., 1993; Thomas et al., 1998), while in the more posterior region, where hindgut will emerge, genes including *Sox17* and *Cdx* members are

expressed (Beck et al., 1995; Silberg et al., 2000) (Figure 3). Furthermore, in some cases, these transcription factors have been shown to reinforce anterior versus posterior identity. For example, *Hex* has been shown to suppress posterior identity in the frog and is required for the formation of anterior endoderm in the mouse (Brickman et al., 2000; Martinez Barbera et al., 2000). In contrast, loss of *Sox17* in mice results in loss of posterior gut endoderm (Kanai-Azuma et al., 2002).

Although early endoderm has been regionalized by the action of the products of these, and other, transcription factors, it remains relatively plastic, since posterior endoderm, for example, can be converted to anterior pancreatic tissue (Afelik et al., 2006). However, transplanted foregut endoderm is better able to form liver than hindgut endoderm, suggesting that these early progenitors are limited in their developmental plasticity (Fukuda-Taira, 1981). The full extent of this plasticity remains relatively unexplored.

The continued morphogenesis of the embryo will lead to germ layer movement and exposure to different signaling cues. In this regard, transplantation studies have shown that primitive streak, ectoderm and mesoderm all provide different inductive cues to the endoderm. For example, when isolated endoderm from E7.5 mice is co-cultured with isolated mesoderm and ectoderm of similar stages, endoderm-specific genes that are normally activated around E8.5 such as *Pdx1*, *somatostatin* and *NeuroD* are induced, an effect that is not seen in control experiments, and suggesting that the mesoderm and ectoderm provide inductive cues to the endoderm. To distinguish these from the possibility that ectoderm and mesoderm are permissive to endoderm formation, anterior endoderm was grafted to posterior mesoderm/ectoderm and vice versa, cultured and the

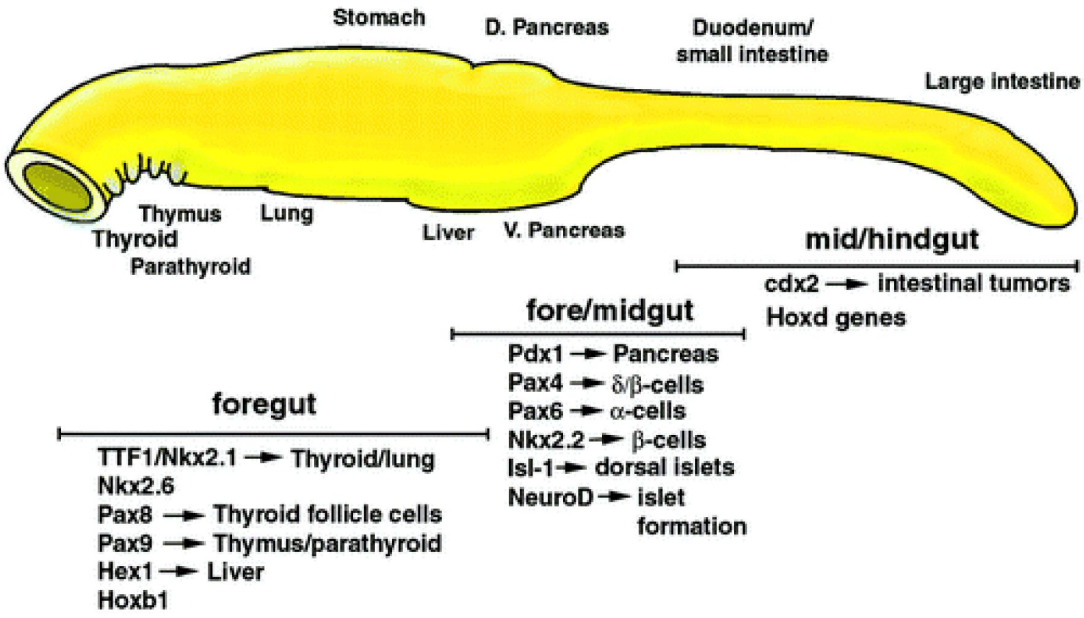


Figure 3: Transcription factors in the early gut tube. A schematic representation of the E9.5 gut tube shows expression of several transcription factors along the gut tube and regions of the gut that contribute to specific organs. Transcription factors that are expressed in overlapping domains along the foregut, midgut, or hindgut are listed below. For simplicity, transcription factors have been grouped according to their relative anterior-posterior expression at this time of development. Some genes have a dorsal-ventral expression difference as well (not shown). Figure and legend adapted with permission from Wells and Melton, 1999. ANNUAL REVIEW OF CELL AND DEVELOPMENTAL BIOLOGY by ANNUAL REVIEWS, INC Reproduced with permission of ANNUAL REVIEWS in the format Republish in a thesis/dissertation via Copyright Clearance Center.

endoderm was examined for markers of anterior and posterior identity. The authors found that the ectoderm/mesoderm has inductive potential, since endoderm acquired the positional identity of the ectoderm/mesoderm. Of note, once the endoderm had acquired its anterior-posterior identity, it was more difficult to produce any subsequent change, suggesting that endoderm plasticity becomes restricted and/or that ectoderm/mesoderm loses its inductive potential. Furthermore the authors demonstrated that endoderm responds to the secreted factor Fibroblast Growth Factor (FGF) 4 in a concentration-dependent manner (Wells and Melton, 2000). These findings are consistent with soluble factors such as FGFs playing a role in endoderm patterning. In this regard, *FGF4* is expressed in the mesoderm of mice and chicks, and can activate genes such as *Cdx2* and repress anterior genes such as *Hex*, in the hindgut (Dessimoz et al., 2006).

A number of mesodermal transcription factors also play key roles in anterior-posterior patterning of intestinal epithelium. This is exemplified by *BarX1*, which is expressed in the stomach mesenchyme during gastric development (Verzi et al., 2009a), and is essential for stomach patterning (Kim et al., 2005; Kim et al., 2007). Other developmental signaling cascades emanating from the mesoderm are also known to regulate anterior-posterior identity in the intestine, including the Hedgehog pathway, as discussed below.

The endoderm itself also provides patterning cues impacting both the endoderm and the underlying mesoderm at later stages. For example, the endoderm from E14 fetal rat small intestine grafted in nude mice developed normal histology and intestinal markers. Grafting endoderm of the presumptive ileum with mesoderm of the presumptive jejunum resulted in jejunal mesoderm acquiring ileal characteristics, with the reverse

experiment having similar results. Furthermore, grafting rat small intestinal endoderm to skin fibroblasts resulted in normal intestinal epithelium and induced the transformation of the fibroblasts to small intestinal mesenchyme (Duluc et al., 1994). Although the authors note that the samples were examined for mesoderm contamination, isolation of pure endoderm at this stage is technically challenging and only recently have appropriate markers been identified (Sherwood et al., 2009). Nevertheless, taken together, these results suggest that the endoderm itself has been regionalized and has inductive potential prior to overt differentiation into intestinal epithelium.

Transcriptional regulators of anterior-posterior identity have also been identified in addition to *BarX1*, discussed above. For example, in the gastrointestinal tract, expression of *Sox2* is limited to the epithelium of the esophagus, lung and stomach primordia. Loss of *Sox2* results in a transformation of both the epithelium and underlying mesenchyme of the stomach to a small intestine identity, suggesting that it regulates gut identity anterior to the pyloric-duodenal junction (Que et al., 2007a; Sherwood et al., 2009). Although some of the mechanisms driving intestinal patterning have begun to emerge, this remains a relatively unexplored area; AP patterning in other tissues, however, has been better described and will be discussed later.

Generation of the mature intestinal epithelium

Once the endoderm has been laid down, the formation of the primitive gut continues with the development of two pockets at the anterior and posterior regions of the embryo, which will eventually meet to form the primitive gut tube by E9.0 (Lawson et al., 1986; Lawson and Pedersen, 1987) (Figure 4). By this stage, the gut tube has already

received patterning cues. Epithelial-mesenchymal interactions will give rise to restricted gene expression along the gastrointestinal tract leading to regionalization of the primitive gut into esophagus, stomach, small and large intestines. Furthermore, restricted gene expression will contribute to budding of areas of the gut tube, which are the anlage of the accessory organs of the digestive system such as the lungs, pancreas, liver and salivary glands (reviewed in Zorn and Wells, 2009) (Figure 5).

The development of the gut continues with the elongation and turning of the gut tube, which is accompanied by growth and differentiation of the intestinal tract. Epithelial invaginations which house the dividing cells then begin forming at E14.5, beginning at the most anterior portion of the small intestine. As these cells divide, the villi are formed. However, in the mouse, it is not until a few weeks after birth that the intestinal invaginations fully develop into mature crypts (Figures 6, 7). At this point, the mature intestinal epithelium is established. In contrast to the finger-like villus projections and invaginating crypts characteristic of the small intestine, the large intestine is devoid of villus projections, which are replaced with a flattened epithelium, as discussed below (Zorn and Wells, 2009).

Gastrointestinal histology and structure

Along the gastrointestinal tract, there are different epithelial linings to accommodate the different functions of the organ. For example, the esophagus is lined by a keratinized (in mice, but not in humans), stratified squamous epithelium, surrounded by a thick muscular layer. The stomach is dilated compared to the rest of the digestive tube

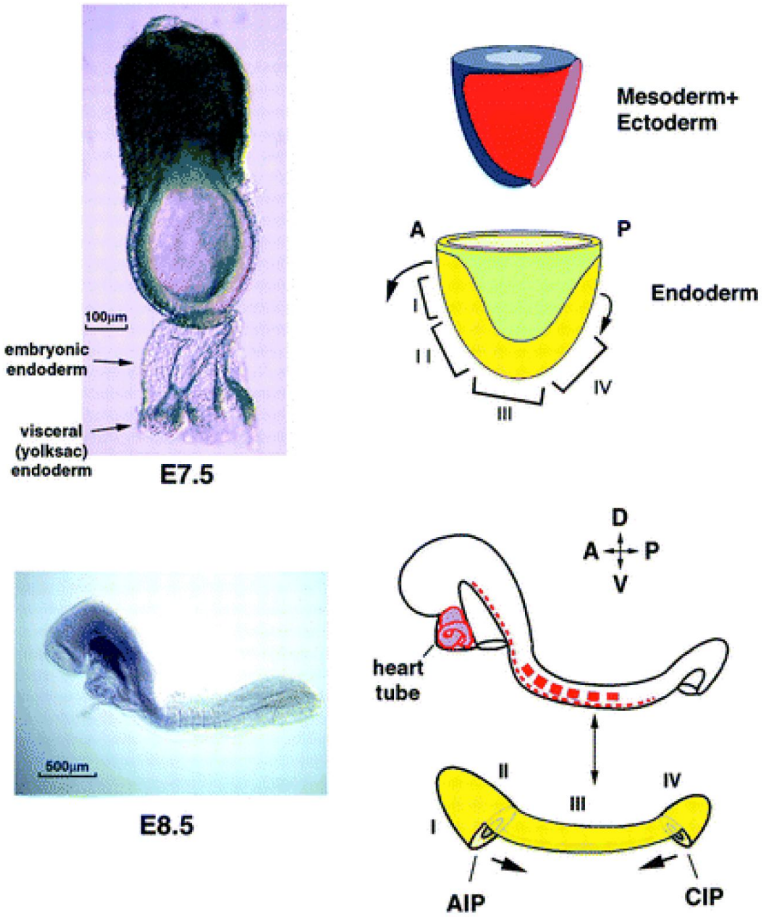
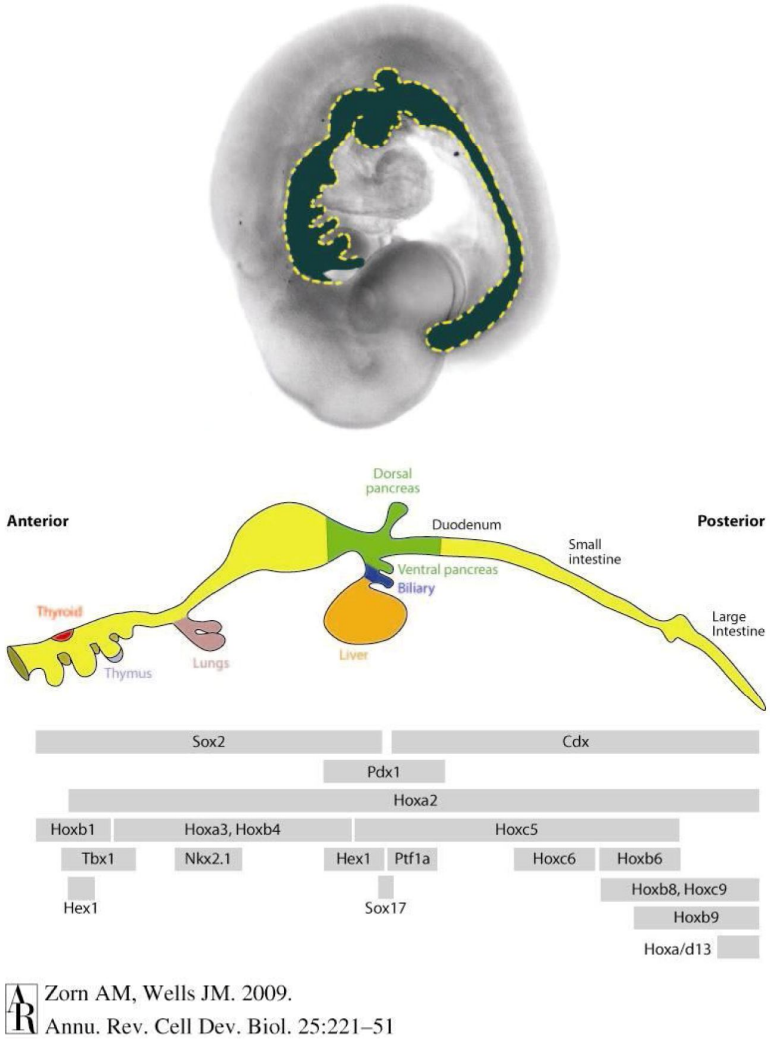


Figure 4: Formation of the gut tube. The *top left panel* shows an E7.5 embryo with the endoderm layer peeled off the underlying mesoderm and ectoderm but still attached at the node. The *bottom left panel* shows an E8.5 embryo with a well-formed foregut. The endoderm of the E7.5 embryo (yellow and green, *top right*) was fate mapped to the forming E8.5 gut tube (yellow, *lower right*). The *right panels* show the endoderm separated from the mesoderm and ectoderm. Roman numerals I–IV represent regions of E7.5 endoderm that fate map to regions I–IV of the E8.5 gut. Regions I-III ultimately contribute to stomach, pancreas, duodenum, and part of the intestine. Region IV (*upper right*) forms the hindgut, which contributes to the large intestine and colon (*lower right*). The foregut tube forms as region I folds over region II (arrow) and migrates in a posterior direction, whereas the hindgut tube forms when region IV folds over and migrates in an anterior direction (arrow). The posterior migration of the anterior intestinal portal (AIP) and the anterior migration of the caudal intestinal portal (CIP), in combination with embryonic turning, close the midgut and form a primitive gut tube by E9. Figure and legend adapted with permission from Wells and Melton, 1999. ANNUAL REVIEW OF CELL AND DEVELOPMENTAL BIOLOGY by ANNUAL REVIEWS, INC Reproduced with permission of ANNUAL REVIEWS in the format Republish in a thesis/dissertation via Copyright Clearance Center.



Zorn AM, Wells JM. 2009.
Annu. Rev. Cell Dev. Biol. 25:221–51

Figure 5: Overlapping expression domains of transcription factors along the A-P axis of the gut tube. The upper panel shows a lateral view of an E9.5 mouse embryo with the gut tube false-colored (*blue*). The middle panel shows a schematic diagram of the bud-stage gut (E10.5 in mouse). The lower panel indicates the relative A-P expression boundaries of several transcription factors. The expression of these factors is temporally dynamic, and these expression domains are not necessarily maintained throughout development. The anterior and posterior expression limits on some of these factors are important in establishing organ domains. Figure and legend adapted with permission from Zorn and Wells 2009. ANNUAL REVIEW OF CELL AND DEVELOPMENTAL BIOLOGY by ANNUAL REVIEWS, INC Reproduced with permission of ANNUAL REVIEWS in the format Republish in a thesis/dissertation via Copyright Clearance Center.

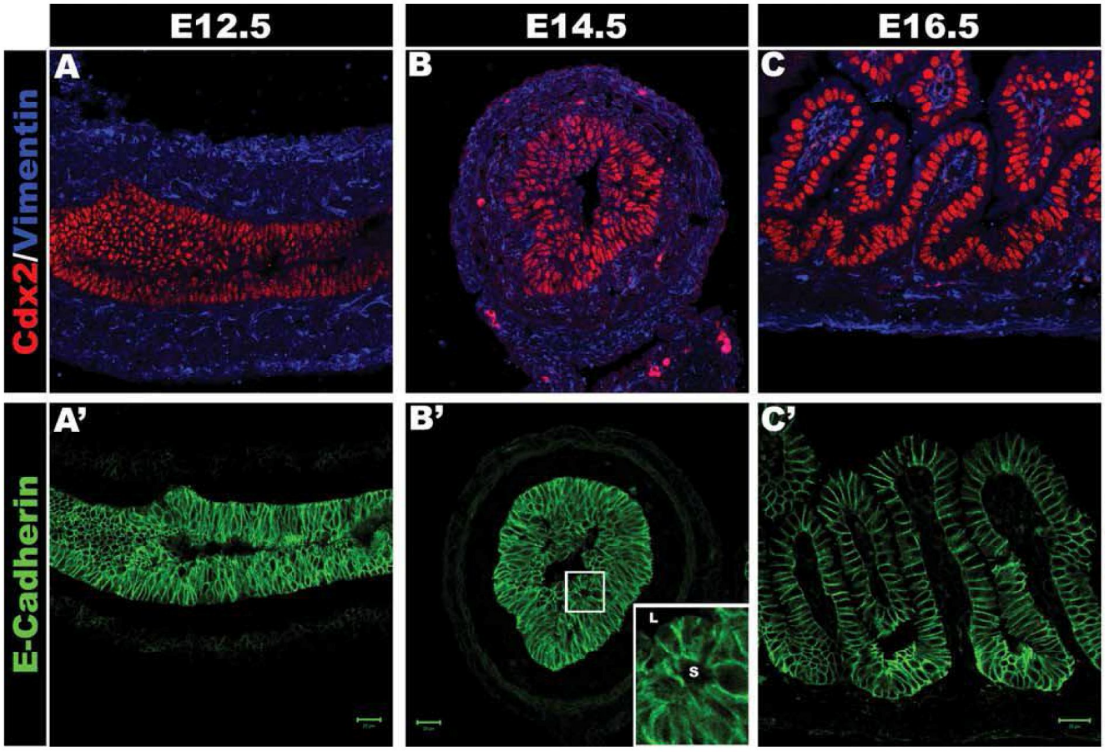


Figure 6: Intestinal epithelial reorganization. Intestinal development at embryonic day (E) 12.5 (A,A'), E14.5 (B,B') and E16.5 (C,C'). A– C: shows the Cdx2-positive epithelium (red) and vimentin-positive mesenchyme. A'–C' shows the E-cadherin positive epithelium. Secondary lumina can be seen at E14.5 (boxed region is magnified in the inset; L indicates primary lumen, s indicates secondary lumen; B') as epithelial reorganization begins. By E16.5, there are clear intervillus regions and villi (C, C'). Figure and legend adapted with permission from Spence, *et al*, 2011. Developmental dynamics : an official publication of the American Association of Anatomists by AMERICAN ASSOCIATION OF ANATOMISTS Reproduced with permission of JOHN/WILEY & SONS, INC. in the format reuse in a dissertation/thesis via Copyright Clearance Center.

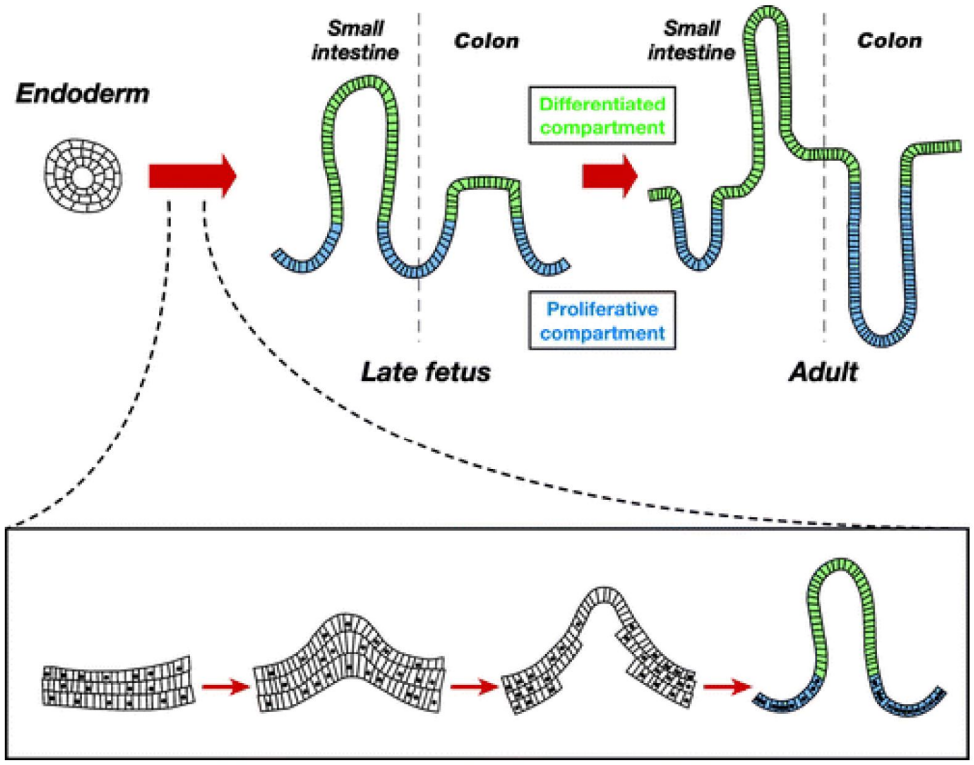


Figure 7: Late fetus vs. adult intestinal epithelium. Structure of the early multilayered endoderm and the compartmentalized late fetal small and large intestines, and development into the final structure of the adult small and large intestine after invagination of the crypts and elongation of the villi. Inset shows detail of the morphogenetic movements that result in the formation of a simple epithelium with two compartments, from a multilayered endoderm. Figure and legend adapted with permission from Sancho, *et al,* 2004. Annual review of cell and developmental biology by ANNUAL REVIEWS, INC Reproduced with permission of ANNUAL REVIEWS in the format Republish in a thesis/dissertation via Copyright Clearance Center.

to accommodate the digestive chyme mixture, which contains ingested food, hydrochloric acid and some digestive enzymes.

The epithelial lining of the stomach consists of three regions. The proximal cardiac region is lined by a keratinized (in mice) squamous layer; the distal pyloric region is lined with a simple glandular epithelium; the middle body region bears semblance to both regions (Figure 8). Surface mucous cells of the stomach produce a protective mucous layer, while parietal cells secrete hydrochloric acid and chief cells secrete the proteolytic precursor, pepsinogen. The pyloric region of the stomach connects to the duodenum of the small intestine through the pyloric sphincter (Ross, 1989).

The small intestine consists of the duodenum, jejunum and ileum, each lined with a simple columnar epithelium, with finger-like villus projections and glandular, invaginating crypts. The intestinal epithelium is the most rapidly renewing tissue in the body. As such, differentiation and self-renewal in the intestine is subject to tight regulation. As discussed below, stem cells are housed in the base of the crypts in both the small and large intestines and give rise to transit-amplifying (TA) cells. TA cells are mitotically active cells, which eventually differentiate into the secretory and absorptive cells of the intestinal epithelium, migrate towards the tip of the villus (or to the base of the crypts in the case of Paneth cells), apoptose and shed (Grossmann et al., 2002) (Figure 9). For most cells in the epithelium, this process takes 5-7 days, with the exception of Paneth cells, which persist for 21 days in mice (Creamer et al., 1961).

Enterocytes, the intestinal absorptive cells, make up the majority of this epithelium. These cells are responsible for final catabolism and absorption of nutrients and much of the water, and express surface enzymes and transport proteins in their

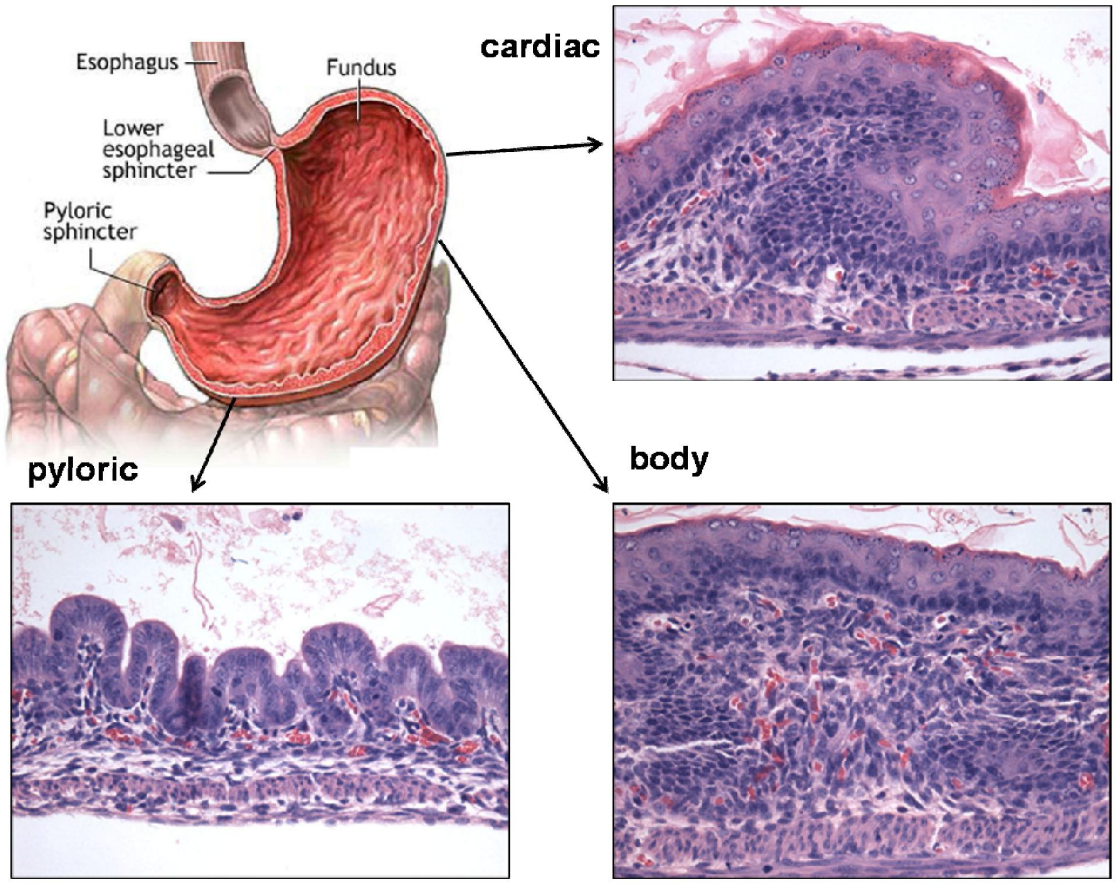


Figure 8: Histology of the stomach. The stomach is divided into three histologically distinct regions. The cardiac region is a stratified, keratinized epithelium; the pyloric region is a glandular, non-keratinized simple epithelium; and the body region is histologically in between the two.

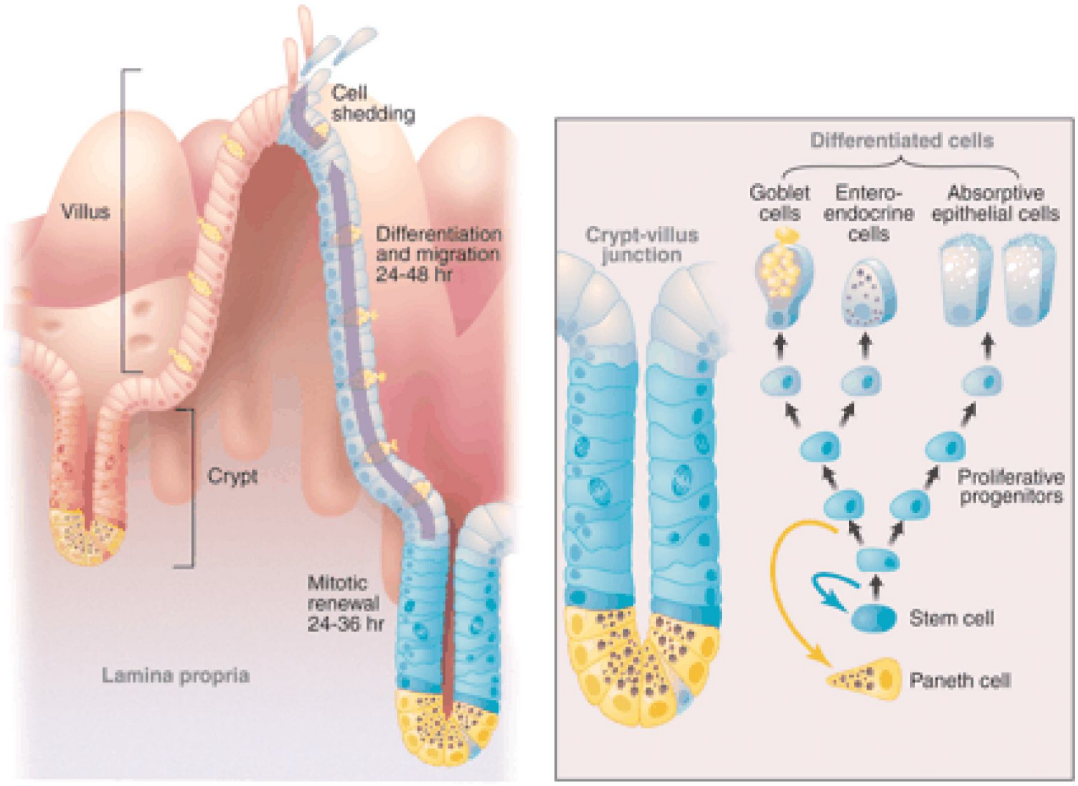


Figure 9: The anatomy of the small intestinal epithelium. The epithelium is shaped into crypts and villi (**left**). The lineage scheme (**right**) depicts the stem cell, the transit-amplifying (mitotic renewal) cells, and the two differentiated branches. The right branch constitutes the enterocyte lineage; the left is the secretory lineage. Relative positions along the crypt-villus axis correspond to the schematic graph of the crypt on the right. Figure and legend adapted with permission from Radtke and Clevers, 2005.

characteristic brush-border microvilli for these purposes (Ross, 1989). The intestinal secretory cells encompass Goblet, Paneth and enteroendocrine cells. Goblet cells secrete a protective mucous layer, and express markers of mucous production such as mucins *TFF3, Gfi1* and *Klf4* (Katz et al., 2002; Podolsky et al., 1993; Shroyer et al., 2005). Paneth cells are not formed until after crypt maturation in mice and, exceptionally, migrate to the bottom of the crypt and secrete antimicrobial peptides. Enteroendocrine cells are a rare cell type and secrete hormones that impact intestinal activity (Ross, 1989). Recently, tuft cells, marked by *DCLK1,* were identified as secretors of opioids and enzymes related to prostaglandin synthesis (Gerbe et al., 2012). Finally, another specialized cell type, the M (Microfold) cell, is located on the surface of lymphatic nodules, which develop after birth (Ross, 1989). Differentiation of these cell types will be discussed below.

Lastly, the large intestine consists of the cecum, the colon and the rectum, with the primary purpose of water absorption. To facilitate this process, the colon has deep invaginating crypts, but no villi. The most abundant cell type in the epithelium is the colonocyte, which mediates water absorption. There are also numerous Goblet cells, which secrete lubricating mucous (Ross, 1989).

Stem cells of the intestinal tract

Renewal of proliferative tissues is typically dependent on a renewable source of stem cell precursors as well as division and differentiation of the daughter cells. This is especially important in the intestine, with its extremely high rate of turnover. Through an asymmetric division, a stem cell is thought to give rise to itself and a replicating daughter

cell. Although both of these cell types likely have a finite number of cell divisions, stem cells are classically thought to undergo cell division less frequently than their highly replicating daughter cells to protect the DNA from mutations arising from mitosis. Daughter cells will go on to divide, produce differentiated cells and undergo apoptosis.

It has been long thought that 4-6 stem cells reside in each crypt of the small and large intestines. As with other tissues, label retention studies were initially used to identify potential stem cell populations. This approach was based on the belief that to protect genome integrity, upon asymmetric cell division, a stem cell would retain the oldest DNA copy, creating an "immortal strand" (Cairns, 1975). As such, administration of a label such as ^{3}H-thymidine or BrdU during stem cell regeneration (such as growth or repopulation during injury) would result in preferential label retention in the immortal DNA strand. Initially, a population of cells at the base of the crypts of the small intestine, in between the Paneth cells, was identified as label-retaining (Figure 10). These were termed crypt-base columnar (CBC) cells, based on their morphology and localization, and were suggested to have multipotent progenitor properties, consistent with stem cells (Cheng and Leblond, 1974). However, contradictory evidence later pointed to a population of a pool of cells at the +4 position relative to the base of the crypt, also capable of long term BrdU label retention in the small intestine (Kaur and Potten, 1986). Less equivocal demonstration that either population was a stem cell was hindered by the difficulty in culturing intestinal cells and the lack of appropriate stem cell markers. As a result, it was only recently possible to demonstrate that any single cell type in the intestine can give rise to an entire crypt-villus unit, and can do so throughout the lifespan of the animal as described below.

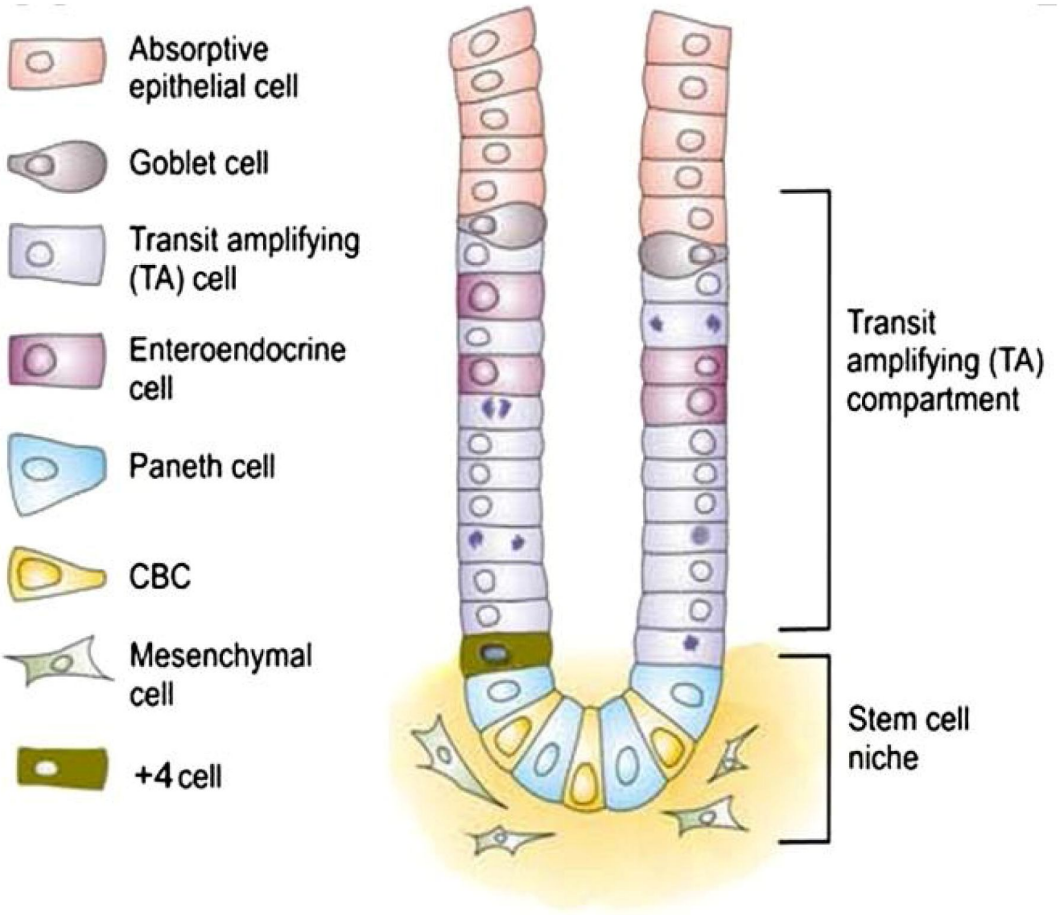


Figure 10: The crypt of the small intestine. Schematic representation of the intestinal crypt, including stem cell niche and transit amplifying (TA) zone as well as differentiated cell types. Note the different locations of the +4 cells and the CBC cells. Figure and legend adapted with permission from Vries, *et al*, 2010. Molecular oncology by ELSEVIER BV. Reproduced with permission of ELSEVIER BV in the format reuse in a thesis/dissertation via Copyright Clearance Center.

As described below, Wnt signaling plays an important role in maintenance of the intestinal crypt. Based on this, the Clevers lab hypothesized that a subset of Wnt target genes should be expressed exclusively in intestinal stem cells. Indeed, they identified the G-protein coupled orphan receptor *Lgr5* as a Wnt target gene expressed in the CBC cells. Through lineage tracing analysis, they were able to show that these Lgr5-positive CBC cells are able to give rise to all differentiated cell types of the epithelium of the colon and small intestine for up to 14 months (Barker et al., 2008; Barker et al., 2007). Furthermore, they developed *in vitro* culture conditions for the intestinal epithelium and showed that a single *Lgr5* cell is able to recapitulate a crypt-villus axis unit *ex vivo*, strongly supporting the notion that Lgr5+ CBC cells are intestinal stem cells (Sato et al., 2011a; Sato et al., 2011b; Sato et al., 2009). *Lgr5* also marks stem cells in the stomach, skin and hair follicles, as well as intestinal tumor initiating cells (Barker and Clevers, 2010; Barker et al., 2009; Barker et al., 2008; Barker et al., 2007; Haegebarth and Clevers, 2009; Jaks et al., 2008; Munoz et al., 2012). Interestingly, while most stem cells are believed to be largely quiescent, CBC cells divide approximately every 20 hours, which may be related to the high turnover of the intestinal epithelium (Barker et al., 2007; Snippert et al., 2010). Finally, Paneth cells appear to be an important part of the *Lgr5* stem cell niche (Sato et al., 2011a), although not an absolute requirement (Kim et al., 2012).

Following the identification of *Lgr5* as a stem cell marker, it was found that *Bmi1*, a member of the Polycomb complex, marked cells at the +4 position in the crypts of the small intestine. Several studies indicated that these may also represent a stem cell population distinct from CBC cells, a notion since refuted (Sangiorgi and Capecchi, 2008; Tian et al., 2011; Yan et al., 2012). Rather, recent evidence suggests that *Lgr5*

cells are actually upstream of *Bmi1* cells. In this regard, transcriptome and proteome analyses of *Lgr5* cells and daughter cells revealed that *Lgr5* cells express markers of the *Bmi1* cell population. To explain these inconsistencies, the authors suggest that *Bmi1* may, in fact, mark a broad range of cells, including the cells of both the +4 and the CBC positions, though not previously identified (Munoz et al., 2012). The authors provide evidence that the previously published *Bmi1* expression domains were not complete. Furthermore, they suggest that upon injury, *Lgr5* negative TA cells revert to a stem cell state to replenish the epithelium (Munoz et al., 2012). Taken together, these results suggest that *Lgr5*+ CBC cells represent the true intestinal stem cell.

SIGNALING PATHWAYS IMPORTANT FOR INTESTINAL DEVELOPMENT AND FUNCTION

Notch signaling: lineage decisions and differentiation

Notch signaling is an important cell-to-cell signaling pathway mediating local cell fate determination (reviewed in Fortini, 2009). Notch was first identified in *Drosophila*, and so named for the serrated appearance of wings in heterozygous flies missing an entire chromosome (Mohr, 1919). The importance of Notch was later shown, again in *Drosophila*, where homozygous mutants were found to have segmentation defects as well as an overabundance of neuronal cells at the expense of epidermis (Poulson, 1937), underscoring the importance of this pathway in dictating appropriate cell fate decisions. Relevant to this thesis is the finding that intestinal differentiation also relies on cell fate determination mediated, in part, by Notch signaling.

Notch receptors bind to ligands of the *Delta-Serrate-Lag-2* (*DSL*) family, which are typically expressed on a neighbouring cell. There are four *Notch (1-4)* receptors and five ligands (*Delta 1, 3, 4, Jagged 1* and *2*) in mice, all of which are expressed in the intestine during development and/or postnatally (Schroder and Gossler, 2002). Preferential expression of either *Notch* or *DSL* in a cell is reinforced by a cell-autonomous pathway which leads to lateral inhibition of the expression of these components in neighbouring cells (Sprinzak et al., 2010). In the canonical pathway, binding of the DSL ligand to a Notch receptor results in two sequential proteolytic cleavages of the receptor, first by a metalloprotease of the ADAM family and subsequently by γ-secretase (Brou et al., 2000; Fehon et al., 1990; Mumm and Kopan, 2000), leading to the release of the Notch intra-cellular domain (NICD) and its subsequent translocation to the nucleus (Jarriault et al., 1995; Struhl and Adachi, 1998). Transcription of Notch target genes is mediated by transcription factors in the CSL family namely CBF-1, Su(H) and Lag-1, which are named for the orthologs in vertebrates, *Drosophila* and *C. elegans*, respectively (Barolo et al., 2000; Brou et al., 1994; Grossman et al., 1994; Henkel et al., 1994; Lai, 2002; Matsunami et al., 1989; Tun et al., 1994). In the absence of the NICD, CSL transcription factors are associated with co-repressors such as NCoR/SMRT, MINT/SHARP/SPEN, SKIP, CIR, CtBP, and Groucho/TLE complexes, leading to repression of target genes though repressor-associated histone deacetylase (HDAC) activity (Barolo et al., 2002; Barolo et al., 2000; Hsieh et al., 1999; Kao et al., 1998; Laherty et al., 1998; Morel et al., 2001; Nagy et al., 1997; Zhou et al., 2000). When NICD is translocated to the nucleus, it interacts with co-activator Mastermind (Mam, also known as MAML) (Wu et al., 2000), allowing

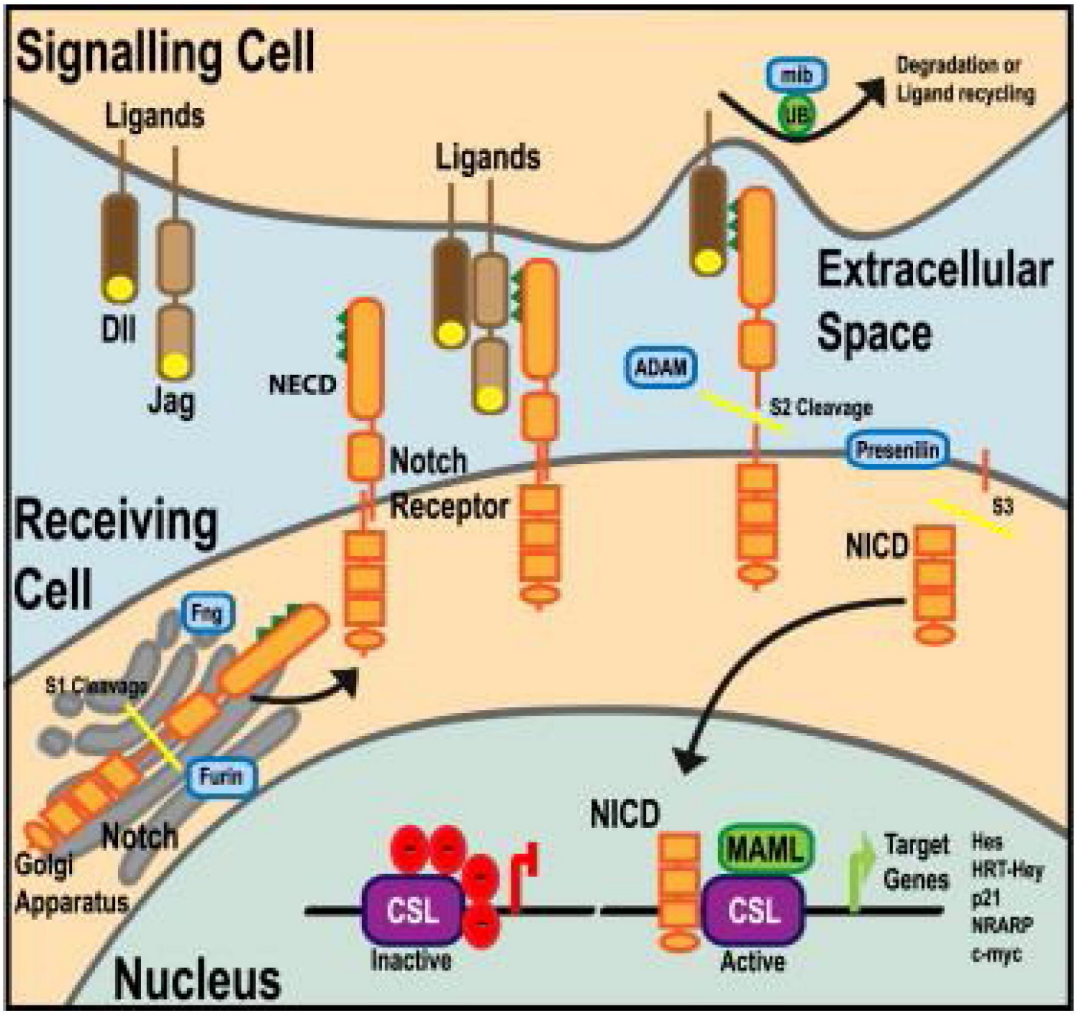


Figure 11: The Notch Pathway. Membrane-bound Notch ligands (Dll1,3,4 and Jag1,2) are characterized by a Delta/Serrate/Lag2 (DSL) motif (yellow) located in the extracellular domain. The Notch receptor (Notch1-4) is processed at the S1 site by a furin protease, sugar-modified by Fringe in the Golgi, and inserted into the membrane as a heterodimer with a large extracellular domain (NECD). Ligand-receptor interaction leads to two consecutive cleavage events (at S2 and S3 sites), respectively carried out by an ADAM protease and presenilin, which release the Notch intracellular domain (NICD). NICD translocates to the nucleus and forms a transcriptional activation complex after binding to Mastermind (MAML) and CSL/RBPJk/Su(H). This ternary complex activates the transcription of a set of target genes including *Hes*, *Hey*, and others. Figure and legend adapted with permission from de la Pompa,and Epstein, 2012. Developmental cell by CELL PRESS. Reproduced with permission of CELL PRESS in the format reuse in a thesis/dissertation via Copyright Clearance Center.

recruitment of positive-acting transcriptional machinery to CSL factors and inducing transcription of target genes such as members of the *Hairy/enhanced/split* (*Hes*) family (Fryer et al., 2002; Hsieh et al., 1997) (Figure 11).

The intestinal stem cell niche is dependent on Notch signaling for both differentiation and maintenance of the crypt. This is evidenced by the impact of loss of *CBF-1* (also called *Rbpj-κ*) which results in mitotic arrest of crypt-derived cells (van Es et al., 2005b), while overexpression of *Notch1* results in expansion of the proliferative pool (Fre et al., 2005). Furthermore, Notch receptors have been shown to impact on the regulation of cell cycle inhibitors p27 and p57 in the intestine (Riccio et al., 2008c).

The regulation of epithelial differentiation by Notch signaling is also evidenced by a number of loss-of-function models. For example, *Rbpj-κ* mutation, γ-secretase inhibition or simultaneous loss of either *Notch1* and *Notch2* or *Dll1* and *Dll4* bias differentiation into secretory Goblet cells (Aberle et al., 1997; Nakamura et al., 2007; Riccio et al., 2008c; van Es JH, 2005). These, and other, studies have led to a model suggesting that Notch signaling mediates a binary decision between secretory and absorptive intestinal cells through the target gene *Hes1* and mutual transcriptional repression by the bHLH transcription factor *Math1* (Kim and Shivdasani, 2011). In this model, TA cells are driven towards an absorptive fate when they express *Hes1*, while cells expressing *Math1* will be biased towards a secretory fate. Consistent with this, loss of *Math1* leads to a depletion of all secretory cell types and an increase in enterocytes while loss of *Hes1* has the opposite effect (Jensen et al., 2000; Yang et al., 2001).

Wnt signaling: maintenance of the intestinal crypt

In addition to Notch, there are several other signaling pathways that are known to be important to normal intestinal development and homeostasis; one such pathway is the Wnt signaling cascade. Wnt signaling is known to play an important role in maintenance of the stem cell niche in many tissues, including the intestinal crypt. In fact, Wnt pathway components are frequently mutated in conditions which impact intestinal homeostasis such as cancer.

The *Drosophila* Wnt homologue *wingless* was identified in a screen for mutations affecting segment formation in the developing larva (Nusslein-Volhard and Wieschaus, 1980). The first mammalian homologue, *Wnt1* (originally called *int1*), was found in a screen for MMTV integration sites (Nusse and Varmus, 1982; Rijsewijk et al., 1987). Since this time, our understanding of the canonical Wnt signaling pathway has evolved significantly.

Wnt proteins are the ligands of Wnt signaling. In mice and humans, there are 19 *Wnts* encoding proteins of approximately 40kDa with conserved cysteine regions. Wnt proteins are highly glycosylated, a modification that is important for both secretion and signaling (Franch-Marro et al., 2008; Takada et al., 2006; Willert et al., 2003). In this regard, purification of the *Wnt3a* gene product also showed a palmitoyl moiety attached at a conserved cysteine residue, the removal of which results in reduced biological activity (Willert et al., 2003), with similar results obtained with other fatty acid modifications of Wnt3A as well as wingless, Wnt5A and Wnt8 (Janda et al., 2012; Komekado et al., 2007; Kurayoshi et al., 2007; Takada et al., 2006). Although Wnt proteins are often described as morphogens, their long range signaling capacity is limited,

and may rely on lipoproteins and other binding partners for transport (Mulligan et al., 2012; Panakova et al., 2005; Zecca et al., 1996). In the intestinal stem cell niche, Wnt proteins seem to act predominantly as short range secretory molecules (Sato et al., 2011b; Sato et al., 2009).

In the absence of a Wnt ligand, the transcriptional effector β-catenin is bound by a destructor complex consisting of Dishevelled, CK1, GSK3, Axin, WTX and APC, which controls the cytoplasmic levels of free β-catenin. Axin, APC and WTX are scaffold proteins which connect β-catenin to the co-receptor LRP5/6. Phosphorylation of β-catenin by the serine-threonine kinases CK1 and GSK3 leads to its ubiquitination and subsequent proteasomal degradation (Aberle et al., 1997) (Figure 12).

Wnt proteins signal via binding to receptors of the *Frizzled* (*Fz*) family, which has ten members encoding seven pass transmembrane proteins with cysteine rich domains for Wnt binding (Bhanot et al., 1996). LRP5/6 co-receptors are single pass transmembrane proteins which are also essential for Wnt signaling (Pinson et al., 2000; Tamai et al., 2000). Receptor-ligand interaction results in a conformational change in the receptor, leading to the binding of Axin and phosphorylation of the LRP5/6 tail by GSK3 and CK1 (Davidson et al., 2005; Mao et al., 2001; Tamai et al., 2004; Zeng et al., 2005). Previous studies suggested that this leads to dissociation of the destructor complex by sequestering Axin, allowing for nuclear translocation of β-catenin (Mao et al., 2001; Tolwinski et al., 2003). More recent work through analysis of endogenous proteins suggests that relocalization of Axin results in movement of the entire destructor complex, including β-catenin, leading to inhibition of β-catenin ubiquitination. This results in saturation of the

destructor complex, allowing newly synthesized β-catenin to escape phosphorylation and degradation and translocate to the nucleus (Li et al., 2012) (Figure 12).

In the absence of a Wnt signal, Lymphoid enhancer factor/T-cell factor (LEF/TCF) transcription factors, thought to be resident at LEF/TCF response elements (LREs) on Wnt target genes, recruit co-repressors such as Groucho which results in transcriptional repression (Cavallo et al., 1998; Roose et al., 1998). Binding of β-catenin to a LEF/TCF member results in recruitment of co-activators such as CBP and Brg1, leading to increased transcription of Wnt target genes (Behrens et al., 1996; Molenaar et al., 1996; van de Wetering et al., 1997).

Several animal models have been produced which illustrate the importance of Wnt signaling in the intestine. Loss of the *TCF4* (also called *Tcf7l2*) transcription factor in *Tcf7l2*$^{-/-}$ mice leads to postnatal lethality, likely due to intestinal insufficiency. Although these mice develop an intestinal epithelium, the intervillus spaces are devoid of proliferative cells, resulting in an epithelium composed entirely of differentiated cells (Korinek et al., 1998). Similarly, ablation of intestinal β-catenin in conditional mutants results in loss of the proliferative compartment in the intestinal epithelium (Ireland et al., 2004). Complementary studies with transgenic mice expressing the secreted Wnt antagonist Dkk1 in the intestinal epithelium has similar effects, suggesting Wnt involvement in regulation of the intestinal stem cell niche (Kuhnert et al., 2004; Pinto et al., 2003). Germline mutations in the negative Wnt regulator APC typically result in intestinal tumourigenesis similar to human Familial Adenomatous Polyposis (FAP) (Kinzler et al., 1991; Luongo et al., 1993; Nishisho et al., 1991; Su et al., 1992), indicative of the role of Wnt signaling in aberrant proliferation. Consistent with this,

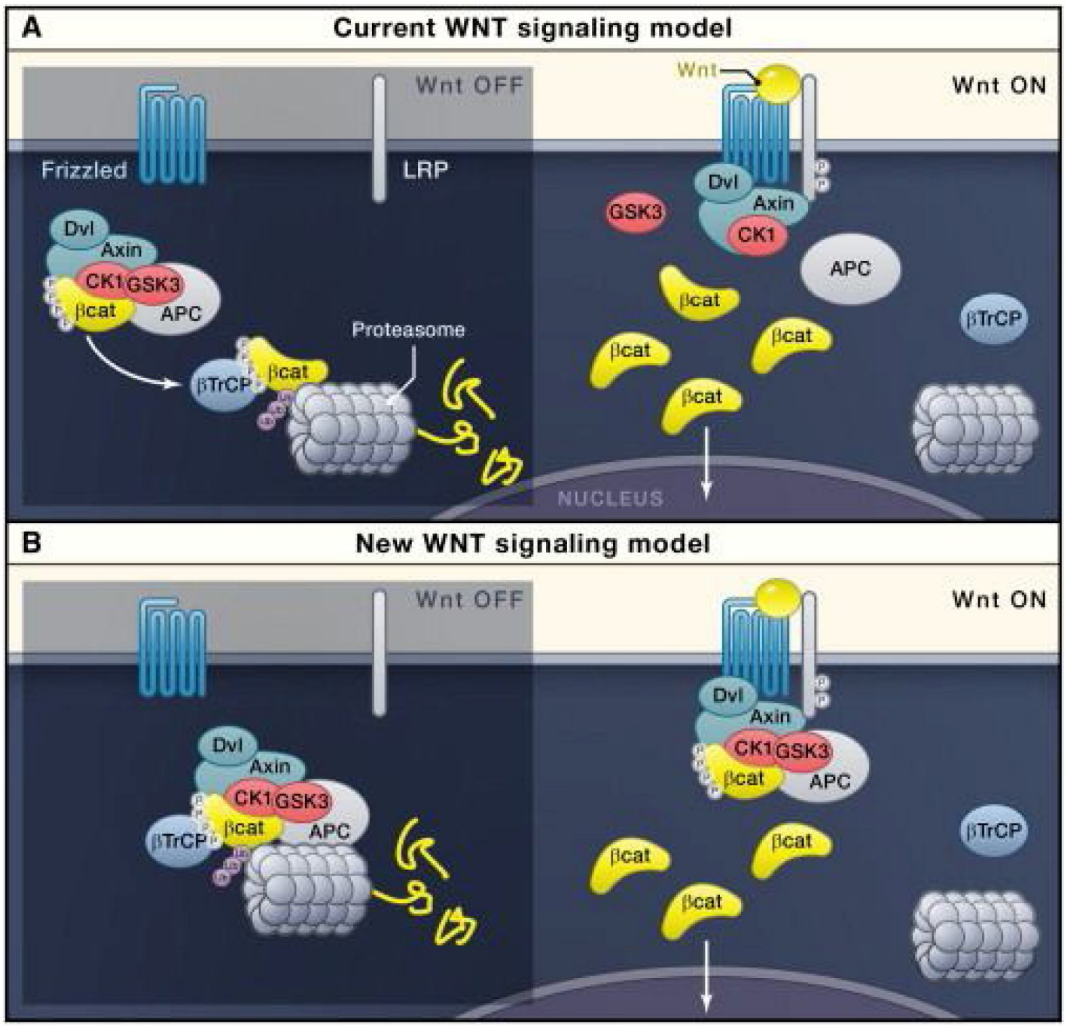


Figure 12: Wnt Signaling at the Receptor and Destruction Complex Level (A) The current Wnt model. In the absence of Wnt, the destruction complex resides in the cytoplasm, where it binds and phosphorylates β-catenin. The latter then leaves the complex to be ubiquitinated by β-TrCP and is then degraded by the proteasome. Wnt induces the association of Axin with phosphorylated LRP. The destruction complex falls apart, and β-catenin is stabilized. (B) A new model based on studying endogenous destruction complex components (Li, *et al*, 2012). In the absence of Wnt, the destruction complex resides in the cytoplasm, where it binds, phosphorylates, and ubiquitinates β-catenin by β-TrCP. The proteasome recycles the complex by degrading β-catenin. Wnt induces the association of the intact complex with phosphorylated LRP. After binding to LRP, the destruction complex still captures and phosphorylates β-catenin, but ubiquitination by β-TrCP is blocked. Newly synthesized β-catenin accumulates. Figure and legend adapted with permission from Clevers and Nusse, 2012. Cell by CELL PRESS. Reproduced with permission of CELL PRESS in the format reuse in a thesis/dissertation via Copyright Clearance Center.

overexpression of stabilized β-catenin also results in intestinal tumourigenesis (Harada et al., 1999). Wnt signaling has also been implicated in the maturation of Paneth cells, differentiation of Goblet cells and patterning of the intestinal epithelium (Gregorieff et al., 2004; Ireland et al., 2004; Pinto et al., 2003; van Es et al., 2005a). Taken together, these results support a role for Wnt signaling in development and homeostasis of the intestinal tract.

Other signaling cascades important to intestinal development

Other developmental signaling pathways are also involved in establishment of the intestinal tract, but are largely outside the scope of this thesis and will therefore be discussed only briefly. For example, Transforming Growth Factor (TGF)β signaling has an important role in the early establishment of endoderm and maintenance of the epithelium later. Briefly, a secreted ligand of the TGFβ superfamily of proteins (TGFβ, BMP or activins), binds to a heterodimer of Type I and Type II serine-threonine kinase receptors (Reissmann et al., 2001). Phosphorylation of the Type I receptor leads to Smad2 and/or Smad3 phosphorylation, association of p-Smad2/3 with Smad4, subsequent translocation to the nucleus, leading to an interaction with transcriptional activators FoxH1 and Mixer, and transcription of target genes (Chu et al., 2004; Dunn et al., 2004; Hart et al., 2002; Kunwar et al., 2003; Yamamoto et al., 2001). The importance of TGFβ in endoderm is exemplified by loss of function studies with the TGFβ ligand Nodal. Nodal is expressed in the primitive streak (Zhou et al., 1993), where it acts to specify lineages (Vincent et al., 2003). While homozygous null *Nodal* mutants do not gastrulate (Gu et al., 1998), other hypomorphic mutants fail to form endoderm (Lowe et

al., 2001; Norris et al., 2002), as is also seen in mutants for other components of the Nodal signaling cascade (Chu et al., 2004; Dunn et al., 2004; Kunwar et al., 2003; Yamamoto et al., 2001). At later stages, TGFβ superfamily members regulate proliferation and differentiation in the intestinal epithelium, and play a complex role in tumourigenesis (Ashcroft et al., 1999; Datto et al., 1999; Eppert et al., 1996; Fearon and Vogelstein, 1990; Grady et al., 1999; Kurokowa et al., 1987; Markowitz et al., 1995; Sodir et al., 2006; Yoshinaga et al., 2008; Zhu et al., 1998).

The Hedgehog signaling pathways is involved in patterning and maintenance of neurectoderm, mesoderm and endoderm derivatives. Briefly, there are three ligands, Sonic hedgehog (Shh), Indian hedgehog (Ihh) and Desert hedgehog (Dhh) in the mouse (Chiang et al., 1996; Echelard et al., 1993). The Patched (Ptch) receptors are twelve pass transmembrane receptors which bind to all three ligands and inhibit the seven pass transmembrane Smoothened (Smo) co-receptor through a mechanism that is poorly defined, but ultimately targets members of the glioblastoma transcription factor family (Gli), which are typically inhibited in the absence of ligand (Alcedo et al., 1996; Laufer et al., 2012; McMahon et al., 2003; Taipale et al., 2002; van den Heuvel and Ingham, 1996). Upon ligand binding, processed Gli is released, translocates to the nucleus and participates in transcription of target genes (Alexandre et al., 1996; Murone et al., 2000; Pan et al., 2006). Among other defects, deletion of *Shh* in mice results in defects impacting the esophagus, trachea and lung, indicative of a role for Shh in foregut development (Litingtung et al., 1998). Furthermore, Shh in the endoderm directs branching morphogenesis of the lung mesenchyme and patterning of the radial gut tube axis, indicative of its role in epithelial-mesenchymal interactions (Litingtung et al., 1998;

Pepicelli et al., 1998; Sukegawa et al., 2000). In the gut tube itself, Shh mutants have loss of smooth muscle and gut tube malrotation, as well as an overgrowth of the stomach and small intestinal epithelia and imperforate anus (Ramalho-Santos et al., 2000), altogether supporting a role for Shh signaling in patterning and differentiation of the intestinal tract.

Retinoic acid (RA) is the active derivative of vitamin A and regulates several processes in development including (but not limited to) eye development and axial patterning. RA is synthesized from dietary retinol in a two step oxidation reaction involving first either alcohol dehydrogenases (ADHs) or retinal dehydrogenase (RDHs) to produce retinaldehyde and second, retinaldehyde dehydrogenases (RALDHs) to generate RA from retinaldehyde. RA signaling is negatively regulated by degradation by the CYP26 family of enzymes. Once in the nucleus, RA binds to retinoic acid receptors (RAR), which are thought to be resident on the DNA with retinoid X (RXR) co-receptors, leading to transcription of target genes (Rhinn and Dolle, 2012). Although not thoroughly understood, RA appears to have a regulatory role in the intestine. For example, withdrawal of vitamin A in weanling rats results in loss of Goblet cells, suggesting that RA may regulate intestinal differentiation (Rojanapo et al., 1980). Indeed, a complementary study with RA treatment of rat organoids *in vitro* or in tissue grafts indicated that RA induces villous outgrowth, epithelial differentiation and crypt fission. These effects may be mediated in part by the mesenchyme (Plateroti et al., 1997) as components for RA synthesis are expressed in this compartment supporting this notion (Frota-Ruchon et al., 2000; Niederreither et al., 2002); this has, however, yet to be formally demonstrated. Finally, RA signaling has been implicated in gut looping,

patterning, morphogenesis and innervation (Pitera et al., 2001; Rhinn and Dolle, 2012; Wang et al., 2006), demonstrating its importance during intestinal morphogenesis.

Transcriptional regulation of gene expression

The concerted involvement of the developmental signaling pathways discussed above eventually contributes to the spatial and temporal regulation of gene expression, necessary for the coordinated and complex cell morphogenesis during development. Gene expression is often tightly regulated to allow for rapid expression of target genes in response to developmental cues regulating events such as migration, differentiation and proliferation. Developmental processes often depend on simultaneous and coordinated changes in expression of several genes in the same cells, or different cells across a tissue; subsequent rapid downregulation of these transcripts is often also important. These multifactorial signaling cascades are often initiated by a common transcription factor, or "master regulator". How the developing embryo achieves such dynamic and tightly regulated gene expression remains an active area of research.

RNA Polymerase II (Pol II) transcribes all protein coding transcripts in the genome, a process that requires it to access the transcriptional start site (TSS) as well as the subsequent recruitment and assembly of the transcriptional elongation complex. The precise mechanisms of gene promoter regulation are incompletely understood. They are thought to encompass a core promoter and proximal promoter regions, with distal regulatory enhancer elements also frequently contributing to regulation of expression. Regulatory DNA sequences of these regions are thought to contribute to the recruitment of specific transcriptional co-activators and co-repressors, ultimately regulating

expression of target genes. Briefly, a pre-initiation complex (PIC) consisting of a TATA binding protein (also known as TFIID), DNA helicases, the Mediator complex and Pol II, along with the general transcription factors TFIIA, TFIIB, TFIIE, TFIIF and TFIIH, is recruited to the core promoter region to unwind DNA and create a transcription bubble, a process which may require the movement of nucleosomes, as discussed below (Baek et al., 2002; Choder and Aloni, 1988; Flanagan et al., 1991; Kim et al., 1994; Mittler et al., 2001). Pol II then initiates transcription at the core promoter and pauses at the proximal promoter region, where the complex is hyperphosphorylated, resulting in elongation (Dahmus, 1976; Hatfield et al., 1983). Elongation is terminated at the end of the transcript and the Pol II complex is recycled for further rounds of transcription (Cho et al., 1999). This process is thought to be regulated by transcription factors bound to both proximal and distal regulatory enhancer regions (for example Banerji et al., 1981; Maston et al., 2012; Soleimani et al., 2012; Vacik et al., 2011).

Pol II-dependent transcription is subject to tight regulation at the level of Pol II access to the DNA and PIC formation, initiation after pausing and productive transcription. These phases of Pol II localization were demonstrated by ChIP studies in several species showing that Pol II can be absent, localized to the 5' end or evenly distributed throughout a gene, respectively (Guenther et al., 2007; Muse, 2007; Venters and Pugh, 2009; Zeitlinger, 2007). Although transcriptional initiation seems to be an important mechanism in single-celled transcriptional regulation, transcription in multicellular organisms appears to be regulated primarily at the level of Pol II pausing (Core and Lis, 2008; Guenther et al., 2007; Muse, 2007; Zeitlinger, 2007). Therefore, once transcription has been initiated, the transcriptional complex must escape from

pausing to productively elongate the nascent transcript and there are several factors which influence this barrier. Pausing may represent a mechanism whereby these genes are poised for transcriptional activation by recruitment of activators. In this regard, nucleosomes consist of 146bp of DNA wrapped around an octamer of core histones (H3, H4, H2A and H2B) (Uberbacher and Bunick, 1989); these are known to inhibit transcription (Archer et al., 1991; Han and Grunstein, 1988; Izban and Luse, 1991; Knezetic and Luse, 1986; Lorch et al., 1987; Workman and Roeder, 1987). Moreover, in yeast and mice, genome-wide studies have indicated that actively transcribed genes are shifted localization of nucleosomes (Changolkar and Pehrson, 2006; Lee et al., 2004), suggesting that histones inhibit transcriptional elongation and that some nascent nuclear factors must alleviate this inhibition.

There are two classes of enzymes affecting chromatin structure: those that remodel histone localization and those that modify histone tails. Histones themselves are extensively modified at the post-translational level, with several different modifications of their tails such as acetylation and methylation which may have effects on transcription. The first description of this phenomenon demonstrated that acetylation of histones increased the rate of transcription of mRNA in a dose dependant manner (Allfrey et al., 1964). It has since been shown that modifications of histone tails at lysine, arginine, serine, threonine, tyrosine, histidine and glutamic acid residues can either recruit transcription factors, or change chromatin conformation, thus affecting transcriptional readout (Bernstein et al., 2005; Liu et al., 2005; Mikkelsen, 2007; Pokholok, 2005; Schubeler et al., 2004). Chromatin remodelers are ATP dependant, multimeric protein complexes subdivided into several families, that bind with high affinity to histones,

allowing for their displacement (Cairns et al., 1994; Delmas et al., 1993; Ebbert et al., 1999; Elfring et al., 1994; Peterson et al., 1994). The mechanisms through which chromatin remodelers regulate gene expression are not entirely clear, however, these effects are thought to be mediated by an increase in transcription factor binding in an ATP-dependent manner (Cote et al., 1994), ultimately leading to the displacement of histones and unwinding of DNA (Lorch et al., 2010). Taken together, these results demonstrate the intricate involvement of chromatin remodeling complexes in transcriptional regulation, which provides the specific patterns of gene expression necessary for developmental processes such as patterning.

CDX REGULATION OF DEVELOPMENT

Anterior-posterior patterning and Hox genes

Development gives rise to many structures which are patterned along the anterior-posterior, left-right or dorsal-ventral axes of the embryo. For example, along the anterior-posterior axis of the developing embryo, there are metametric structures of paraxial mesoderm, the somites, which give rise to the axial skeleton and its associated musculature. These derivatives are subject to patterning according to their position along the anterior-posterior axis of the embryo. The manner by which the embryo acquires anterior-posterior patterning of derivatives of the mesoderm and neurectoderm has been the subject of many studies, and the *Hox* genes products are classic regulators of these processes.

Early work in *Drosophila* found that mutations associated with duplicated abdominal segments and legs, which localized to the *Bithorax* (*BX-C*) gene complex, resulted from a transformation of posterior segments to a more anterior identity, a phenomenon termed homeosis. The products of this gene cluster were found to regulate patterning of the thoracic and abdominal segments of the fly (Lewis, 1978). Later studies identified the *Antennapedia* (*ANT-C*) complex as functioning in a manner analogous to *BX-C* (Lewis et al., 1980). Identification of a common, conserved, DNA and protein sequence termed the homeobox suggested that products of the *ANT-C* and *BX-C* complexes act in a similar manner (McGinnis et al., 1984a; McGinnis et al., 1984b; Regulski et al., 1985). It was later determined that gene products of the homeobox family encode DNA-binding homeodomain (HD) transcription factors (*Hox* genes in vertebrates) (Laughton, 1984; Mazo et al., 1990; Zappavigna et al., 1991). These likely arose from an ancestral duplication of a common *HOM-C* cluster (Akam, 1987).

In mammals, there are 39 *Hox* homologues, divided into four clusters, *HoxA, B, C* and *D*, all of which are thought to have arisen from a *HOM-C* in primitive insects (Schughart et al., 1989). Each *Hox* cluster contains paralogous groups, numbered 1-13 according to their physical localization in the cluster and relatedness of their homeobox sequences. No single cluster, however, contains all 13 paralogs. Interestingly, the *Hox* genes are arrayed 3' to 5' in the genome, which correlates with the timing of onset and anterior limit of their expression (Dressler and Gruss, 1989; Duboule and Dolle, 1989; Dubrulle and Pourquie, 2004; Durston et al., 2011; Izpisua-Belmonte et al., 1991a; Izpisua-Belmonte et al., 1991b). Accordingly, *Hox* genes from the first paralog groups are expressed earliest in the anterior-most portion of the primitive streak and are encoded

by the most 3' regions of the *Hox* cluster. Likewise, group 13 members have the latest onset of expression, exhibit more posterior-restricted expression domains and are localized at the 5' extremity of the *Hox* clusters (Figure 13). As in *Drosophila*, mammalian *Hox* gene products regulate patterning along the anterior-posterior axis of the embryo.

The mechanisms for *Hox* regulation of anterior-posterior patterning are not completely understood. The domains of *Hox* expression begin at varying axial levels and extend to different degrees, resulting in a nested, overlapping array of gene expression. As a result, any one metameric structure, such as a somite, displays a unique combination of *Hox* transcripts suggestive of a "Hox code" regulating positional identity (Kessel and Gruss, 1990). Furthermore, to explain certain Hox mutant phenotypes, it has been hypothesized that a mechanism of "posterior prevalence" may be involved. This supposes that more posteriorly expressed *Hox* genes are dominant to anterior ones; this explains why loss of Hox function affects only domains anterior to its domain of expression (Duboule and Morata, 1994) (Figure 14).

Drosophila Caudal

Several other homeodomain proteins are also known to regulate anterior-posterior patterning of the embryo. *Caudal (cad)* is a homeobox-containing gene isolated in *Drosophila* which exhibits a posterior high gradient of expression in the gut, gonads and genital disc (Mlodzik et al., 1985; Mlodzik and Gehring, 1987). At the time that *cad* was isolated, products of the *Hox* genes had just been identified as transcriptional regulators of segmentation, but were usually expressed in a very specific spatial-temporal manner

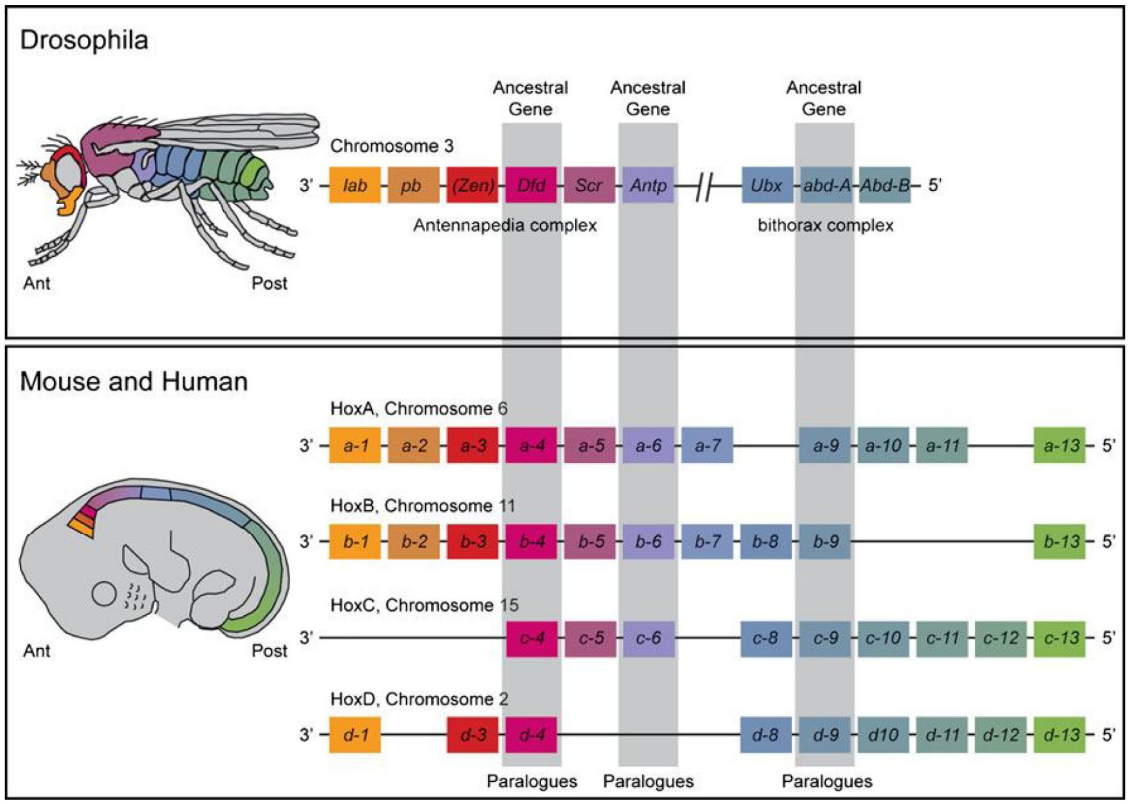


Figure 13: Hox genes in mouse and human with their phylogenetic counterparts in Drosophila. 39 *Hox* genes are involved in the mouse and human vertebral column, found in four clusters of *Hox* A, B, C and D on four chromosomes (6, 11, 15 and 2), designated by Arabic numbers within each cluster and arranged as paralogs, so that the lower numbered *Hox* paralogs such as *Hox* a-1 and *Hox* d-1 are located on the anterior 3′ position of the chromosomes, and the higher numbered paralogs such as *Hox* a-13 and *Hox* d-13 are on the 5′ posterior position of the chromosomes. There is also temporal and structural colinearity with the embryonic axis so that the lower numbered paralogs are expressed earlier and more anterior on the embryonic axis than the higher numbered paralogs. (See colour match between genes and their expression domains on the embryonic axis.) Three sets of paralogs and their corresponding ancestral genes are designated by the grey bars. Figure and legend adapted with permission from Pang and Thompson, 2010.

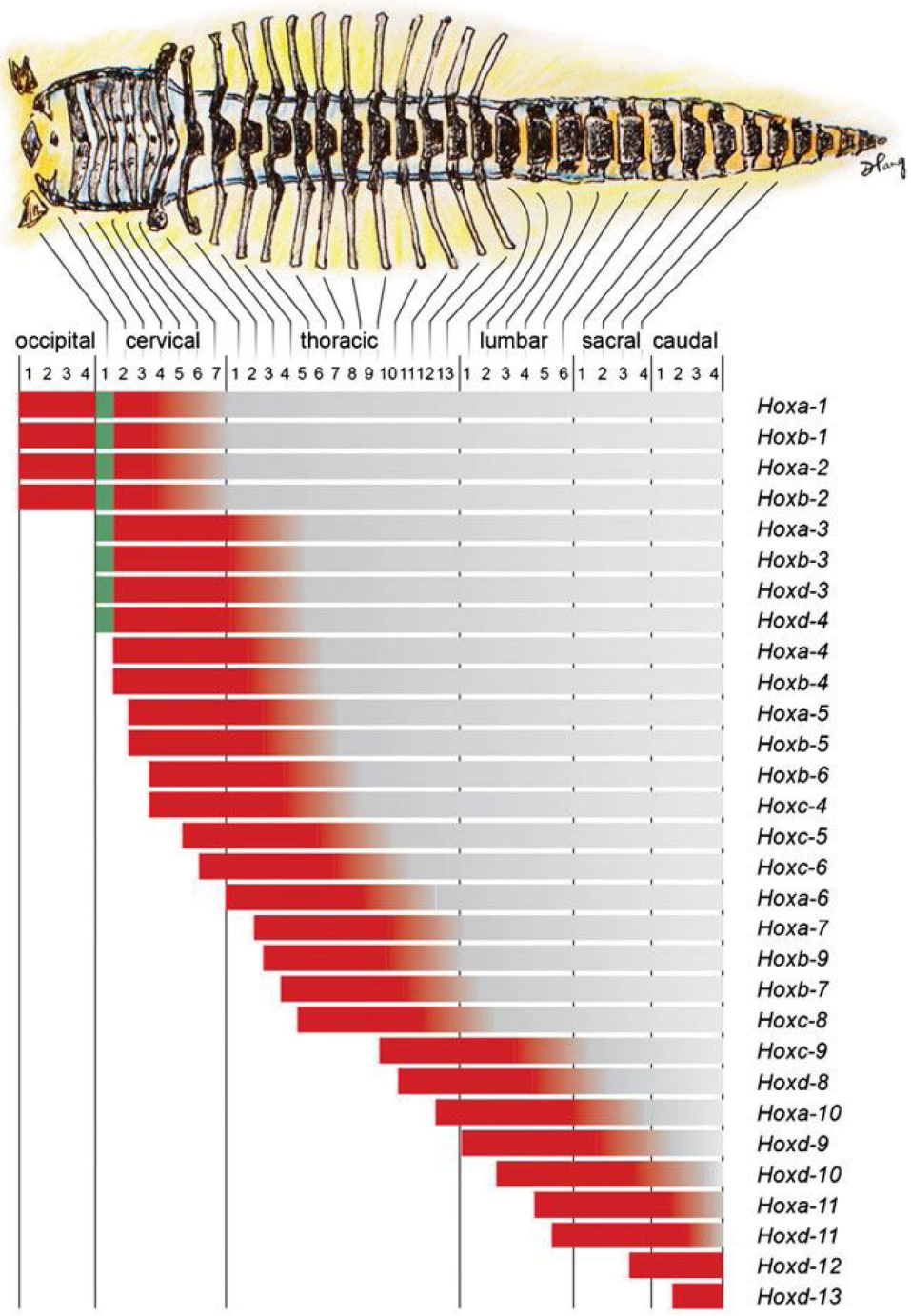


Figure 14: Expression domains of Hox genes in the mouse embryonic vertebral column. Each prevertebral segment has its own combination of Hox gene expression domains (Hox code). Figure and legend adapted with permission from Pang and Thompson, 2010.

(Lewis, 1978, 1982; Struhl, 1981, 1982, 1983). The early and global expression domain of *cad* suggested a broader role in patterning processes. Indeed, genetic mutation of *cad* results in segmentation defects and disruption of metameric pattern formation along the anterior-posterior axis of the fly embryo (Macdonald and Struhl, 1986), at least in part due to its direct regulation of pair-rule gene *fushi tarazu* (Dearolf et al., 1989). In a complementary study, it was shown that heat inducible overexpression of *cad* results in posteriorization of anterior structures, and disruption of patterning, presumably due to misregulation of segmentation genes such as *fushi tarazu*, *engrailed* and *Deformed* (Mlodzik et al., 1990). CAD therefore plays an important role in patterning the anterior-posterior axis in *Drosophila*, behaving in a manner analogous to *Hox* gene products in the caudal embryo.

Mammalian Cdx genes

Murine homeodomain transcription factors related to CAD are encoded by the *Cdx* genes, *Cdx1* (Duprey et al., 1988), *Cdx2* (James and Kazenwadel, 1991) and *Cdx4 (Gamer and Wright, 1993)*. *Cdx3* was originally cloned from a hamster pancreas cDNA library (German et al., 1994), but was later determined to be *Cdx2* (Mallo et al., 1997; Suh et al., 1994). All three *Cdx* genes exhibit overlapping expression domains in the posterior mouse embryo (Figure 15).

Onset of *Cdx1* expression begins around E7.5 in the primitive streak region in all three germ layers and it perdures in the tailbud until approximately E12 (Hierholzer and Kemler, 2009). In the ectoderm, *Cdx1* is briefly expressed in the future hindbrain and

spinal cord, while in the mesoderm it is transiently expressed in the myotomal compartment of the somites, the proximal developing limb buds and the mesonephros (Meyer and Gruss, 1993). *Cdx1* expression reinitiates in the endoderm commencing at E12.5, where it is seen in a posterior-high gradient with highest expression in the distal prospective colon with expression persisting throughout the lifespan of the animal (Duprey et al., 1988; Meyer and Gruss, 1993; Silberg et al., 2000). In the intestinal epithelium, *Cdx1* is expressed differentially along the crypt-villus axis, with more abundant levels in the stem-cell associated crypt region than in the differentiated villus cells (Silberg et al., 2000; Subramanian et al., 1998) (Figure 16).

Cdx2 expression is reported to begin at E8.5 in the posterior portion of the embryo proper, in the hindgut, the posterior neural tube and the tailbud. By E12.5, expression of *Cdx2* in the embryo is limited to the gut tube endoderm caudal to the foregut/midgut boundary, where it persists throughout adulthood. There is also an early onset of expression in the trophectoderm at E3.5, which is maintained in the placenta until E12.5 (Beck et al., 1995; Chawengsaksophak et al., 2004). In the intestinal epithelium, *Cdx2* expression peaks in the cecum and diminishes in either direction (James et al., 1994; Silberg et al., 2000). *Cdx2* expression along the crypt-villus axis is uniform, but the protein exhibits a crypt-high gradient of phosphorylation, which may impact on its transcriptional activity (Rings et al., 2001) (Figure 16).

Cdx4 has more restricted expression domain, beginning at E7.0 in the allantois and the primitive streak. It is transiently expressed in the posterior hindgut endoderm, neurectoderm, lateral plate and presomitic mesoderm until it is extinguished around

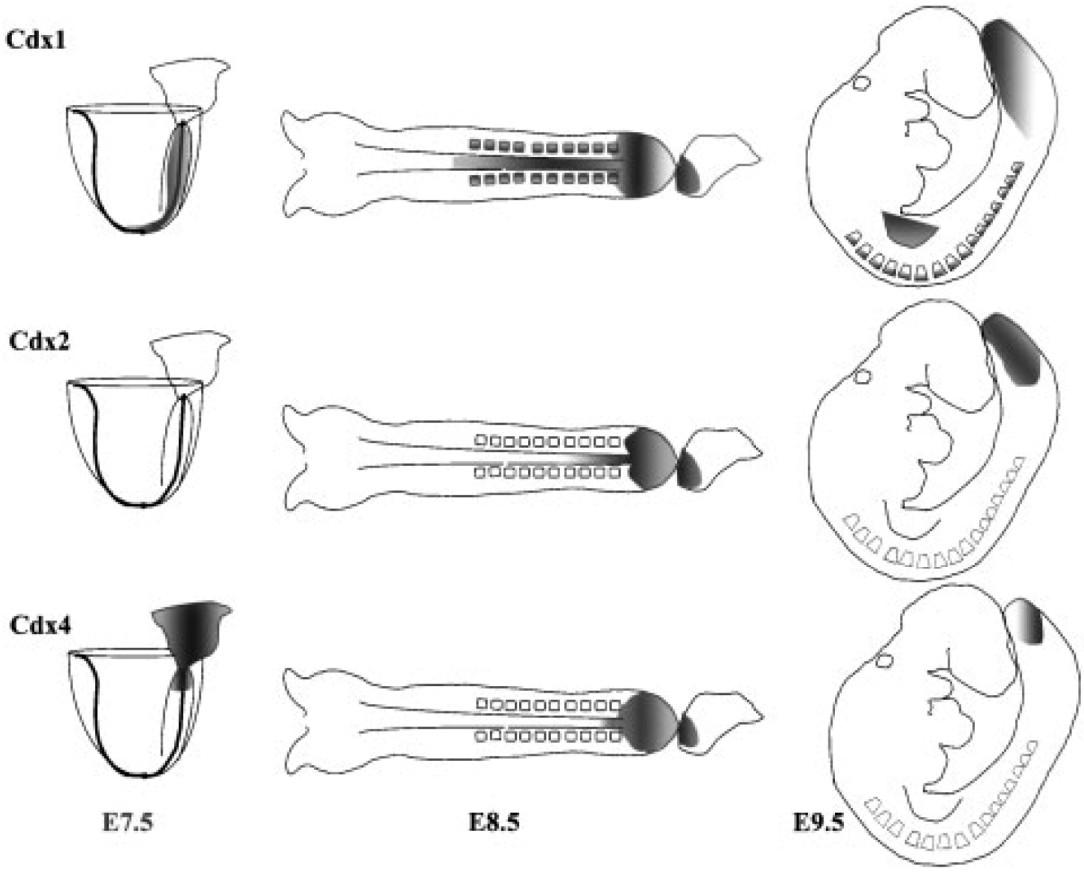


Figure 15: Representation of Cdx expression in E7.5–E9.5 mouse embryos. Relative level of expression is denoted by shading for each gene. Extraembryonic expression is not indicated. Figure and legend adapted with permission from Lohnes, 2003. BioEssays by INTERNATIONAL COUNCIL OF SCIENTIFIC UNIONS ; COMPANY OF BIOLOGISTS Reproduced with permission of JOHN/WILEY & SONS LTD. in the format reuse in a dissertation/thesis via Copyright Clearance Center.

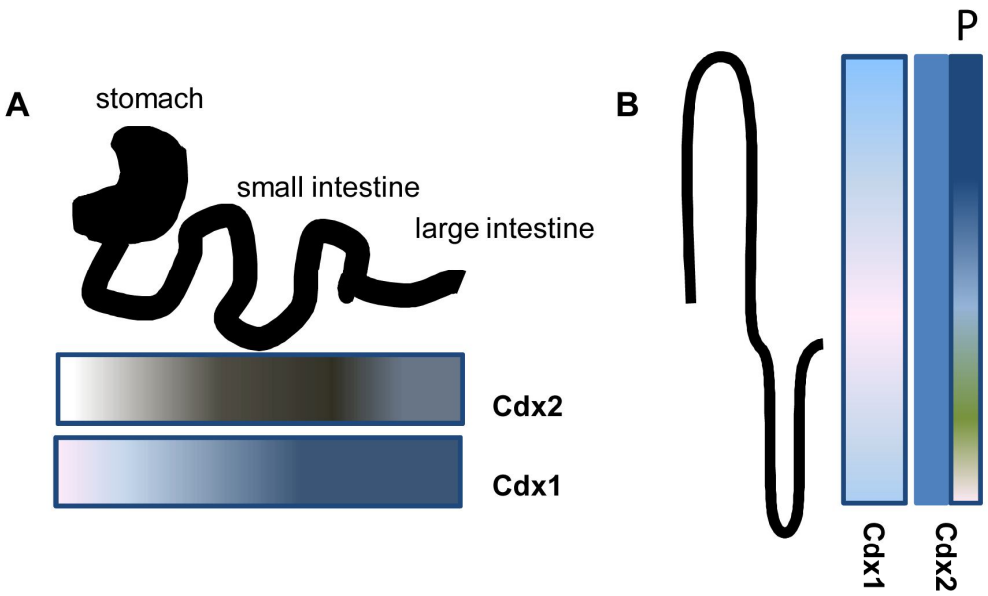


Figure 16: Intestinal expression of Cdx1 and Cdx2. Along the anterior-posterior axis (A), Cdx1 is expressed most predominantly in the distal colon, diminishing towards the anterior intestine. Cdx2 is expressed most robustly in the cecum, dissipating in either direction. (B) Along the crypt-villus axis, Cdx1 expression is maximal in the base of the crypts, while Cdx2 is uniformly expressed, but differentially phosphorylated (P), as reflected by differences in shading.

E10.0 (Gamer and Wright, 1993). Unlike *Cdx1* and *Cdx2*, *Cdx4* is not detected in the intestinal epithelium and further discussion is outside of the scope of this thesis.

Cdx structure

Cdx transcription factors are characterized by their homeodomains, which are highly conserved between family members. However, outside of these sequences, they are quite divergent, with only three small conserved regions identified (Figure 17): a hexapeptide motif located just upstream of the homeodomain; an amino terminal signal peptide involved in processing; and a 9 amino acid region of unknown function between this and the hexapeptide motif (Gamer and Wright, 1993). A poorly defined transactivation domain is also located in the N-terminal region, which encompasses the first two conserved regions (Lynch et al., 2003; Taylor et al., 1997; Trinh et al., 1999).

Cdx loss-of-function mutants

The roles of the Cdx members in the mouse have been investigated using loss of function models. *Cdx1*$^{-/-}$ mutants are viable and fertile and exhibit anterior homeotic transformations of the cervical and upper thoracic vertebrae. Wild-type mice have seven cervical (C), thirteen thoracic (T) and six lumbar (L) vertebrae, while *Cdx1*$^{-/-}$ offspring exhibit malformations and transformations affecting several vertebrae in the cervical and upper thoracic region. For example, in *Cdx1*$^{-/-}$ mutants, the first cervical vertebra, C1, is typically fused to the base of the skull, while C2 has acquired characteristics of C1 (Figure 18). *Cdx1*$^{-/-}$ mice are otherwise normal, including their gastrointestinal tracts (Subramanian et al., 1995).

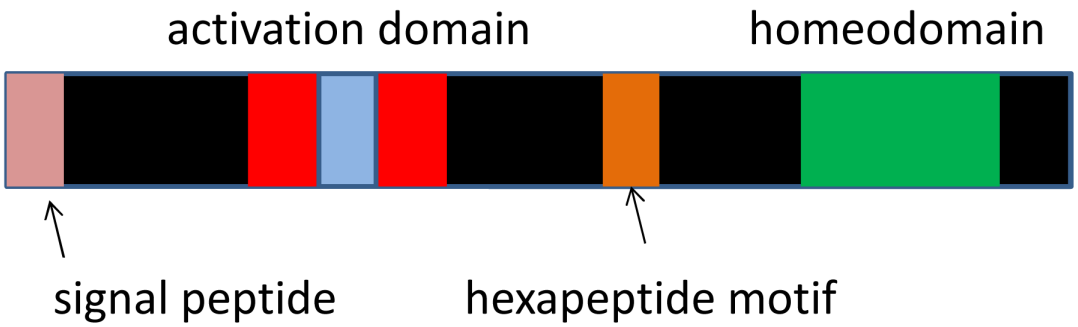


Figure 17: Cdx protein domains. Schematic representation of Cdx proteins and conserved domains including a signaling peptide (pink), the hexapeptide motif, (orange) and the homeodomain (green). The blue section represents a conserved 9 residue long region of unknown function. The activation domain (red) is located in the N terminal region.

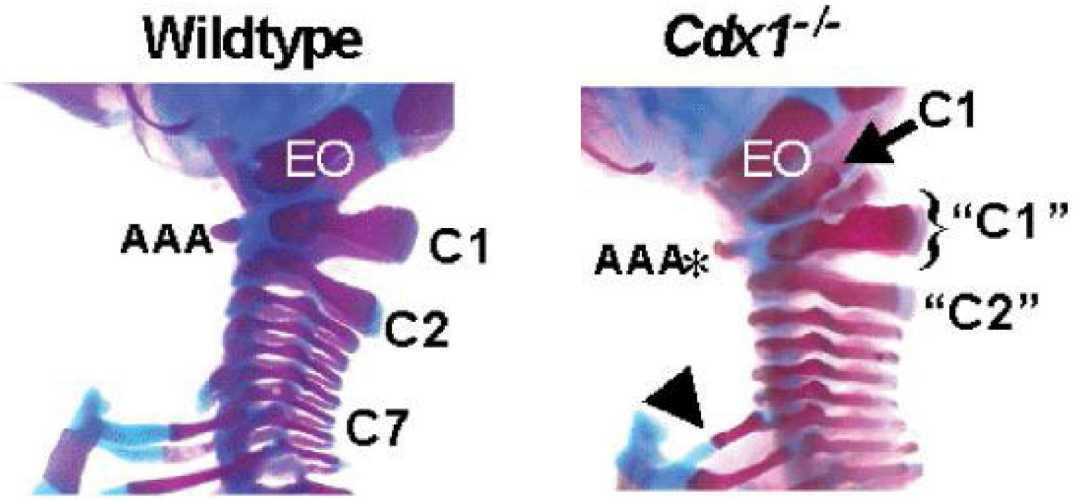


Figure 18: Cdx regulates vertebral patterning. Cervical section of E18.5 skeletons for WT (left) and *Cdx1*$^{-/-}$ (right) mice. Note that C1 is fused to the exoccipital and C2 acquires characteristics of C1 in *Cdx1*$^{-/-}$ mice. AAA- anterior arch of atlas, C- cervical, EO- exoccipital, AAA*- transformed anterior arch of atlas. Figure adapted with permission from Lohnes, 2003. BioEssays by INTERNATIONAL COUNCIL OF SCIENTIFIC UNIONS ; COMPANY OF BIOLOGISTS Reproduced with permission of JOHN/WILEY & SONS LTD. in the format reuse in a dissertation/thesis via Copyright Clearance Center.

The vertebral homeoses are consistent with posteriorized *Hox* gene expression in somites in Cdx1 mutants (Charite et al., 1998; Isaacs et al., 1998; Knittel et al., 1995; Schyr et al., 2012; Shashikant et al., 1995; Tabaries et al., 2005). Cdx-dependent regulation of *Hox* expression is likely through direct Cdx binding to response elements (CDREs) in the promoter regions of relevant targets. For example, it has been shown that CDREs in the *Hoxb8* promoter are necessary for normal *Hoxb8* transgenic expression and Cdx members can bind to this element directly *in vitro* (Charite et al., 1998). In addition, Cdx1 is required for proper initiation of *Hoxc8* expression during early embryogenesis through binding to CDREs in the proximal promoter region (Schyr et al., 2012; Shashikant et al., 1995). Finally, some Cdx null defects can be rescued by ectopic expression of *Hox* genes in both mice and zebrafish (Davidson et al., 2003; Young et al., 2009). Altogether, these results support regulation of *Hox* gene expression by Cdx members.

Cdx2$^{-/-}$ mutants are peri-implantation lethal due to an early role for Cdx2 in the trophectoderm needed for implantation. *Cdx2* heterozygotes are viable and fertile, but have a shortened or kinky tail and anterior homeoses of the cervical and thoracic vertebrae similar to *Cdx1*$^{-/-}$ mutants, indicative of overlapping function between these two proteins. Pertinently, *Cdx2*$^{+/-}$ adults between 12 and 28 weeks of age have rare tumours in the peri-cecal region of the intestinal tract and occasionally in the small intestine (Chawengsaksophak et al., 1997). Histological examination of these lesions revealed regions of epithelium reminiscent of the entire gastrointestinal tract, including stratified, keratinized esophageal, glandular stomach, small and large intestines. These hamartomas resemble a localized homeotic transformation of the gastrointestinal epithelium,

suggested to represent intercalary regeneration triggered by loss of expression of the remaining wild type Cdx2 allele (Beck et al., 1999; Chawengsaksophak et al., 1997; Tamai et al., 1999). In complementary studies, overexpression of either Cdx1 or Cdx2 under the regulatory control of stomach or esophagus specific promoters resulted in a transformation from stomach epithelium to small intestinal epithelium (Kong et al., 2011; Mutoh et al., 2002; Mutoh et al., 2009; Mutoh et al., 2004). Taken together, these findings support a role for Cdx members in establishment of posterior intestinal identity and endoderm patterning.

More recent evidence for *Cdx2* function in the intestine has been forthcoming from other model systems. In zebrafish, the functional equivalent to *Cdx2*, *Cdx1b*, has been shown to regulate intestinal cell proliferation and terminal differentiation (Chen et al., 2009; Flores et al., 2008). Morpholino knockdown of *Cdx1*, *Cdx2* and *Cdx4* in *Xenopus* results in misregulation of regionally expressed midgut and hindgut genes, resulting in loss of intestinal identity and disruption of gut coiling (Faas and Isaacs, 2009). The limitations of morpholino knockdown studies and the early embryonic lethality of the *Cdx2* null allele in mice have, however, precluded a better understanding of the function of Cdx2 in the endoderm, necessitating the development of conditional mutants.

Cdx2 conditional mutants

Several groups have now generated conditional Cdx2 mutants through Cre-loxP technology (Gu et al., 1994; Sauer, 1998) (Figure 19). To achieve this, the second exon of

Cdx2, which encodes most of the DNA binding homeodomain, was flanked by loxP sites ("floxed") using homologous recombination-based approaches. Transgenic mice bearing a Cre expression construct driven by spatially and temporally restricted promoter regions can be crossed with these floxed mice, resulting in Cre-mediated excision of sequences intervening two directly-repeated *loxP* sites. Additional temporal control can be achieved through use of a Cre-ER^T fusion protein, where Cre is fused to an estrogen receptor ligand binding domain which has been modified to respond to the mixed ER agonist tamoxifen (Metzger et al., 1995) and not endogenous estrogen.

Our lab has made use of a ubiquitously expressed *Actin* Cre-ER^T transgenic mouse line (Santagati et al., 2005) to effect temporal loss of Cdx2 function. *Cdx2*$^{f/f}$; *Actin* Cre-ER^T mice treated with tamoxifen by oral gavage at E5.5 are devoid of Cdx2 protein at E8.5, severely truncated at E9.5 and die around E11, indicating a role for Cdx2 in axis elongation (Savory et al., 2009a), and consistent with earlier work using tetraploid aggregates (Chawengsaksophak et al., 2004). Cdx2 manifests this function, in part, through direct regulation of key factors in axial elongation including *T*, *Wnt3a* and *Cyp26A1*. These findings also demonstrated that Cdx2 can operate through non-*Hox* pathways and that it integrates axial elongation and patterning (Savory et al., 2009a).

Treatment of mice as above precluded analysis of Cdx2 function in the intestinal tract due to lethality. Cdx2 can be conditionally inactivated in the intestine in a similar manner, using for example, a *villin* Cre-ER^T (el Marjou et al., 2004), as described later.

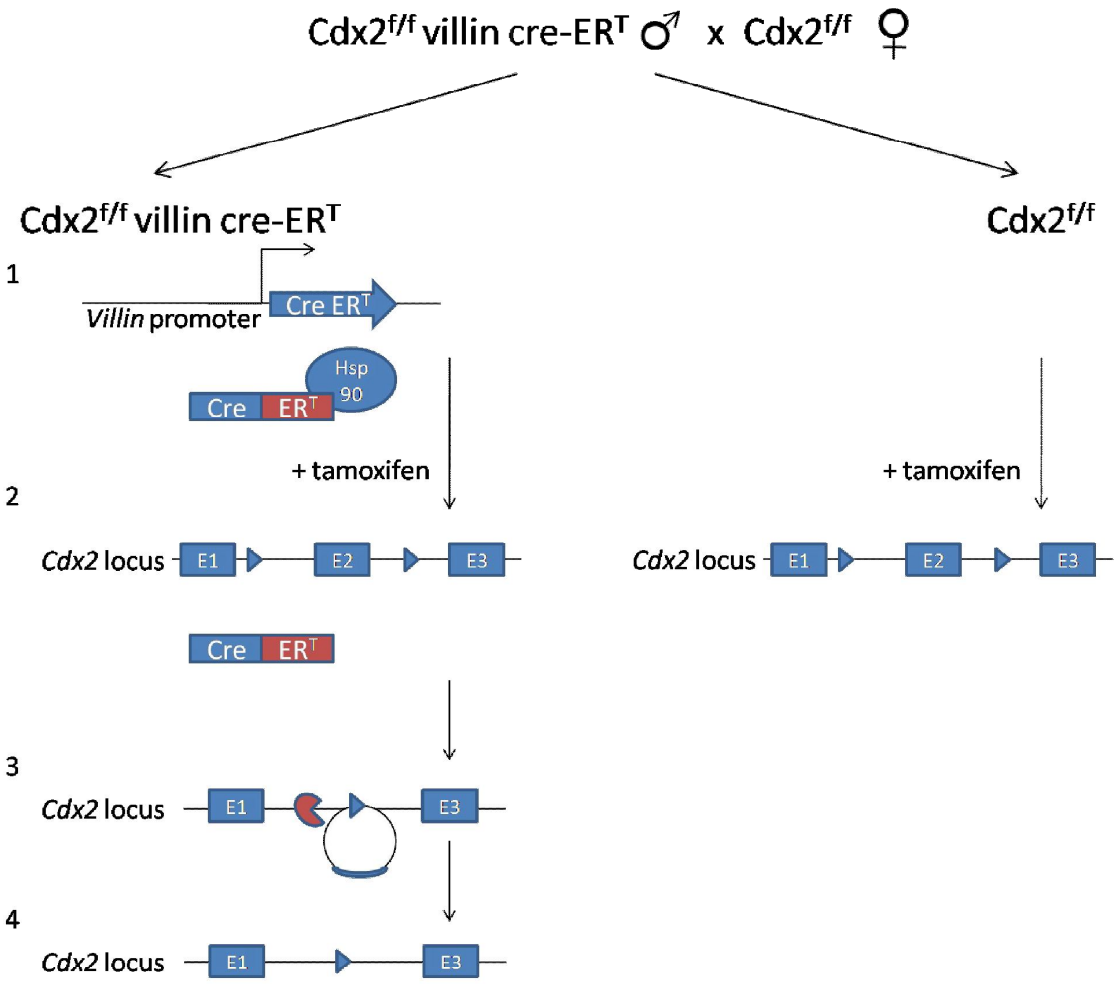


Figure 19: Cdx2 conditional knockout strategy. A *Cdx2$^{f/f}$ villin* Cre-ER^T male is bred with a *Cdx2$^{f/f}$* female to produce offspring with or without *villin cre* in a predicted 1:1 ratio. [1] *Cre* is transcribed under the spatial control of the villin promoter. The Cre protein is fused to a modified estrogen receptor ligand binding domain (ER^T) that will only respond to tamoxifen. In the absence of tamoxifen, association with Hsp90 results in cytoplasmic sequestration of the chimeric protein. [2] A portion of the *Cdx2* locus is flanked by two loxP sites (small blue triangles). Pregnant females are dosed with tamoxifen to release Hsp90 from the Cre-ER^T chimeric protein, resulting in its translocation to the nucleus and recombination between *loxP* sites and functional inactivation of Cdx2 [3-4]. Similarly, *Cdx1$^{-/-}$Cdx2$^{f/f}$* females can be bred with *Cdx1$^{-/-}$Cdx2$^{f/f}$ villin* cre-ER^T males to produce *Cdx1-Cdx2* double mutants. Littermate controls not expressing the transgene are unaffected by tamoxifen administration (right side)

Regulation of Cdx expression

Cdx expression is governed by a number of signaling pathways, including canonical Wnt, FGF and RA, as well as via autoregulation and cross-regulation. Of the three family members, regulation of *Cdx1* expression is perhaps the best understood. A 3.6kb promoter region can recapitulate expression in transgenic mice in all tissues except the definitive endoderm (Lickert and Kemler, 2002). In this regard, *Cdx1* is directly regulated by Wnt, RA and Cdx1 through two LREs and an RARE upstream of the transcriptional start site (Allan et al., 2001; Beland et al., 2004; Houle et al., 2000; Houle et al., 2003; Lickert et al., 2000; Lickert and Kemler, 2002; Pilon et al., 2007; Prinos et al., 2001). Consistent with this, *Cdx1* expression is induced by exogenous RA and compromised in *RAR* mutants (Houle et al., 2000). Moreover, loss of the putative RARE in the *Cdx1* promoter reduces *Cdx1* expression during embryogenesis (Houle et al., 2003). Additionally, combined *Cdx1* and *RAR* mutations in murine models suggest that RA acts upstream of *Cdx1* (Allan et al., 2001).

Several groups have shown that *Cdx1* is also downstream of Wnt signaling (Beland et al., 2004; Ikeya and Takada, 2001; Lickert et al., 2000). Interestingly, RA synergizes with Wnt signaling *in vitro*, as measured in cell-based promoter assays (Prinos et al., 2001), and also seen in mice (Houle et al., 2003). Transgenic expression analyses suggest that Wnt is required for normal onset of initiation and RA is needed for maintenance of early *Cdx1* expression in the embryo (Houle et al., 2000; Prinos et al., 2001); Furthermore, loss of the LREs *in vivo* abrogates the induction of *Cdx1* expression in response to RA, suggesting cooperation *in vivo* (Pilon et al., 2007); this relationship is less clear in the intestinal tract (Lickert and Kemler, 2002). Loss of both the endogenous

LREs and the RARE in the mouse results in a marked loss of *Cdx1* expression, and recapitulation of the *Cdx1*$^{-/-}$ phenotype.

Cdx1 autoregulation is achieved through Cdx1 binding to LEF/TCF members at the LREs on the *Cdx1* promoter (Beland et al., 2004; Lickert et al., 2000) (Figure 20). Consistent with this, *Cdx1* and *Wnt* interact genetically to regulate *Cdx1* expression, suggesting the two are involved in regulation of *Cdx1 in vivo* (Beland et al., 2004). Of note, LEF/TCF is also known to have an effect on endodermal *Cdx1* expression *in vivo* (Lickert et al., 2000).

Transgenic reporter analyses have shown that a region from -5151bp to +3912 of the *Cdx2* locus is sufficient to recapitulate expression from gastrulation to E10, with the exception of the trophectoderm and the intestinal tract. Endodermal and intestinal expression is partially recapitulated by a -9177bp to +102bp transgenic construct; the distal-most 1.4kb sequences are also responsive to endodermal transcription factors such as HNF4α and GATA6 in cell-based reporter assays, although additional sequences are required for complete intestinal expression (Benahmed et al., 2008). Like *Cdx1*, *Cdx2* is also downstream of Wnt signaling (Sherwood et al., 2011; Shimizu et al., 2005). Activation of canonical Wnt signaling through ectopic β-catenin expression or GSK3 inhibition causes an upregulation in *Cdx2* expression within 6 hours in intestinal explants, suggestive of direct regulation (Sherwood et al., 2011). Wnt signaling is upstream of *cdx4* and *cdx1a* (*Cdx4* and *Cdx2* in mouse) expression in zebrafish (Shimizu et al., 2005) and *Cdx4* in mouse (Pilon et al., 2006); regulation is achieved through modulating co-regulators of Wnt effector Tcf3 at the *cdx4* promoter in zebrafish (Ro and Dawid, 2011).

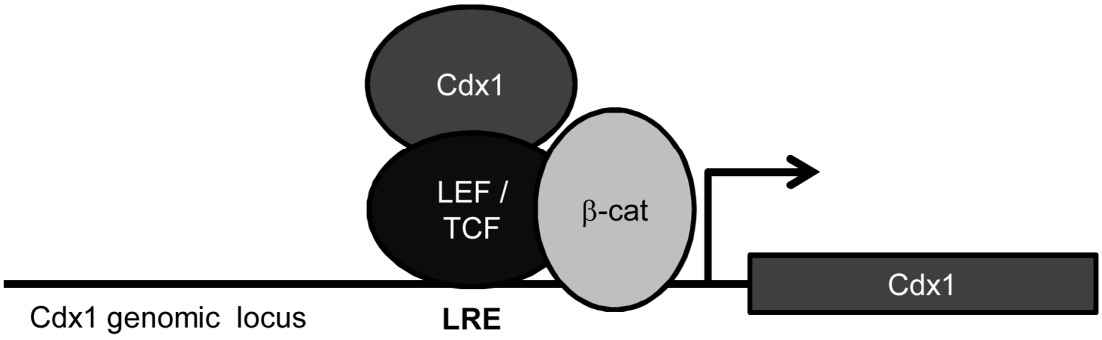


Figure 20: Cdx1 autoregulation. Regulation of the *Cdx1* promoter is achieved through TCF/LEF binding to a LEF/TCF response element (LRE) in the proximal Cdx1 promoter while Cdx1 binds to the LEF/TCF protein and its transcriptional co-regulator β-catenin (β-cat).

FGF signaling is necessary for expression of *Cdx1, 2* and *4* in *Xenopus* (Cocciolone et al., 2000). In *Xenopus tropicalis,* FGF regulation of *Xcad3* (*Cdx4* in mouse) maps to the first intron, where multiple transcription factors, including Lef/Tcf, Ets and Sox members, operate in concert (Haremaki et al., 2003). In the chick hindbrain, *CdxA* and *CdxB* (*Cdx1* and *Cdx4* in the mouse) are also necessary to convey the FGF signal to *Hox* genes (Bel-Vialar et al., 2002).

Functional Overlap and Specificity of Cdx members

Outside of the homeodomain, Cdx members have considerably divergent sequences (including in the transactivation domain) suggesting functional specificity. This is further supported by the different timing of onset, duration and location of their expression domains, as discussed above. To investigate functional specificity, several groups have modeled Cdx function in tissue culture, yielding conflicting results. For example, on some promoters, such as the *Cdx4* or *apical sodium-dependent bile acid transporter* promoters, Cdx2 more readily transactivates reporter expression than Cdx1 (Ma et al., 2012; Savory et al., 2011b). Conversely, *intestinal alkaline phosphatase (IAP)* is activated by Cdx1 and inhibited by Cdx2 in intestinal cells (Alkhoury et al., 2005). Moreover, expression of the calcium channel *MS4AI2* is induced by Cdx2, but not Cdx1 (Koslowski et al., 2009). However, in other models, Cdx members appear to act similarly. *Solute carrier family 5, member 8 (SLC5A8),* for instance, is comparably activated by Cdx1 and Cdx2 in two different colonic cell lines, and *SLC5A8* is comparably lost when either Cdx1 or Cdx2 is silenced in cell lines or *in vivo* (Svaren and Horz, 1997). Furthermore, in embryonal carcinoma cells, the promoter of the planar cell

polarity gene *Ptk7* is comparably induced by Cdx1 and Cdx2 (Savory et al., 2011a). Taken together, these results suggest that Cdx1 and Cdx2 can operate in similar or distinct manners in a context-dependent fashion.

The question of Cdx specificity has also been addressed in animal models. A "gene swap" mouse model, wherein the endogenous *Cdx1* locus was replaced by *Cdx2* cDNA has no discernible phenotype (Savory et al., 2009b). This is in marked contrast to $Cdx1^{-/-}$ mutants, which have anterior homeotic transformations of the cervical and thoracic vertebrae (Subramanian et al., 1995), indicating that Cdx2 can fully compensate for loss of Cdx1 in axial patterning. These results suggest that it is the timing and domain of expression, rather than specific function that dictates Cdx-dependent skeletal patterning.

Functional overlap of Cdx members in axial patterning and elongation has also been suggested from studies of Cdx compound mutants, each of which exhibits more severe phenotypes than the cognate single mutants (Mason and Struhl, 2005; van den Akker et al., 2002; van Nes et al., 2006). For example, while $Cdx1^{-/-}$ mice have homeoses of the first three cervical vertebrae, $Cdx1^{-/-}Cdx2^{+/-}$ offspring exhibit additional vertebral defects (van den Akker et al., 2002). Cdx4 null mutants are normal and healthy but exhibit lesions when combined with other Cdx mutants (van Nes et al., 2006). Finally, $Cdx1^{-/-}Cdx2^{-/-}$ conditional mutants are more severely impacted than $Cdx1^{-/-}$ or $Cdx2^{-/-}$ conditional mutants (Savory et al., 2009a; Savory et al., 2011a), further demonstrating functional overlap. Functional overlap between Cdx members in the intestine, however, has not yet been investigated *in vivo*.

Other Hox-independent Cdx functions

Until recently, it was thought that Cdx acted largely upstream of *Hox* genes to regulate AP vertebral patterning of the embryo. In addition to this, *Cdx* gene products are known to play other roles during embryonic development and intestinal homeostasis. In the preimplantation embryo, Cdx2 regulates segregation of the inner cell mass and trophectoderm at the blastocyst stage, a finding which is presumed to underlie the implantation failure in *Cdx2*$^{-/-}$ mutants (Strumpf et al., 2005). Although Cdx2 is required for the maintenance of the trophectoderm, it appears as though it operates downstream of early events which regulate the trophectoderm-inner cell mass lineage decision, although the relevant target genes remain to be identified (Ralston et al., 2010; Ralston and Rossant, 2008).

In the intestine, Cdx2 has been shown to operate as a master regulator of intestinal gene expression in zebrafish and frog (Chen et al., 2009; Faas and Isaacs, 2009; Flores et al., 2008). Cdx2 has also been proposed to have a function in the endo-lysosome pathway and in epithelial cell polarity in the intestine, although the molecular basis for these roles is not currently well defined (Gao and Kaestner, 2010). Moreover, loss of both CDX1 and CDX2 have been associated with human colorectal tumours (Choi et al., 2006; Hinoi et al., 2001; Mallo et al., 1997; Silberg et al., 1997; Sivagnanasundaram et al., 2001; Vider et al., 1997), although the basis for this currently remains unknown.

RATIONALE

The processes underlying patterning of the intestinal epithelium are poorly understood. Since Cdx members are expressed in the developing endoderm and play a prominent role in patterning of other lineages, we sought to determine better their role in the developing intestine. Elucidation of these processes has also been complicated by the fact that many of the genes relevant to intestinal development are expressed in both the definitive and visceral endoderm and/or mesoderm, often making interpretation of gene knockout phenotypes difficult. In addition, reciprocal interactions between the endoderm and adjacent mesoderm are essential to anterior-posterior axis regionalization and endoderm differentiation (Lewis and Tam, 2006). In this regard, examination of endoderm patterning is relatively unexplored. Moreover, although Cdx members have been long known to regulate *Hox* transcription, mechanisms and targets of Cdx function in the intestine currently remain elusive.

HYPOTHESIS

Cdx1 and Cdx2 play key roles in intestinal patterning and differentiation.

OBJECTIVES

1. Determine the effect of loss of Cdx2 and the combined loss of both Cdx1 and Cdx2 in intestinal development.
2. Determine the molecular mechanism(s) through which they elicit these effects.

CHAPTER 2: Cdx2 regulates patterning of the intestinal epithelium

Cdx2 regulates patterning of the intestinal epithelium

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Running title: Cdx2 and intestinal patterning

Keywords: Cdx1, Cdx2, intestine, epithelium, endoderm, AP patterning, homeosis, functional redundancy, ParaHox, Pdx1

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Author contributions:

Stephanie Grainger designed and carried out experiments, wrote manuscript and made figures.

Joanne G.A. Savory generated *Cdx2*$^{f/f}$ mice.

David Lohnes helped to design experiments and to write the manuscript.

ABSTRACT

Cdx1, Cdx2 and *Cdx4* encode homeodomain transcription factors that are involved in vertebral anterior-posterior (AP) patterning. *Cdx1* and *Cdx2* are also expressed in the intestinal epithelium during development, suggesting a role in this tissue. Intestinal defects have not been reported in *Cdx1* null mutants, while *Cdx2* null mutants die at embryonic day 3.5 (E3.5), thus precluding assessment of the null phenotype at later stages. To circumvent this latter shortcoming, we have used a conditional Cre-lox strategy to inactivate Cdx2 in the intestinal epithelium. Using this approach, we found that ablation of Cdx2 at E13.5 led to a transformation of the small intestine to a pyloric stomach-like identity, although the molecular nature of the underlying mesenchyme remained unchanged. Further analysis of Cdx1-Cdx2 double mutants suggest that Cdx1 does not play a critical role in the development of the small intestine, at least after E13.5.

INTRODUCTION

The vertebrate digestive system is composed of the gut tube, which develops from both the definitive and visceral endoderm, and its accessory organs such as the pancreas, liver and salivary glands (Kwon et al., 2008; Wells and Melton, 1999; Zorn and Wells, 2009). Definitive endoderm is formed as epiblast cells are recruited through the anterior portion of the primitive streak, and is subsequently regionalized in an anterior to posterior order, with cells exiting first recruited to the prospective foregut and those exiting last contributing to the hindgut. The epithelial lining of the intestinal tract subsequently undergoes patterning reflective of position along the anterior-posterior (AP) axis. For example, the esophagus becomes lined by a stratified keratinized epithelium, which continues into the cardiac region of the stomach, while the pyloric region of the stomach is lined with a non-keratinized simple columnar epithelium which assumes a scalloped appearance at the site of the gastric pits. The surface of the small intestine is comprised of finger-like villi and invaginating crypts which define the crypt-villus axis. The simple columnar epithelium of the small intestine consists largely of absorptive enterocytes, with mucus-secreting Goblet cells, hormone-secreting enteroendocrine cells, immune-stimulating microfold (M) cells and anti-microbial Paneth cells comprising the remaining cell types. The colon also consists of a simple columnar epithelium with crypts, but has neither villi nor Paneth cells (Wells and Melton, 1999; Zorn and Wells, 2009).

Signaling cues emanating from the underlying mesoderm are thought to play an important role in patterning of the intestinal epithelium (Duluc et al., 1994). This is reflected in the differential expression of transcription factors along the AP axis of the gut tube. In this regard, although *Hox* genes are key players in AP patterning of the

neurectoderm and mesoderm (Deschamps et al., 1999; Krumlauf, 1994; McGinnis and Krumlauf, 1992), *Hox* null mutant mice exhibit relatively minor intestinal patterning defects (Aubin et al., 1997; Boulet and Capecchi, 1996; Manley and Capecchi, 1995; Warot et al., 1997; Zacchetti et al., 2007), suggesting that other players are involved in endoderm patterning. As one such example, *BarX1* is expressed in the stomach mesenchyme during gastric development (Verzi et al., 2009b), and is essential for stomach patterning (Kim et al., 2005; Kim et al., 2007). The endoderm itself is also a source of intrinsic patterning cues. For instance, the transcription factor *Sox2*, which is expressed in the esophagus, lung and stomach primordia, is involved in establishing the pyloric-duodenal junction (Sherwood et al., 2009) as well as the boundary between anterior, keratinized, forestomach and glandular hindstomach (Que et al., 2007b). Other endodermal markers are also regionalized along the AP axis, including *Foxe1*, which is restricted to the esophagus, *Odd1* in the stomach, and several genes of the *Hoxd* cluster which are restricted to the intestine (Sherwood et al., 2009; Yasugi and Mizuno, 2008).

Transcription factors encoded by the *ParaHox* genes are also implicated in GI development. *Parahox* and *Hox* genes are thought to have evolved from a duplication of an ancestral *ProtoHox* cluster. Members of the *ParaHox* gene cluster, such as *Gsh1*, *Pdx1* and *Cdx2*, are expressed principally in the endoderm and derivatives thereof (Ferrier et al., 2005; Illes et al., 2009). For instance, *Pdx1* is expressed with an anterior-high gradient along the intestinal tract and is essential for proper formation of the pancreas and foregut (Monaghan et al., 1993; Stoffers et al., 1997). However, programs involved in the patterning of the hindgut are less well defined. In this regard, *Cdx1* and *Cdx2* are also differentially expressed along the AP axis of the gut with a posterior-high

gradient, and have been implicated in both intestinal development and in the function of the mature gut (Beck, 2004; Guo et al., 2004; Silberg et al., 2000).

Cdx genes encode homeodomain transcription factors related to *Drosophila caudal*. The three *Cdx* murine homologues, *Cdx1*, *Cdx2* and *Cdx4,* exhibit overlapping patterns of expression in the posterior embryo and have overlapping function regarding their role in patterning of the paraxial mesoderm (Beck, 2004; Lohnes, 2003; Savory et al., 2009a; van den Akker et al., 2002; van Nes et al., 2006). Both *Cdx1* and *Cdx2,* but not *Cdx4,* are also expressed in the hindgut endoderm and mature intestine. *Cdx2* expression in the prospective gut begins at E8.5 in the hindgut rudiment. By E12.5, *Cdx2* expression is limited to the endoderm caudal to the foregut/midgut juncture with expression highest in the proximal colon and diminishing in either direction; this expression perdures in the adult (Beck et al., 1995; Chawengsaksophak et al., 2004). *Cdx1* expression initiates in the hindgut endoderm at E12.5 with a posterior-high gradient peaking in the distal prospective colon. Like *Cdx2*, this expression also persists throughout the lifespan of the animal (Duprey et al., 1988; Meyer and Gruss, 1993; Silberg et al., 2000).

Cdx1 null mice have not been reported to have an intestinal phenotype (Subramanian et al., 1995), while *Cdx2* null mice die around E3.5 due to an implantation failure. *Cdx2* heterozygotes are viable and fertile, however the intestinal tracts of adults exhibit occasional Cdx2-deficient lesions in the colon and small intestine. These lesions exhibit areas of metaplasia comprised of esophageal- and gastric-like epithelium, suggestive of a transformation to a more anterior fate (Chawengsaksophak et al., 1997). These observations are consistent with findings from other model systems indicative of

critical roles for Cdx2 in the intestine. For example, in zebrafish, the Cdx2 orthologue cdx1b regulates intestinal cell proliferation and terminal differentiation (Chen et al., 2009; Flores et al., 2008). Morpholino knockdown of *Cdx1, Cdx2* and *Cdx4* in *Xenopus tropicalis* results in misexpression of midgut genes such as *darmin* and the hindgut marker *XSox17b,* leading to an altered intestinal identity as evidenced by loss of *intestinal fatty acid binding protein* (*IFABP*) expression and disruption of gut coiling (Faas and Isaacs, 2009). More recently, conditional deletion approaches have illustrated a critical role for Cdx2 in the mouse, with ablation of Cdx2 in the endoderm leading to transformation of the small intestine to an esophageal phenotype and agenesis of the colon. Notably, this transformation occurs independent of major alterations in the enteric Hox code (Gao et al., 2009).

In the present study, we used a tamoxifen-regulated Cre driven by the intestinal epithelium-specific *villin* promoter (el Marjou et al., 2004) to inactivate a floxed *Cdx2* allele. Although *villin* expression begins around E9.0 (Ezzell et al., 1989), we found that recombination prior to E13.5 resulted in repopulation of the intestinal epithelium with cells apparently heterozygous for Cdx2, suggesting Cdx2 null cells are at a competitive disadvantage. Treatment at E13.5, however, resulted in efficient deletion and aberrant patterning of the epithelium of the small intestine, underscoring a role for Cdx2 in the gut at a relatively late stage. We also present evidence that Cdx2 function is required to delimit *Pdx1* along the intestinal tract. Finally, in contrast with prior observations (Gao et al., 2009), we found that *Cdx1* expression was not affected by loss of Cdx2, and that Cdx1 does not play a critical role in development of the small intestine, at least from E13.5.

MATERIALS AND METHODS

Generation of Cdx2 and Cdx1-Cdx2 intestinal loss-of-function mutants

Cdx2$^{f/f}$, *Cdx1*$^{-/-}$, and *Villin*-Cre ER^{T} mice have been previously described (el Marjou et al., 2004; Savory et al., 2009a; Subramanian et al., 1995). *Cdx2* deletion was effected by crossing *Cdx2*$^{f/f}$ females with *Cdx2*$^{f/f}$-*Villin*-Cre ER^{T} males and treating pregnant females with 5mg of tamoxifen at E13.5 by oral gavage. Cdx1-Cdx2 double mutants were generated in a similar manner by crossing *Cdx1*$^{-/-}$ mice into the *Cdx2*$^{f/f}$ and *Cdx2*$^{f/f}$-*Villin*-Cre ER^{T} backgrounds. Littermates lacking the Cre ER^{T} transgene were used as controls in both instances.

Histology and immunohistochemistry

E18.5 intestinal tracts were dissected free of accessory organs in phosphate-buffered saline treated with diethylpyrocarbonate (PBS-DEPC), fixed in 4% paraformaldehyde (PFA) in PBS-DEPC at 4°C overnight, dehydrated through an ethanol series, embedded in paraffin and sectioned at 5 μm for histological and immunohistochemical analysis. Hematoxylin and eosin, Periodic acid- Schiff, Alcian Blue and immunostaining were carried out using standard methods. Primary antibodies used were: anti-Pdx1 monoclonal (1/100 dilution, Developmental Studies Hybridoma Bank); rabbit polyclonal anti-Cdx1 and anti-Cdx2 (Savory et al., 2009b); anti-PepsinogenII (1/1000 dilution, Binding Site Ltd, Birmingham, England); anti-H^{+}/K^{+} ATPase (β subunit; 1/1000 dilution, Abcam); and anti-Ki67 (1/1000 dilution, Abcam). Secondary antibodies were HRP-conjugated

goat anti-mouse, goat anti-sheep or goat anti-rabbit, as appropriate (1/1000, Santa Cruz Biotechnologies). Slides were mounted using Permount (Fisher) and scanned at 40X using a Zeiss Mirax Midi Scanner (Zeiss).

Western blot analysis

Stomach, small or large intestine from E18.5 fetuses were collected in 1ml of lysis buffer (20mM Tris pH 8.0, 25mM NaCl, 1.5mM $MgCl_2$, 1mM EGTA, 1% Triton X-100, 10% glycerol, 1mM DTT and protease inhibitors), minced and sonicated and supernatants cleared by centrifugation. Fifty micrograms of protein were resolved on a 12% SDS-PAGE gel, transferred to PVDF membrane (Millipore) which was then blocked with 5% skim milk in PBS with 0.1% Tween-20 and incubated with anti-Cdx2 (Savory et al., 2009b) or β-actin (1/5000 dilution, mAbcam8226) antibodies overnight. Secondary antibodies (HRP-conjugated anti-rabbit or anti-mouse IgG; 1/10 000 dilution, Santa Cruz Biotechology) were detected by ECL (Perkin Elmer) according to the manufacturer's instructions. Signal was quantified using ImageJ software using the β-actin signal as a loading control.

In situ hybridization

Sections were cut at 10μm and slides were processed as described above. Probes were synthesized using the DIG RNA labeling system (Roche) according to the manufacturer's recommendations. *In situ* hybridization was carried out as previously described (Jensen and Wallace, 1997) and slides mounted using Permount (Fisher).

Reverse-transcriptase polymerase chain reaction (RT-PCR)

RNA was extracted from E18.5 stomach, small intestine or large intestine using Trizol reagent (Invitrogen) and used to generate cDNA by standard procedures. cDNA was subsequently amplified by PCR using oligonucleotides for Muc1, Sox2, Ghrl, IFABP, Keratin5, p63, BarX1 or β-actin. PCR products were resolved by electrophoresis on a 1.5% agarose gel, stained using SYBR Safe Nucleic Acid Stain I (Invitrogen) and quantified using LAS Image-4000 luminescent image analyzer and the Multi Gauge program (Fujifilm) using β-actin expression to normalize for loading.

RESULTS

Conditional inactivation of Cdx2 in the intestine

To circumvent the lethality inherent to *Cdx2* loss-of-function (Chawengsaksophak et al., 1997), we generated a floxed allele (*Cdx2*$^{f/f}$) whereby *loxP* sites flanked the homeodomain-encoding second exon of the *Cdx2* locus (Savory et al., 2009a). To investigate the role of Cdx2 in the intestine, we used a *villin*-Cre ER^{T} transgenic which expresses a tamoxifen-regulated Cre recombinase in the intestinal epithelium (el Marjou et al., 2004). Since expression of this transgene begins at E9.0, we initially treated mice at either E9.5 or E11.5 with 2mg of tamoxifen. The resulting offspring were viable and survived into adulthood with apparently normal intestinal tracts (data not shown). Immunohistochemical analysis of these offspring revealed a uniform level of residual Cdx2 protein throughout the intestinal tract which, however, appeared to represent approximately 50% of that seen in wild-type controls. Consistent with this, PCR analysis revealed the presence of both recombined and floxed alleles (Figure 21). These observations suggest that ablation at these stages resulted in a residual population of cells heterozygous for Cdx2 that were able to outcompete *Cdx2* null cells and repopulate the intestinal tract.

We subsequently found that a single 5mg dose administered at E13.5 led to effective deletion of *Cdx2* in the small intestine, as assessed by both immunohistochemistry and Western blot analysis. However, a considerable amount of Cdx2 expression persisted in the large intestine (Figure 22). Western blot analysis also

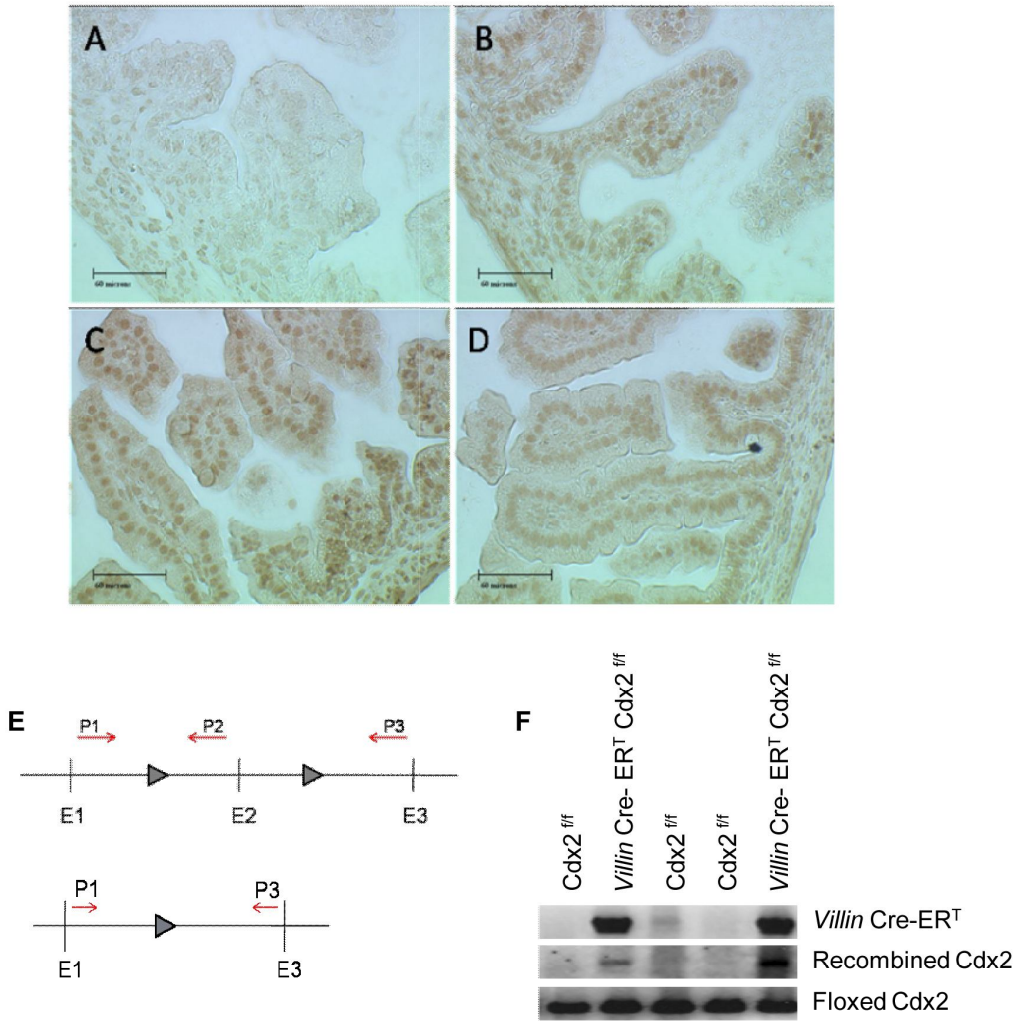

Figure 21: Inefficient recombination Villin Cre-ER^T prior to E13.5. Immunohistochemical analysis for Cdx2 in E18.5 small intestines from (A) no antibody control; (B) following treatment with 2mg tamoxifen at E11.5; (C) wild type; (D) following 2 mg tamoxifen at E9.5. Scale bars correspond to 60 µm. (E) Primer design for PCR-based genotyping of the Cdx2 locus. (F) PCR products indicating Cre transgene (upper panel), recombined allele (middle panel) and floxed allele (lower panel).

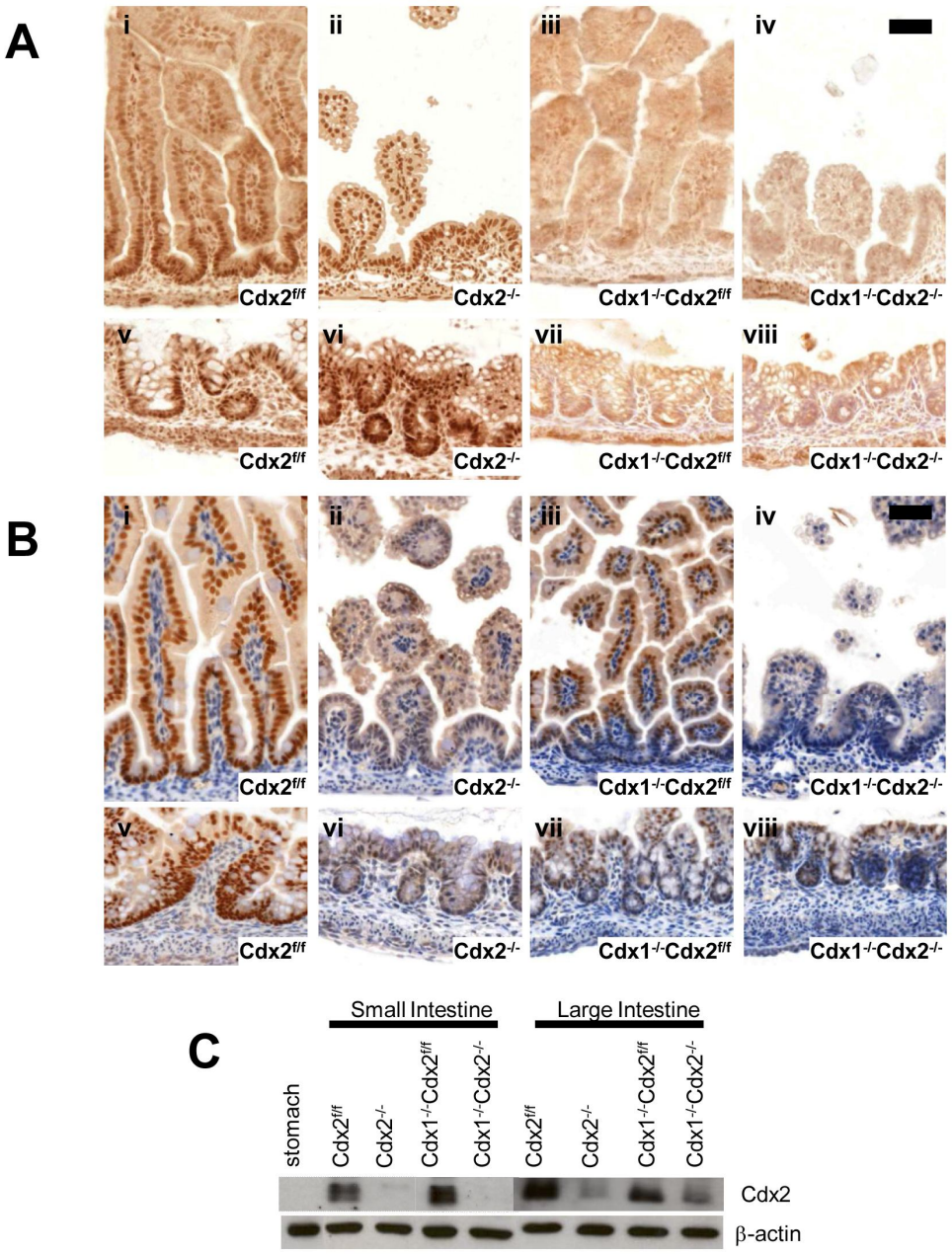

Figure 22: Conditional inactivation of Cdx2. Immunohistochemistry for Cdx1 (A) or Cdx2 (B) in small intestine (upper panels) and colon (lower panels) in *Cdx2$^{f/f}$* (i and v), *Cdx2$^{-/-}$* (ii and vi), *Cdx1$^{-/-}$Cdx2$^{f/f}$* (iii and vii) and *Cdx1$^{-/-}$Cdx2$^{-/-}$* (iv and viii) E18.5 fetuses. Scale bars represent 50 μm. (C) Western blot analysis for Cdx2 in wild-type, Cdx2 null and Cdx1-Cdx2 double null small or large intestine. β-actin was used as a loading control while stomach extracts were used as a negative control.

failed to detect a truncated N-terminal protein predicted to arise from the targeting strategy. This suggests that any such product was unstable and not likely to confound interpretation of results. These data suggest that this dosing regimen yields effective ablation of Cdx2 in the small intestine, hereafter referred to as *Cdx2*$^{-/-}$.

Generation and analysis of Cdx2 and Cdx1 - Cdx2 null small intestine

Cdx members appear to functionally overlap in several developmental programs, including vertebral patterning (Faas and Isaacs, 2009; Savory et al., 2009b; van den Akker et al., 2002; van Nes et al., 2006) and hematopoiesis (Davidson and Zon, 2006). Functional equivalence has been less rigorously addressed in the intestine, and data from tissue culture models suggest Cdx1 and Cdx2 may be actually functionally distinct in some cases (Alkhoury et al., 2005; Bai et al., 2003; Gautier-Stein et al., 2003; Moucadel et al., 2002; Oh et al., 2002). In this regard, recent work demonstrated that ablation of Cdx2 in definitive endoderm results in loss of Cdx1 (Gao et al., 2009). While this places Cdx2 upstream of Cdx1, it also suggests that the resultant phenotype may be a consequence of the loss of both Cdx1 and Cdx2. In contrast, we found that ablation of Cdx2 at E13.5 did not compromise Cdx1 expression in the intestine (Figure 22A; compare i to ii and v to vi). However, Cdx1 null mutants exhibited a modest induction of Cdx2 in the large intestine (approximately 30% by densitometry, Figure 22C), consistent with cross-regulation among Cdx members (Beland et al., 2004; Prinos et al., 2001; Xu et al., 1999).

Based on the above observations, we anticipated that Cdx1 may compensate for the absence of Cdx2 in the intestine, and therefore derived both *Cdx2*$^{-/-}$ and *Cdx1*$^{-/-}$*Cdx2*$^{-/-}$

double mutants for comparison. Both genotypes were recovered at a Mendelian frequency at E18.5, but did not survive beyond P0. Full term mutants exhibited slightly distended abdomens, but appeared otherwise normal on external examination (Figure 23A). The gastrointestinal tracts of *Cdx2*$^{-/-}$ and *Cdx1*$^{-/-}$*Cdx2*$^{-/-}$ offspring exhibited a similarly distended duodenum (Figure 23B, C), with the small and large intestine being of normal length (Figure 23B, D, E). Other gastrointestinal organs appeared to be normal, including the stomach and accessory organs (Figure 23B, data not shown).

Histological analysis of E18.5 fetuses revealed abnormal morphogenesis of the Cdx2 mutant small intestine, with a severe reduction in villus length and poor organization (Figure 24; compare i and iii to ii and iv); *Cdx1*$^{-/-}$*Cdx2*$^{-/-}$ offspring exhibited similar defects (Figure 24; compare ii to iv and vi to viii). Both single and double mutant epithelia also exhibited vacuolated cells, suggestive of apoptosis, although no difference was found relative to littermate controls in TUNEL assays (data not shown). In addition, the basal polarity typical of the nuclei of wild-type enterocytes was lost in the mutants (Figure 24; compare ix to x and data not shown). The cecums and colons of both *Cdx2*$^{-/-}$ and *Cdx1*$^{-/-}$*Cdx2*$^{-/-}$ mice appeared histologically normal (Figure 24 v-viii), likely due to the residual level of Cdx2 in the distal GI tract (Figure 22B, C), and were therefore not further assessed. Finally, and in contrast to prior work (Gao et al. 2009), there was no evidence of imperforate anus in Cdx2 single or Cdx1-Cdx2 double null mutants (Fig. 24xi and data not shown).

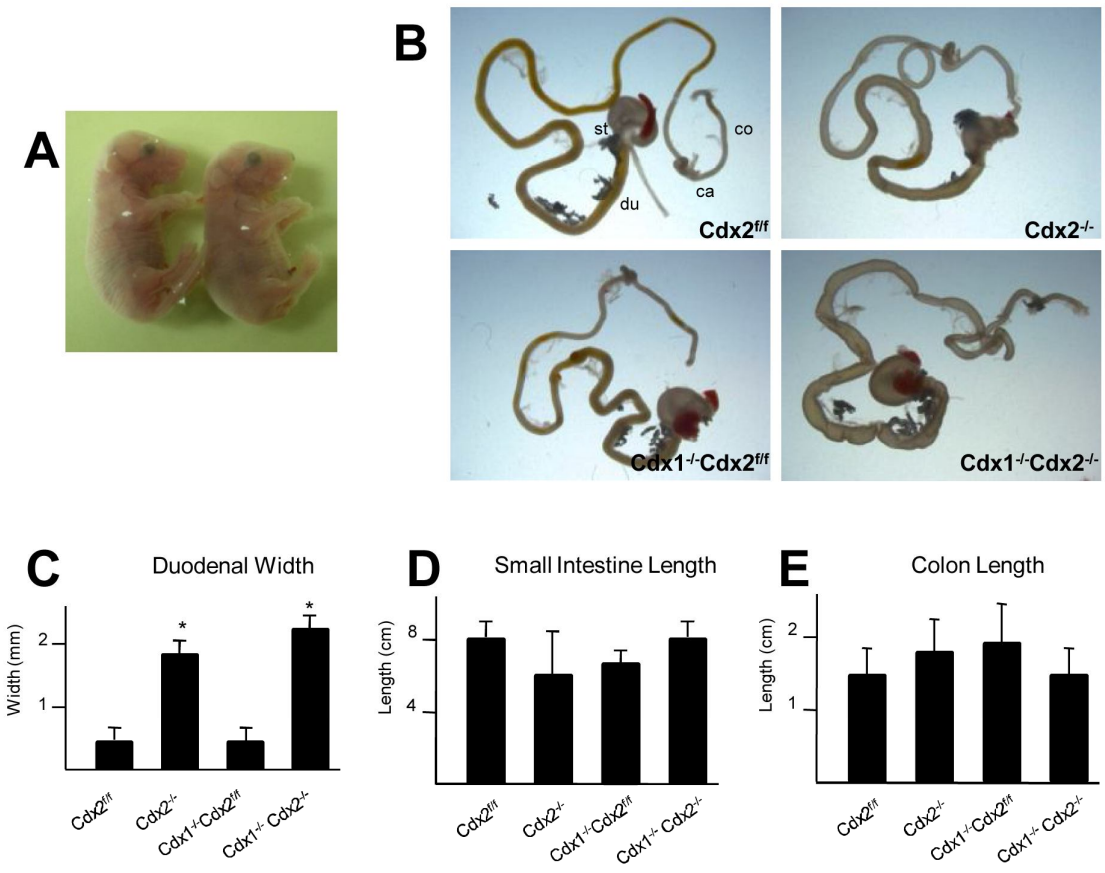

Figure 23: Intestinal morphology is compromised in *Cdx2*$^{-/-}$ mice. (A)*Cdx2*$^{f/f}$ (left) and *Cdx2*$^{-/-}$ (right) fetuses. Note the distension of the abdomen in the Cdx2$^{-/-}$ specimen. (B) E18.5 intestinal tracts. Duodenal width (C) was increased in Cdx2 mutants, while the length of the small intestine (D) and colon (E) was not significantly affected. Error bars represent standard deviation from the mean. *$P < 0.05$ by Student's *t*-test. Abbreviations: st, stomach; du, duodenum; ca, caecum; co, colon.

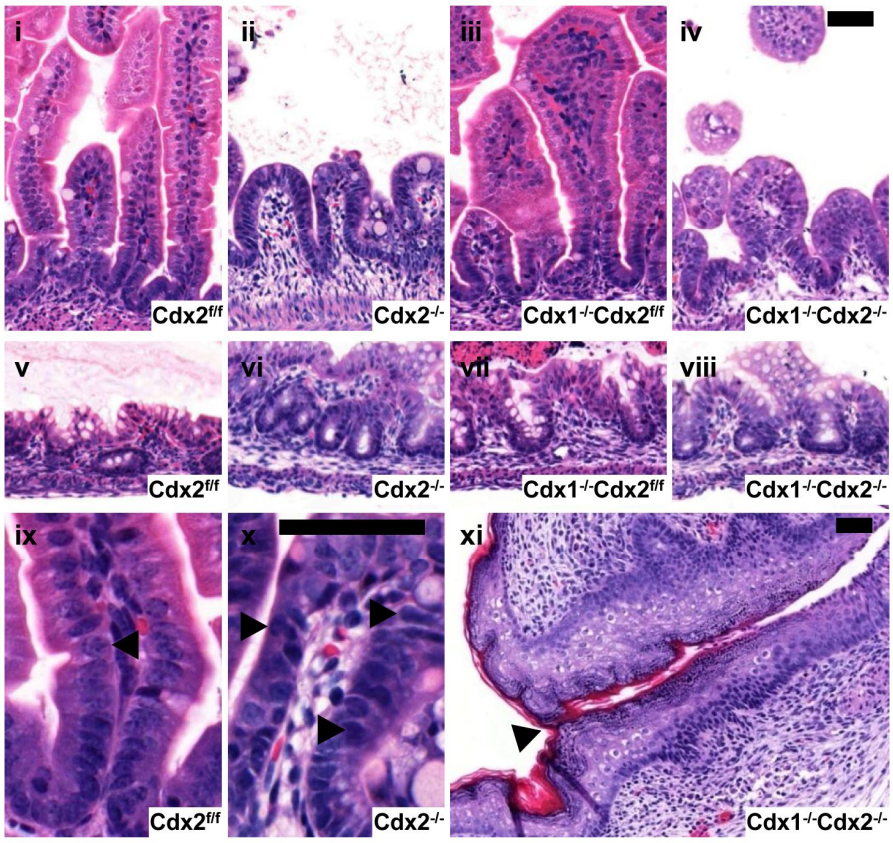

Figure 24: Cdx2 is essential for morphogenesis of the small intestine. Hematoxylin and eosin staining of small intestine (i, ii, iii, iv, ix, x) or colon (v, vi, vii, viii) from *Cdx2*$^{f/f}$, *Cdx2*$^{-/-}$, *Cdx1*$^{-/-}$*Cdx2*$^{f/f}$ or *Cdx1*$^{-/-}$*Cdx2*$^{-/-}$ E18.5 fetuses. Note the altered villus morphology in *Cdx2* single and *Cdx1-Cdx2* double mutant small intestines. Note also the abnormal apical-basal polarity of nuclei in *Cdx2* mutant intestinal epithelial cells (compare ix to x). Arrows denote nuclei that have lost apical-basal polarity compared to controls. xi is a longitudinal section showing the rectum and anus of a *Cdx1-Cdx2* mutant. Arrow denotes the anus from the external side. Scale bar represents 50 μm.

Cdx2 is essential for patterning of the small intestine

Previous studies have suggested that Cdx2 plays an essential role in intestinal patterning (Beck et al., 1999; Chawengsaksophak et al., 1997; Gao et al., 2009; Stringer et al., 2008). To further investigate this, we used differential staining with Periodic acid-Schiff (PAS), which stains mucins of both the intestinal Goblet cells and the gastric pit cells of the stomach, and Alcian Blue, which stains only mucins secreted by intestinal Goblet cells (Culling et al., 1975). These studies revealed that *Cdx2*$^{-/-}$ small intestines exhibited ectopic PAS staining, most notably at the apical edge of the villi (Figure 25; compare i and iii to ii and iv). There was also robust PAS staining in large rounded cells which resembled Goblet cells and which were also stained with Alcian Blue. Alcian Blue, however, did not stain the apical edge of the mutant villi (Figure 25; compare vi and viii to vii and ix). Staining patterns for *Cdx1*$^{-/-}$*Cdx2*$^{-/-}$ offspring were indistinguishable from Cdx2 single mutants (Figure 25; compare ii and vii to iv and ix). It is also notable that these mutant staining patterns resemble that of pyloric stomach (Figure 25; compare ii and iv to v).

H^+/K^+ ATPase is expressed in gastric parietal cells (Shull, 1990) as seen by immunohistochemistry in control stomach (Figure 26A). In the Cdx2 and Cdx1-Cdx2 mutant small intestine, but not in controls, regions of staining were also observed in the proximal duodenum (Figure 26C; arrowheads, compare to 26B). The staining patterning seen in the mutant intestine is also consistent with the localization of H^+/K^+ ATPase in cytoplasmic tubulovesicles resulting in a punctate expression pattern (Okamoto and Forte, 2001).

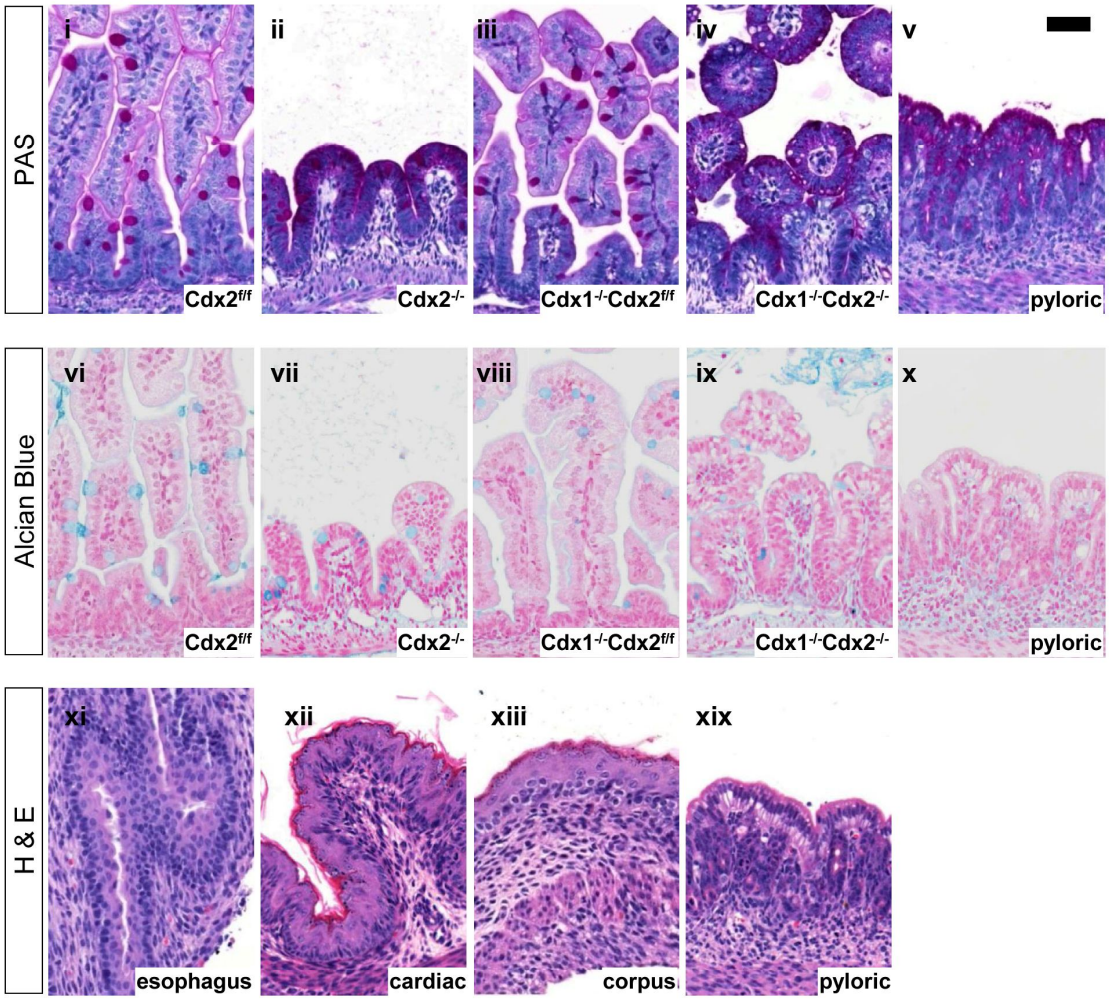

Figure 25: Cdx2 mutant intestine exhibits characteristics of glandular stomach. Periodic acid-Schiff (PAS, i–v) and Alcian Blue (vi–x) staining of small intestines from *Cdx2*$^{f/f}$ (i and vi), *Cdx2*$^{-/-}$ (ii and vii), *Cdx1*$^{-/-}$*Cdx2*$^{f/f}$ (iii and viii) or *Cdx1*$^{-/-}$*Cdx2*$^{-/-}$ (iv and ix) E18.5 fetuses. Wild-type pyloric stomach (v and x) is shown for comparison. Hematoxylin and eosin (lower panels) staining of wild-type esophagus or cardiac, corpus and pyloric regions of wild-type stomach as indicated. Compare these esophageal and stomach sections to Cdx2 and Cdx1-2 mutant small intestines in Fig. 24 (ii and iv). Scale bars represent 50 μm.

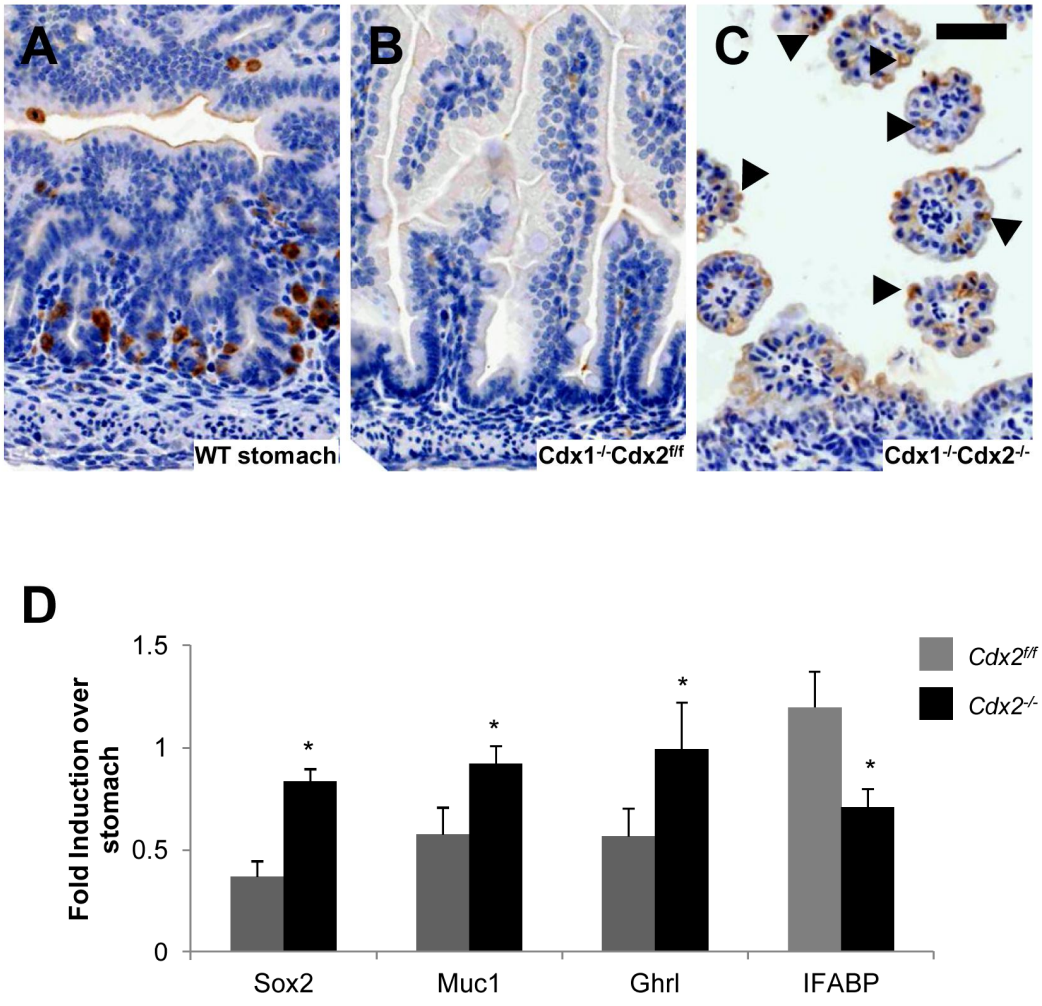

Figure 26: Expression of gastric markers in Cdx2 mutants. Immunohistochemical staining for H^+/K^+ ATPase in wild-type stomach (**A**) *Cdx1*$^{-/-}$*Cdx2*$^{f/f}$ (**B**) and *Cdx1*$^{-/-}$ *Cdx2*$^{-/-}$ (**C**) E18.5 small intestines. Arrowheads in **(C)** indicate punctuate tubulovesicle-like staining. Scale bar represents 50mm. (**D)** RT-PCR analysis from E18.5 small intestine or wild-type stomachs for *Sox2*, *Muc1*, *Ghrl* or *IFABP*. Values represent mean fold expression in *Cdx2*$^{f/f}$(gray) and *Cdx2*$^{-/-}$ (black) small intestines relative to wild-type stomach. Error bars represent standard deviation from the mean. Experiment was repeated in triplicate with similar trends. * $P<0.05$ by student's t-test.

To further characterize the *Cdx2*$^{-/-}$ intestinal phenotype, we investigated additional markers characteristic of stomach or intestine by semi-quantitative RT-PCR. *Sox2* is initially expressed in the epithelium of the esophagus and stomach and extends to the duodenum, with expression attenuated in the posterior stomach at term (Que et al., 2007b). *Muc1* is a transmembrane protein found in the apical surface of glandular epithelia of the stomach (Braga et al., 1992) while *Ghrelin* (*Ghrl)* is expressed in gastric mucosal cells (Kojima et al., 1999). *IFABP* is expressed in intestinal epithelial cells (Agellon et al., 2002). We found an enrichment of these three gastric markers and a concomitant decrease in *IFABP* expression in the Cdx2 null small intestine compared to wild-type littermate controls (Figure 26D).

The presence of ectopic gastric mucins and the gain in the expression of stomach markers, together with the reduction in expression of *IFABP*, is consistent with a partial transformation of the Cdx2 mutant small intestine to a stomach-like identity. In agreement with this, the villi of both the Cdx2 and Cdx1-Cdx2 mutants were morphologically similar to the pyloric region (but not the cardiac or corpus regions) of the stomach (Compare Figures 24xi, xii, xiii, xix to Figure 24 ii, iv). Neither single nor double mutant intestines bore any morphological resemblance to the esophagus (Compare Figure 24ii and iv to Figure 25xi-x), nor was expression of esophageal markers such as *p63* or *keratin5* detected (Figure 27) in contrast to previous findings (Beck et al., 1999; Chawengsaksophak et al., 1997; Gao et al., 2009). Finally, epithelial-mesenchymal signaling plays a key role in endoderm development (Kim et al., 2005; Kim et al., 2007). However, and also in contrast to prior findings (Stringer et al., 2008), we did not find any evidence for ectopic *BarX1* expression in the mutants (Figure 28). Taken together, these

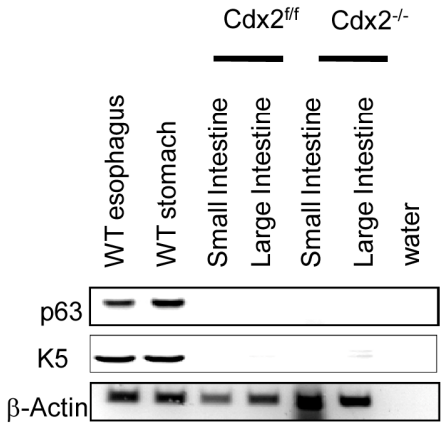

Figure 27: p63 or K5 expression is undetectable in Cdx2 mutant intestine. RNA was isolated from esophagus, stomach, small and large intestine of wild type and Cdx2 mutant fetuses and used as a template for RT-PCR analysis of expression of p63 (upper panel) or K5 (middle panel). β-actin amplification (lower panel) was used as a positive control.

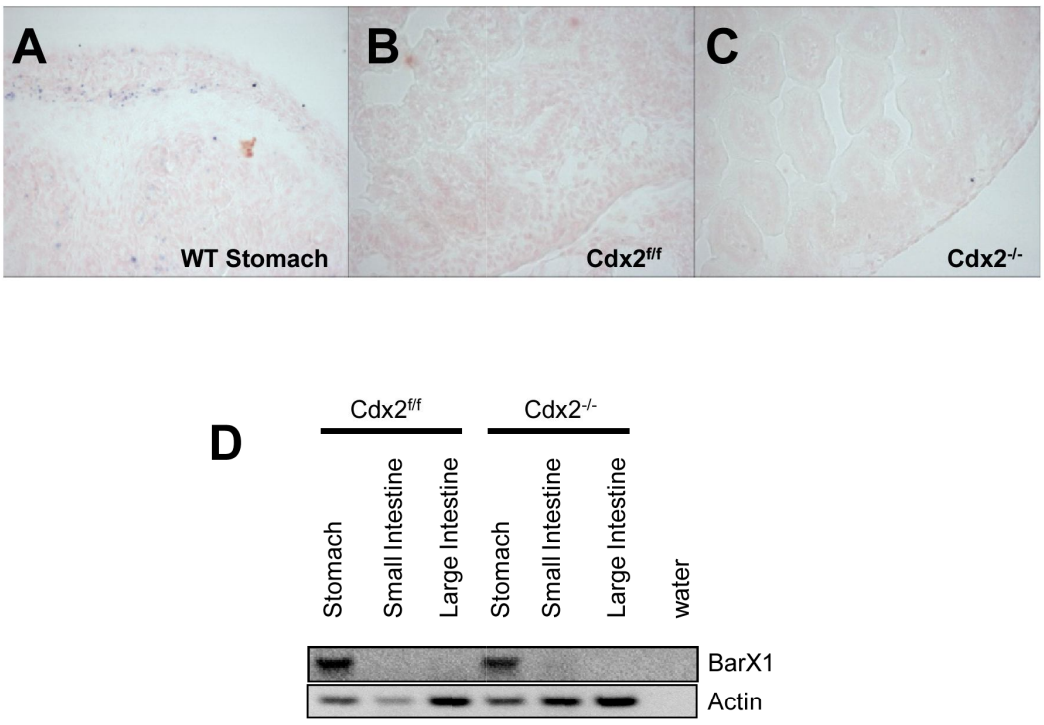

Figure 28: Normal expression of *BarX1* in Cdx2 mutants. (A)*In situ* hybridization of *BarX1* in E18.5 (A) wild type stomach, (B) *Cdx2$^{f/f}$* and (C) *Cdx2$^{-/-}$* small intestines. (D) RT-PCR analysis of *BarX1* expression.

results are consistent with loss of Cdx2 at E13.5 leading to a partial transformation of the epithelium of the small intestine to a pyloric identity, with some persisting intestinal characteristics.

Cdx2 restricts cell cycle in the intestine

In adult mice, intestinal stem cells which reside near the base of crypts give rise to rapidly dividing transit amplifying cells which in turn exit mitosis and differentiate into the cell types comprising the intestinal epithelium. In the developing fetus, actively dividing cells are localized to the intervillus spaces since crypts have yet to form (Crosnier et al., 2006). Consistent with this, in the wild-type small intestine Ki67, which marks actively dividing cells, was observed at the base of inter-villus spaces (Figure 29Ai, B). In the *Cdx2*$^{-/-}$ small intestine, however, Ki67 staining was observed in a more superficial and expanded domain (Figure 29Aii, B); *Cdx1*$^{-/-}$*Cdx2*$^{-/-}$ mutants exhibited an identical pattern of expression (data not shown). These results suggest that Cdx2 is required to restrict cycling cells in the small intestine. It remains to be seen if this is due to an impact on transit-amplifying (or stem) cells or if it is secondary to the loss of the normal intestinal architecture. It is, however, interesting to note that a similar pattern of Ki67 expression is seen in the pyloric stomach (Figure 29Aiii), consistent with anterior transformation of the mutant intestine.

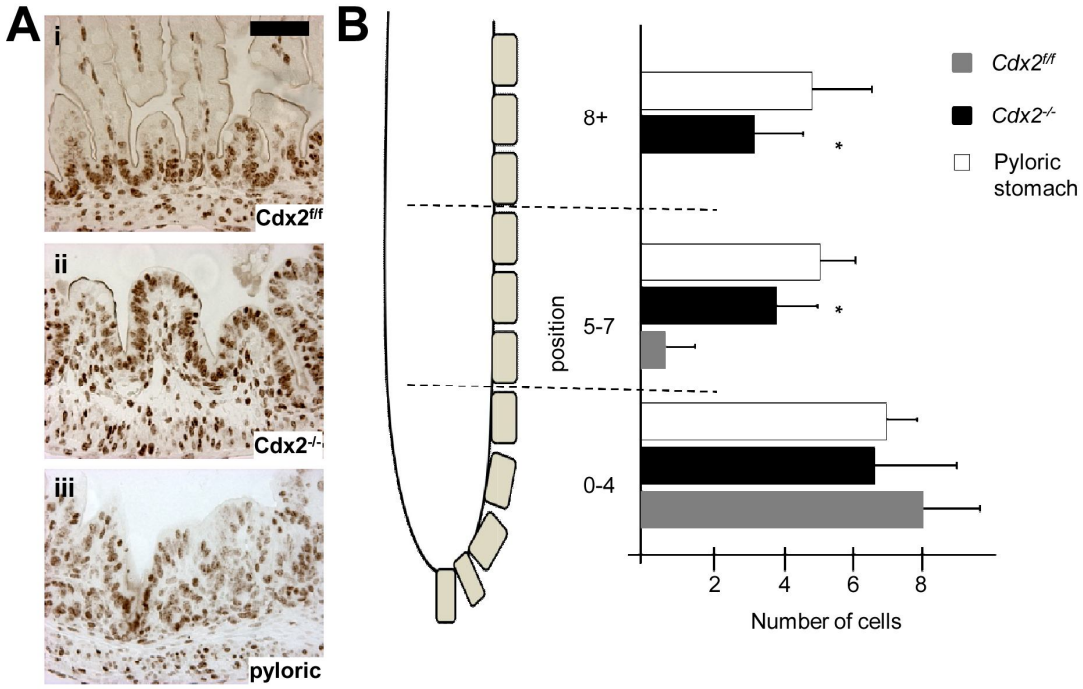

Figure 29: Cdx2 regulates the localization of cycling cells. (**A**) Ki67 immunohistochemistry of E18.5 *Cdx2*$^{f/f}$ (i) and *Cdx2*$^{-/-}$ (ii) small intestine and wild-type pyloric stomach (iii). **(B)** Ki67 positive nuclei were counted according to position with the centre of the intervillus region as 0. Bars shown represent the mean count from 9 intervillus spaces from 3 *Cdx2*$^{f/f}$ (gray) and 3 *Cdx2*$^{-/-}$ (black) specimen. Error bars represent standard deviation from the mean. * $P<0.05$ by student's t-test. Scale bar represents 50μm.

Loss of Cdx2 alters Pdx1 expression

The murine ParaHox family member Pdx1 has critical roles in the intestinal tract, as evidenced by the lack a pancreas and additional defects in Pdx1 null mutants (Jepeal et al., 2005; Jonsson et al., 1994; Larsson et al., 1996; Offield et al., 1996). A relationship between Pdx1 and Cdx2 is supported by the finding that the sea urchin homologues of *Cdx2* and *Pdx1*, *spCdx* and *spLox*, act in an autoregulatory loop to specify hindgut endoderm (Cole et al., 2009). We therefore assessed *Pdx1* expression in Cdx2 mutants to see if a similar regulatory relationship existed.

Pdx1 is normally expressed in the epithelia of the distal stomach and the duodenum; this pattern was conserved in *Cdx1*$^{-/-}$*Cdx2*$^{-/-}$ and *Cdx2*$^{-/-}$ offspring (Figure 30). However, ectopic Pdx1 expression was also observed in the distal small intestine in the mutant backgrounds (Figure 30viii and x) consistent with anteriorization of the intestine, and suggesting that a Pdx1-Cdx2 cross-regulatory loop related to that described in the sea urchin may be conserved in the mouse.

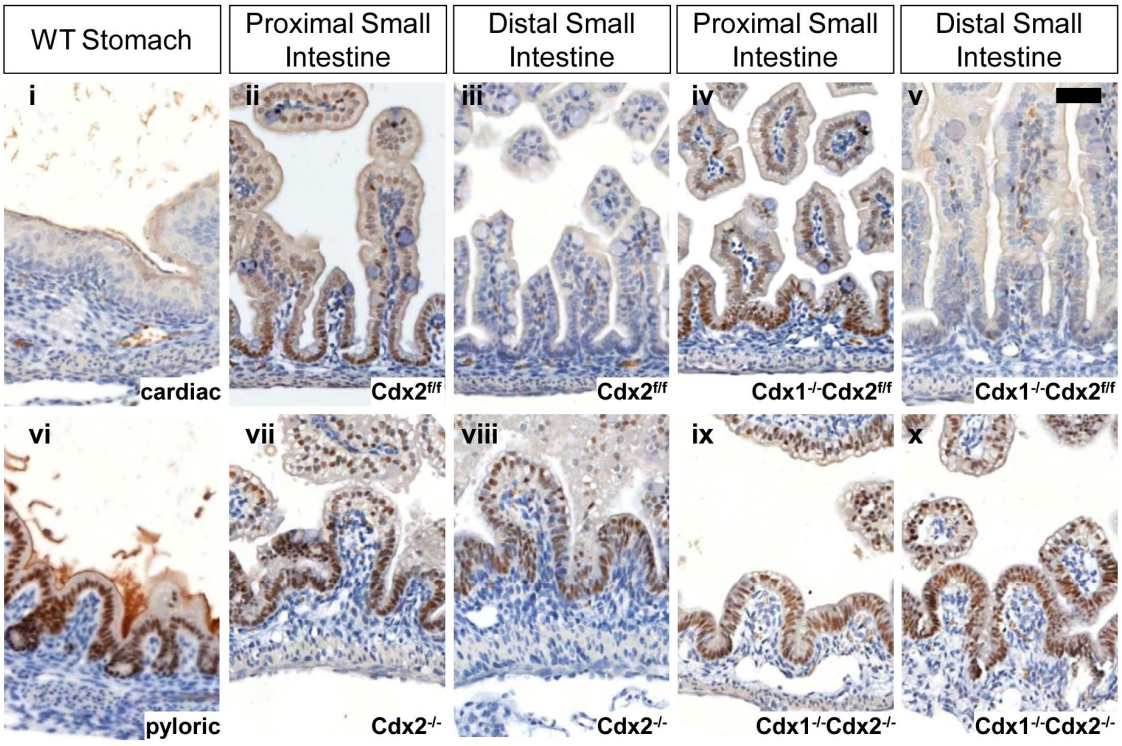

Figure 30: Cdx2 regulates Pdx1 expression. Pdx1 immunohistochemistry in *Cdx2$^{f/f}$* (**ii**, **iii**), *Cdx2$^{-/-}$* (**vii**, **viii**), *Cdx1$^{-/-}$Cdx2$^{f/f}$* (**iv**, **v**) and *Cdx1$^{-/-}$Cdx2$^{-/-}$* (**ix**, **x**) proximal (**ii**, **iv**, **vii**, **ix**) and distal (**iii**, **v**, **viii**, **x**) E18.5 small intestines, and wild-type E18.5 cardiac (**i**) and pyloric (**vi**) stomach. Note the ectopic Pdx1 expression in the distal small intestine of Cdx2 (viii) and Cdx1-Cdx2 (x) mutants. Scale bar represents 50μm.

DISCUSSION

To investigate the role of Cdx2 in intestinal development, we used a ligand-activated Cre under the control of the *villin* promoter to effect deletion of Cdx2 in the intestinal epithelium at E13.5. Immunohistochemistry and western blot analysis revealed that this approach resulted in efficient deletion of Cdx2 in the small intestine, however, considerable protein remained in the large intestine. This may be due to relative expression levels of the *villin*-Cre transgene along the GI tract (el Marjou et al., 2004; Subramanian et al., 1995). The resultant Cdx2 mutants exhibited a distended duodenum with severely disrupted villus morphology. Differential staining and expression analyses indicated that the mutant small intestine exhibited several markers typical of the gastric epithelium, consistent with prior findings (Beck et al., 2003; Beck et al., 1999; Chawengsaksophak et al., 1997) and indicative of a critical role for Cdx2 in patterning of the small intestine at E13.5 (or later). Our findings are also in general agreement with recent work showing that loss of Cdx2 in the early definitive endoderm results in an anterior transformation of the small intestine to an esophageal identity (Gao et al., 2009).

Cdx2 ablation differentially impacts on intestinal patterning in a temporal manner

We found that loss of Cdx2 at E13.5 led to a partial transformation of the small intestine to a glandular stomach-like identity. For example, PAS and Alcian blue staining suggests the presence of ectopic stomach mucins, intermingled with normal intestinal Goblet cells. An increase in expression of stomach markers such as *Ghrl*, *Sox2* and *Muc1*, and a reduction in the intestinal marker *IFABP*, was also observed. Taken

together, these findings are suggestive of a partial transformation of the epithelium to a mixed lineage of pyloric stomach and small intestine. In contrast to this, Cdx2 disruption in the definitive endoderm around E8.5 results in transformation of the small intestine to an esophageal identity and agenesis of the colon (Gao et al., 2009). These different outcomes are not likely due to compensation by Cdx1, as we found that loss of Cdx1 in the Cdx2 mutant background had no discernible impact. Moreover, temporal ablation of Cdx2 using an *Actin*-CreER^{T} transgene (Santagati et al., 2005) at E8.5 resulted in transformation of the small intestine to an esophageal-like keratinized epithelium (our unpublished observation). These observations suggest that loss of Cdx2 differentially impacts on intestinal patterning in a temporal manner, with early loss resulting in a more anterior transformation, and that intestinal patterning exhibits considerable plasticity to at least E13.5.

Information as regards the plasticity of the endoderm and its derivatives is largely restricted to pancreas and liver, but is consistent with a protracted period of plasticity. For example the homeobox transcription factor HB9 can alter endoderm from a pancreatic to a gut fate if expression is forced beyond its normal period during pancreatic development (Li and Edlund, 2001). In addition, loss of *Ptf1a* results in conversion of pancreatic precursors to an intestinal fate, despite early expression of pancreatic specific transcription factors suggesting that these precursors can be reprogrammed (Kawaguchi et al., 2002). During chronic tissue damage in the adult mouse, hepatocytes and pancreatic islet cells sometimes sporadically arise from other cell types, such as the bile duct-associated cells (Bonner-Weir and Weir, 2005; Fausto and Campbell, 2003; Taub, 2004). Finally, in the intestinal tract itself, Barrett's esophagus represents another

example tissue reprogramming, in this case from squamous-keratinized epithelial tissue to a columnar, duodenal-like, epithelium. Interestingly, this metaplasia may be driven by ectopic expression of Cdx2 (Souza et al., 2008).

Our study provides precedent regarding Cdx2 function as relates to the plasticity of the intestinal endoderm. Although it remains to be determined which programs are impacted, one potential candidate is the Wnt signaling pathway, which has a differential impact on intestinal patterning, with more anterior structures such as the stomach, liver and pancreas requiring repression of the Wnt signal (Kim et al., 2005; Kim et al., 2007; McLin et al., 2007), while development of the posterior portion of the gut tube is Wnt-dependent (Gregorieff et al., 2004). In this regard, Cdx members have been shown to be both Wnt targets and also to regulate the Wnt signal (Lickert et al., 2000; Pilon et al., 2007; Prinos et al., 2001; Shimizu et al., 2005), consistent with a Cdx-Wnt pathway in intestinal development.

The prior finding of agenesis of the colon in Cdx2 mutants suggests that Cdx2 null cells are incapable of contributing to distal GI tract (Gao et al., 2009). The nature of the Cre transgene employed in the current study likely lead to a failure to recapitulate this phenotype. However, consistent with this prior observation, we did find that cells heterozygous for Cdx2 appeared to be at a competitive advantage over Cdx2 null cells, as evidenced by repletion of the intestinal tract exclusively with the former when following tamoxifen-mediated recombination at earlier stages. Finally, we have seen no evidence for a blockage at any level of the intestine This suggests that the distension of the duodenum in Cdx mutants is specific, rather than secondary to a blind-ending colon as previously suggested (Gao et al., 2009)

Cdx2 and mesodermal patterning of the GI tract

Spontaneous loss of Cdx2 expression has been reported to occur occasionally in the intestinal epithelium of Cdx2 heterozygotes, and this event has been associated with ectopic expression of *BarX1* in the underlying mesoderm (Stringer et al., 2008). Similar effects on mesodermal gene expression were not observed in either the current, or other (Gao et al., 2009), models of Cdx2 loss-of-function. In the latter instance, the lack of ectopic *BarX1* expression could be precluded by the transformation of the Cdx2 mutant intestine to an esophageal fate. However, the present study suggests a partial transformation of the small intestine to a pyloric fate. It is possible that the incomplete nature of this transformation precludes effects on underlying mesoderm. Alternatively, the stage of Cdx2 loss-of-function may impact on epithelial-mesenchymal interactions.

In the purple sea urchin *Stronglyocentrotus purpuratus*, knockdown of *spLox* (the homologue of Pdx1) revealed an interaction between *spLox* and *spCdx* (the homologue of Cdx2), suggesting that these two ParaHox members are involved in a feedback loop wherein spLox first induces the expression of *spCdx* in the hindgut, which in turn represses *spLox* to establish hindgut identity (Cole et al., 2009). The ectopic Pdx1 expression seen in the Cdx2 mutant intestine is consistent with conservation of this relationship. However, inactivation of Cdx2 at earlier stages has no such impact on Pdx1 (Gao et al. 2009) suggesting either that the ectopic Pdx1 expression observed in the current study is indicative of a temporal Cdx2-dependent event or simply reflective of the transformation of the small intestine to a posterior stomach identity.

Cdx1 function in the small intestine

Cdx1 and Cdx2 functionally overlap in vertebral patterning (Faas and Isaacs, 2009; Savory et al., 2009b; van den Akker et al., 2002) and hematopoiesis (Davidson and Zon, 2006). Their co-expression in the intestinal epithelium suggests the potential for similar functional overlap. In this regard, early ablation of Cdx2 in the definitive endoderm results in the loss of onset of expression of *Cdx1* (Gao et al., 2009), and the phenotype observed in this prior work relative to the current study, could be suggestive of a Cdx1-Cdx2 double mutant phenotype.

As Cdx1 expression was not affected by Cdx2 loss at E13.5, we generated Cdx1-Cdx2 double mutants to investigate the contribution of Cdx1 to intestinal development at this stage. The finding that Cdx1-Cdx2 double mutants were indistinguishable from Cdx2 single mutants, together with the lack of an intestinal phenotype in Cdx1 single mutants (Subramanian et al., 1995) argues that Cdx1 may be dispensable for the development of the small intestine, as previously suggested (Bonhomme et al., 2008). We cannot exclude, however, an earlier role for Cdx1 in endodermal progenitor cells (Hierholzer and Kemler, 2009). It is also conceivable that Cdx1 plays a critical distal GI tract where its function may be masked by residual Cdx2 expression. Finally, it is conceivable that Cdx1 (and Cdx2) play critical roles in the adult intestinal tract.

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CHAPTER 3: Cdx regulates Dll1 in multiple lineages

Cdx regulates Dll1 in multiple lineages

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Running title: Dll1 and Cdx operate in a common pathway

Keywords: Cdx1, Cdx2, Dll1, intestine, somitogenesis, patterning, Notch, differentiation

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Author contributions:

Stephanie Grainger designed and carried out all experiments in the intestine and genetic non-complementation and *in situ* hybridization in embryos, as well as wrote manuscript and made figures.

Jennifer Lam carried out most embryo experiments and generated EMSA data.

Joanne G.A. Savory generated $Cdx2^{f/f}$ mice.

Alan J. Mears helped with microarray analysis.

Filippo M. Rijli provided *Actin* cre-ER^T mice.

David Lohnes helped to design experiments and to write the manuscript.

ABSTRACT

Vertebrate *Cdx* genes encode homeodomain transcription factors related to *caudal* in *Drosophila*. The murine *Cdx* homologues *Cdx1*, *Cdx2* and *Cdx4* play important roles in anterior-posterior patterning of the embryonic axis and the intestine, as well as axial elongation. While our understanding of the ontogenic programs requiring Cdx function has advanced considerably, the molecular bases underlying these functions are less well understood. In this regard, *Cdx1-Cdx2* conditional mutants exhibit abnormal somite formation, while loss of Cdx1-Cdx2 in the intestinal epithelium results in a shift in differentiation towards the Goblet cell lineage. The aim of the present study was to identify the Cdx-dependent mechanisms impacting on these events. Consistent with prior work implicating Notch signaling in these pathways, we found that expression of the Notch ligand *Dll1* was reduced in Cdx mutants in both the intestinal epithelium and paraxial mesoderm. Cdx members occupied the *Dll1* promoter both *in vivo* and *in vitro*, while genetic analysis indicated interaction between Cdx and Dll1 pathways in both somitogenesis and Goblet cell differentiation. These findings suggest that Cdx members operate upstream of *Dll1* to convey different functions in two distinct lineages.

INTRODUCTION

The endoderm, ectoderm and mesoderm germ layers are formed during gastrulation, with each contributing to distinct cellular lineages. For example, the vertebrae of the axial skeleton and their associated muscles and tendons, the skeletal muscles of the body wall and limbs, as well as the dermis of the back are derived from transient blocks of paraxial mesoderm, known as somites. Somites are produced in a periodic fashion via condensation of paraxial presomitic mesoderm, with a new somite pair being formed approximately every 120 minutes in the mouse. Once generated, somites undergo subsequent differentiation into dermatome, myotome, and sclerotome, which are the anlagen of the dermis, skeletal muscle of the trunk and limbs, and vertebrae, respectively (Dequeant and Pourquie, 2008).

The periodicity of somite condensation and their placement along the axis are under tight spatio-temporal regulation. The positioning of somite condensation is believed to rely on the interaction of caudal-high gradients of fibroblast growth factors (FGFs) and Wnt proteins, which are opposed by a gradient of retinoic acid (RA) produced in more anterior regions. These opposing cues are believed to establish a determination front which dictates the location of somite condensation along the AP axis (Olivera-Martinez and Storey, 2007; Wahl et al., 2007). The periodicity of somite segmentation is governed by a molecular "clock", the activity of which is reflected by the oscillating expression of a number of genes. For example, members of the Notch pathway including *Lfng*, *Hes1*, and *Hes7*; Wnt pathway components such as *Axin2;* and Fgf pathway members *Spry2* and *Dusp6* (Feller et al., 2008; Gomez et al., 2008; Gridley,

2006) oscillate during somitogenesis. Expression of many of these genes is typically initiated in the caudal region of the tailbud and propagate anteriorly through the presomitic mesoderm to the determination front, where segmentation is initiated (Olivera-Martinez and Storey, 2007).

Like the mesoderm, the endoderm also undergoes extensive patterning along the AP axis, leading to the mature gastrointestinal tract. This patterning is reflected by the regionalization of the intestinal epithelium into esophagus, stomach, small and large intestines, as well as the development of accessory organs such as the pancreas, liver and salivary glands (Barrow, 2006; Kwon et al., 2008; Rajewsky, 2006; Wells and Melton, 1999). Endoderm patterning is incompletely understood, but relies on a number of transcription factors including Cdx2, as well as signaling molecules such as Wnt, Shh and RA, which emanate from both the endoderm and the underlying mesoderm (Bayha et al., 2009; Bergsten et al., 2001; Gao et al., 2009; Grainger et al., 2010; Wells and Melton, 1999; Zacchetti et al., 2007).

The mature small intestinal epithelium is composed of villi and invaginating crypts which define the crypt-villus axis, and is maintained by intestinal stem cells in two distinct populations at or near the base of each crypt (Barker et al., 2007; Sangiorgi and Capecchi, 2008). These stem cells give rise to rapidly proliferating transit-amplifying cells which subsequently exit mitosis and differentiate into the mature cells of the intestinal epithelium. These are grouped into either absorptive cells (enterocytes) or the secretory cells (Goblet, enteroendocrine and Paneth cells) (Wells and Melton, 1999).

The intestinal mucosa is the most rapidly renewing epithelium in the body. Turnover occurs every 5-7 days in the mouse, with the exception of the Paneth cells

which reside in the base of the crypt for approximately 21 days (Wells and Melton, 1999). This rapid turnover necessitates tight coordination of proliferation and differentiation of precursor populations, which is regulated by a transcription network that includes Wnt and Notch pathways, among others (Crosnier et al., 2006; de Lau et al., 2007; Fre et al., 2005).

In the canonical pathway, Notch receptors bind Delta-Serrate-Lag-2 (DSL) ligands, which include the Delta-like (Dll) homologues in mammals. Ligand binding to the Notch receptor initiates two proteolytic cleavages leading to the release and nuclear translocation of the Notch intracellular domain (NICD). NICD then participates in the transcriptional regulation of target genes via association with CSL transcription factors (D'Souza et al., 2008; Fortini, 2009). In the intestine, both the maintenance of the intestinal stem cell niche and differentiation of the transit-amplifying (TA) cell population are dependent on Notch signaling (Pellegrinet et al., 2011; Wells and Melton, 1999). In particular, Notch activation of *Hes1* favours enterocyte differentiation, while high *Math1* activity results in differentiation into the secretory lineages. Consistent with this, loss of *Math1* results in a depletion of secretory lineages without affecting enterocytes (Yang et al., 2001), while loss of *Hes1* results in an increase in secretory lineages at the expense of enterocytes (Jensen et al., 2000). Cross-talk between these two transcription factors is also evidenced by the finding that Hes1 represses *Math1* expression (Jensen et al., 2000; Zheng et al., 2000). Involvement of Notch signaling in intestinal differentiation is further underscored by the outcome of *Pofut1* or *RBP-J* mutation, gamma secretase inhibition or through simultaneous loss of either *Notch1* and

Notch2 or *Dll1* and *Dll4*, all of which bias TA cell differentiation into Goblet cells (Guilmeau et al., 2008; Pellegrinet et al., 2011; Riccio et al., 2008c; van Es JH, 2005).

Cdx genes encode homeodomain transcription factors related to *Drosophila caudal*. The three *Cdx* murine homologues, *Cdx1*, *Cdx2* and *Cdx4*, exhibit overlapping patterns of expression in the posterior embryo and play overlapping roles in vertebral patterning, axial elongation, and neural tube closure (Beck, 2004; Beck et al., 1995; Lohnes, 2003; Savory et al., 2011a; Savory et al., 2009b; van den Akker et al., 2002; van Nes et al., 2006). Cdx2 also plays key roles in patterning of the definitive endoderm and, together with Cdx1, is essential for maintenance of the intestinal epithelium in the adult (Beck et al., 1999; Gao et al., 2009; Grainger et al., 2010; Verzi et al., 2010b; Verzi et al., 2011).

Loss of Cdx2, or both Cdx1 and Cdx2, results in an increase in Goblet cells in the small intestine as well as defects in somitogenesis (Crissey et al., 2011; Savory et al., 2009a; Savory et al., 2011a; Verzi et al., 2011). Consistent with a role for Notch signaling in these processes, we found that expression of *Dll1* was compromised in both the intestinal epithelium and in the tail bud of Cdx mutant embryos, with concomitant impact on downstream differentiation effectors in the intestine. Non-allelic non-complementation studies revealed a genetic interaction between *Cdx* and *Dll1* mutant alleles in both paraxial mesoderm and in the intestinal epithelium. Finally, we identified two potential Cdx response elements in the *Dll1* promoter, and found that Cdx proteins occupy this region *in vivo*. These findings are consistent with *Dll1* operating downstream of Cdx members, possibly through a direct regulatory interaction. This study illustrates

that Cdx function can manifest, in part, through a common molecular pathway in distinct lineages.

MATERIALS AND METHODS

Mice

Cdx1$^{-/-}$, *Cdx2*$^{f/f}$, *Dll1*$^{f/f}$, *actin*-Cre ER^{T} and *villin*-Cre ER^{T} mice have been previously described (Brooker et al., 2006; el Marjou et al., 2004; Santagati et al., 2005; Savory et al., 2009a; Subramanian et al., 1995). *Cdx2* deletion was effected by Tamoxifen (Tam) administration at E13.5 in *villin*-Cre ER^{T} (Grainger et al., 2010)(Grainger et al.) or at E5.5 in *actin*-Cre ER^{T} (Savory et al., 2009a) backgrounds. Embryos were subsequently harvested at E6.5-E9.5 for investigation of somitogenesis, while gastrointestinal tracts were harvested at E18.5. Non-transgenic littermates were used as controls in both instances.

Histological analysis

E18.5 intestinal tracts were sectioned and processed for histological staining as previously described (Grainger et al., 2010). Slides were mounted using Permount (Fisher) and images captured using a Zeiss Mirax Midi Scanner (Zeiss). Goblet cells were quantified as PAS-positive cells relative to the total number of nuclei captured from 5 random fields from each sample. Data was accrued from a minimum of 3 independent samples.

In situ hybridization

In situ hybridization (ISH) of gastrointestinal sections was carried out as previously described (Grainger et al., 2010) using probes for *Hes1* and *Dll1* (Schroder and Gossler, 2002). The probe for *Math1*, corresponding to the first 500 bp of the transcript, was derived by RT-PCR. Whole mount ISH was performed as previously described, with probes for *Mox1*, *Uncx4.1* and *Paraxis* (Houle et al., 2000; Savory et al., 2011a). Embryos were photographed using a Leica MZ16FA microscope.

Quantitative Polymerase Chain Reaction (qPCR) and semi-quantitative reverse-transcriptase PCR (RT-PCR)

RNA was extracted from embryonic (E)18.5 small intestine using Trizol reagent (Invitrogen) and used to generate cDNA by standard procedures. cDNA was subsequently amplified by semi-quantitative RT-PCR or qPCR using oligonucleotides specific for *Dll1*, *Math1*, *TFF3*, *IFABP* or *β-actin* with SsoFast EvaGreen Supermix (qPCR, BioRad) or GoTaq (RT-PCR, Promega), according to the manufacturer's recommendations. qPCR was performed using the MX3005P (Agilent Technologies) and results were analyzed using the $2^{-\Delta\Delta Ct}$ method (Schefe et al., 2006), normalized to *β-actin*. For specificity, the dissociation curve was considered for each amplicon. RT-PCR was performed over a series of cycles and samples within the linear range used for analysis. Data in both cases is reflective of at least 3 different biological sample sets (specific PCR conditions and primer sequences available in supplementary information).

Chromatin immunoprecipitation (ChIP)

ChIP was performed as previously described (Pilon et al., 2006) using chromatin generated from wild-type E8.5 embryos or E18.5 intestinal tracts. PCR was directed over regions encompassing potential CDREs, or distal (control) intervals by standard methods. Oligonucleotide sequences used for amplification are available in Appendix Table 1.

Electrophoretic mobility shift assay (EMSA)

EMSA was carried out as previously described (Houle et al., 2000; Pilon et al., 2006). GST or GST-Cdx2 fusion proteins were used (Beland et al., 2004) with double-stranded oligonucleotides harbouring either candidate Cdx response elements or mutated sequences thereof. Oligonucleotides corresponding to a Cdx response element from the *Hoxb8* locus (Charite et al., 1998), or a DR5 retinoic acid response element served as positive and negative controls, respectively. Antibody supershift was carried out using 1 μg of αCdx2 (Savory et al., 2009b). Oligonucleotide sequences are available in Appendix Table 2.

RESULTS

Loss of Cdx impacts Goblet cell differentiation

Cdx2 has been proposed to play a role in differentiation of the intestinal epithelium and maintenance of intestinal character (Alkhoury et al., 2005; Boyd et al., 2010; Chan et al., 2009; Crissey et al., 2011; Flores et al., 2008; Gao et al., 2009; Grainger et al., 2010; Mutoh et al., 2006; Mutoh et al., 2005). To further investigate this relationship, we circumvented the early lethality associated with loss of Cdx2 by effecting intestine-specific inactivation of a floxed *Cdx2* allele (Savory et al., 2009a). This was achieved using a Tamoxifen-inducible Cre driven by the *villin* promoter (el Marjou et al., 2004) as previously described (Grainger et al., 2010). To assess the contribution of Cdx1 to intestinal differentiation, we bred the *Cdx1*$^{-/-}$ mouse line (Subramanian et al., 1995) into the *Cdx2*$^{f/f}$ *villin* Cre-ER^{T} background and deleted *Cdx2* as above.

To investigate the impact of loss of Cdx function on intestinal differentiation, we assessed Goblet cell number using differential staining with Periodic-Acid Schiff (PAS) or Alcian Blue (Culling et al., 1975). Wild-type fetal intestinal epithelium displayed robust PAS staining of Goblet cells throughout the intestinal tract with a similar pattern for Alcian blue (Fig. 31 and data not shown). *Cdx2* mutant intestine exhibited ectopic PAS staining along the apical edge of the epithelium, which, together with other data, is suggestive of transformation to a pyloric stomach as previously described (Grainger et al., 2010). In addition, these mutants exhibited normal Goblet cells (Fig. 31A, white

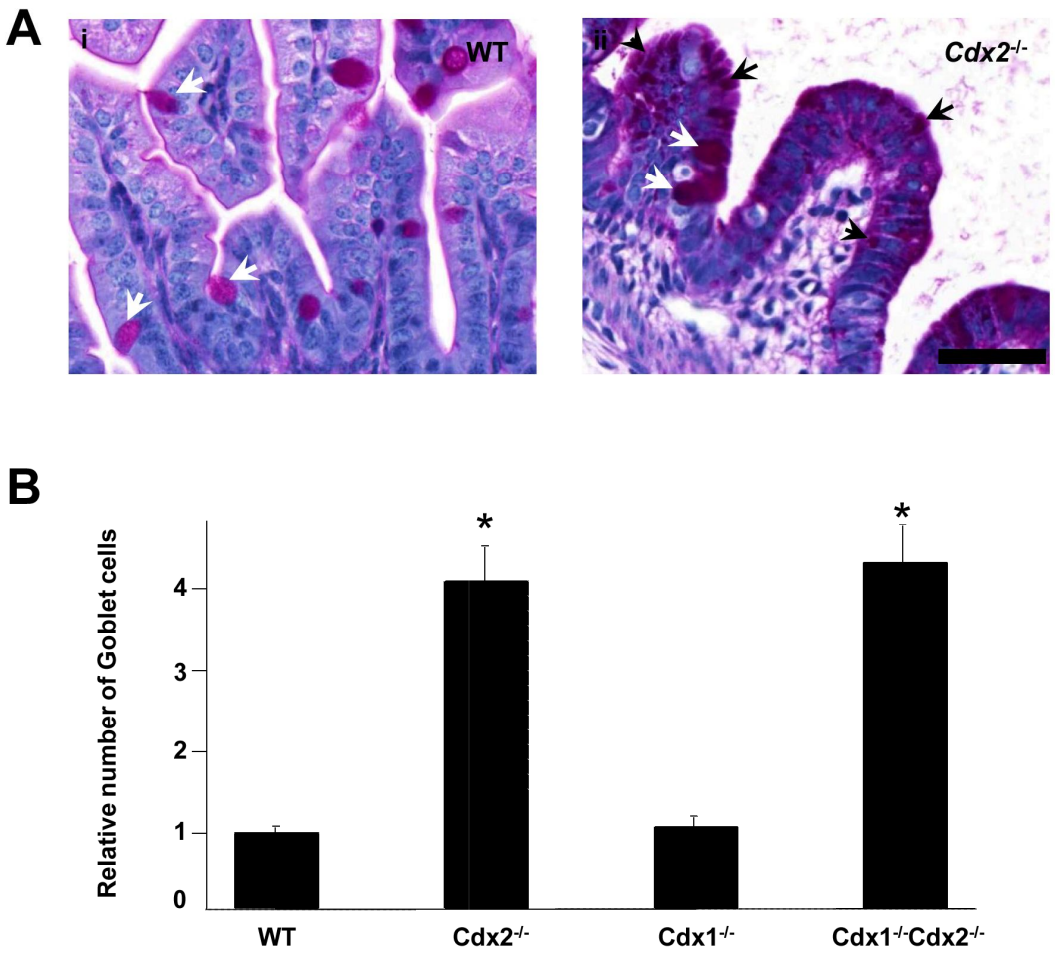

Figure 31: Loss of Cdx2 affects Goblet cell lineage in the small intestine. (A) Periodic Acid Schiff (PAS) staining of E18.5 small intestine from wild-type (WT) (i) and *Cdx2*$^{-/-}$ (ii) littermates. Note the normal (white arrows) and smaller-sized (black arrows) Goblet cells. Scale bars represent 50 µm. (B) Quantification of the number of Goblet cells in WT, *Cdx2*$^{-/-}$, *Cdx1*$^{-/-}$ and *Cdx1*$^{-/-}$*Cdx2*$^{-/-}$ E18.5 small intestinal tracts. Error bars represent s.d. from the mean of 5 independent fields. These results were consistent for n=3 animals from each genotype. * $p<0.05$ by Student's t-test.

arrows) and a distinct population of smaller Goblet cells (Fig. 31A, black arrows) which stained robustly with PAS, but less so with Alcian blue (data not shown). Quantification of Goblet cells in the small intestine of *Cdx2* single mutants showed an approximate four-fold increase compared to littermate controls (Fig. 31B). There was no difference in the number of Goblet cells in *Cdx1-Cdx2* double mutants compared to *Cdx2* single mutants (Fig. 31B), suggesting that Cdx2, but not Cdx1, suppresses Goblet cell differentiation.

Loss of Cdx2 affects lineage decision events in the small intestine

Differentiation of the intestinal epithelium proceeds through an early cell-fate decision event towards either the secretory or absorptive lineage (van der Flier and Clevers, 2009). Semi-quantitative RT-PCR and qPCR was performed to investigate the effect of Cdx loss on the expression of genes indicative of this bifurcation. Disruption of *Cdx2* alone or in combination with *Cdx1* resulted in a strong reduction in expression of the enterocyte marker *IFABP* (Agellon et al., 2002) (Fig. 32B, C) and a concomitant increase in expression of the Goblet cell markers *TFF3, Gfi1* and *Klf4* (Katz et al., 2002; Podolsky et al., 1993; Shroyer et al., 2005) (Fig. 32B, C and data not shown), indicative of an increase in the number of Goblet cells at the expense of enterocytes.

The secretory versus absorptive cell fate decision is largely controlled by the Notch signaling cascade through the downstream target *Hes1*, together with the bHLH transcription factor Math1 (Fig. 32A) (Yang et al., 2001). In this model, Notch-dependent induction of *Hes1* favors differentiation towards an absorptive fate (Jensen et al., 2000). Conversely, *Math1*, which is repressed by Hes1 (Jensen et al., 2000; Zheng et al., 2000),

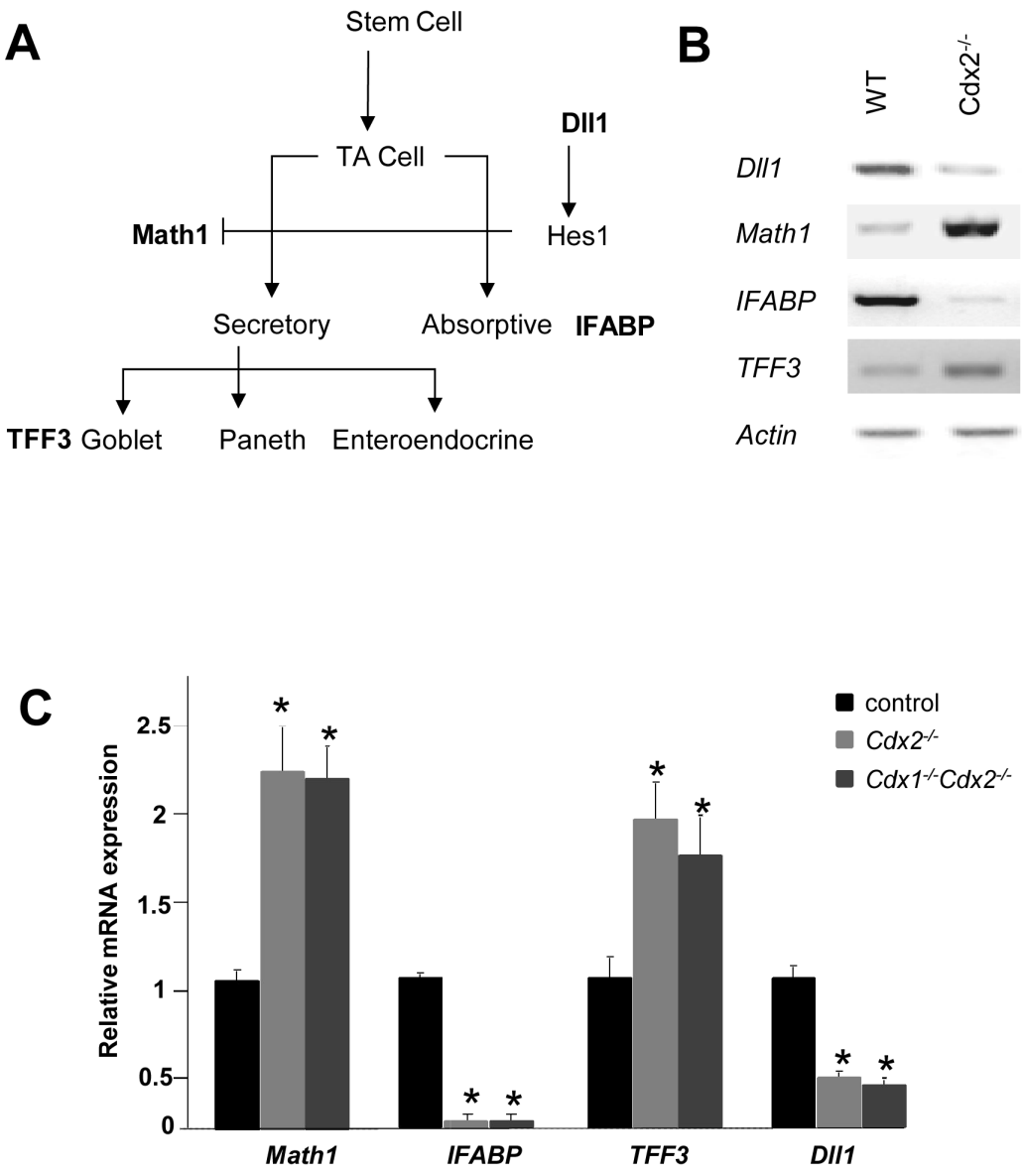

Figure 32: Loss of Cdx2 impacts on players in intestinal differentiation. (A) Schematic representation of intestinal epithelial differentiation. (B) Semi-quantitative reverse-transcriptase polymerase chain reaction (RT-PCR) for WT and *Cdx2*$^{-/-}$ small intestines from littermates at E18.5. (C) Quantitative PCR data from control, *Cdx2*$^{-/-}$ and *Cdx1*$^{-/-}$*Cdx2*$^{-/-}$ E18.5 intestines. Error bars represent s.d. from the mean expression levels of 3 independent samples. * $p<0.05$ by Student's t-test.

biases cells towards a secretory fate (Yang et al., 2001). In *Cdx2* mutants, *Math1* expression in the small intestine was upregulated as assessed by both qPCR (Fig. 32B, C) and *in situ* hybridization (Fig. 33C). The finding that *Cdx* mutants exhibited a reduction in *Hes1* levels (Fig. 33A) suggested a basis for the observed increase in *Math1*. Subsequent analyses revealed that loss of Cdx2 alone, or together with Cdx1, resulted in a reduction in the expression of *Dll1* (Figs. 32B, C and 33B). Expression of the Notch ligands *Jag1/2* and *Dll3/4*, as well as the *Notch1* and *Notch4* receptors, were unaffected by the loss of Cdx2 (data not shown). These findings suggested that Cdx2 impacts on Goblet cell differentiation through regulation of *Dll1* expression.

Cdx1-Cdx2 mutants exhibit aberrant somitogenesis

Prior work has shown that Cdx function is also required for normal somitogenesis (Savory et al., 2009a; Savory et al., 2011a). In *Cdx1-Cdx2* double mutants, somites appear irregularly shaped with indistinct boundaries. *In situ* hybridization analysis of *Cdx1-Cdx2* mutants using probes against *Mox1, Uncx4.1* and *Paraxis* suggested that somites were specified and condensed, but that segmentation and rostral-caudal polarization were defective (Fig. 34 and our previously published data) (Savory et al., 2011a).

Dll1 mutants exhibit somite defects similar to the *Cdx* mutants described above (Hrabe de Angelis et al., 1997). As *Dll1* expression is affected by ablation of Cdx2 in the intestine, and expression of *Dll1, Cdx1* and *Cdx2* are largely concordant in the caudal

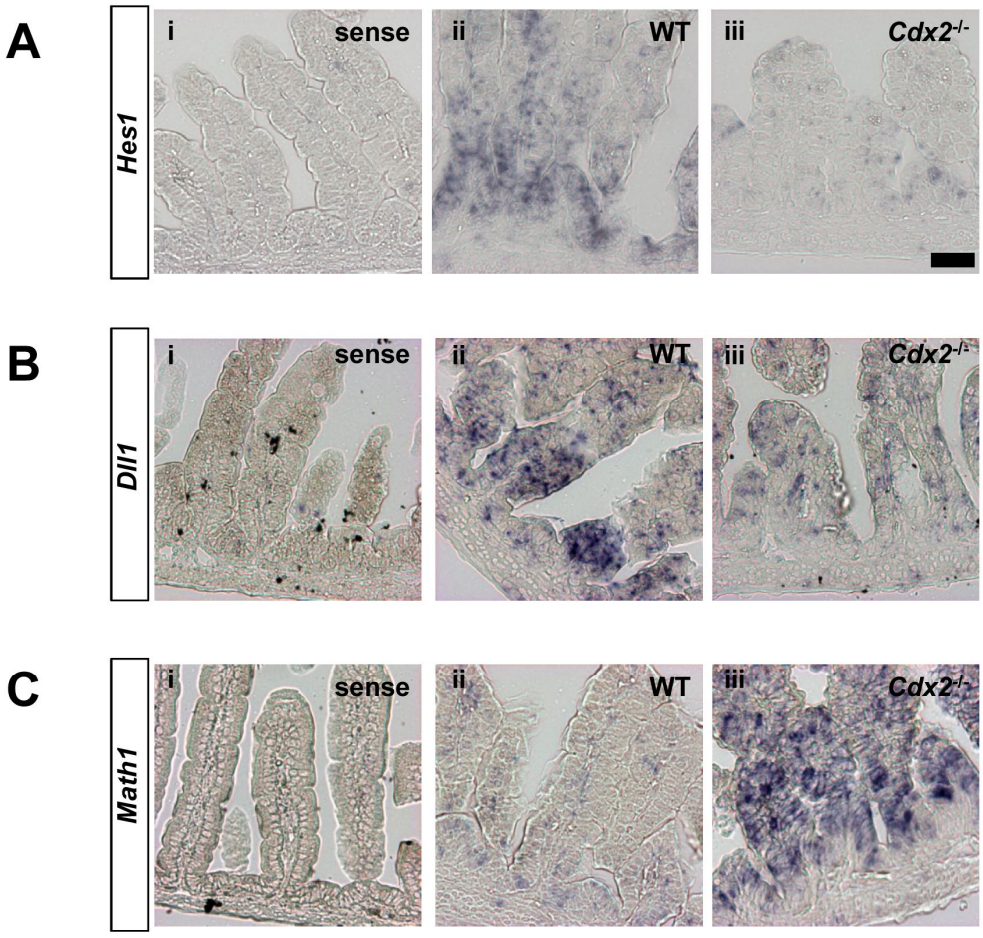

Figure 33: Loss of Cdx2 affects the Notch pathway. *In situ* hybridization of E18.5 intestines for *Hes1* (A), *Dll1* (B) and *Math1* (C) in sense controls (i), WT (ii) and *Cdx2*$^{-/-}$ (iii). Note the loss of expression in *Cdx2* mutant intestine (iii) at E18.5 in A and B and increase in expression in C. Scale bar represents 50 μm.

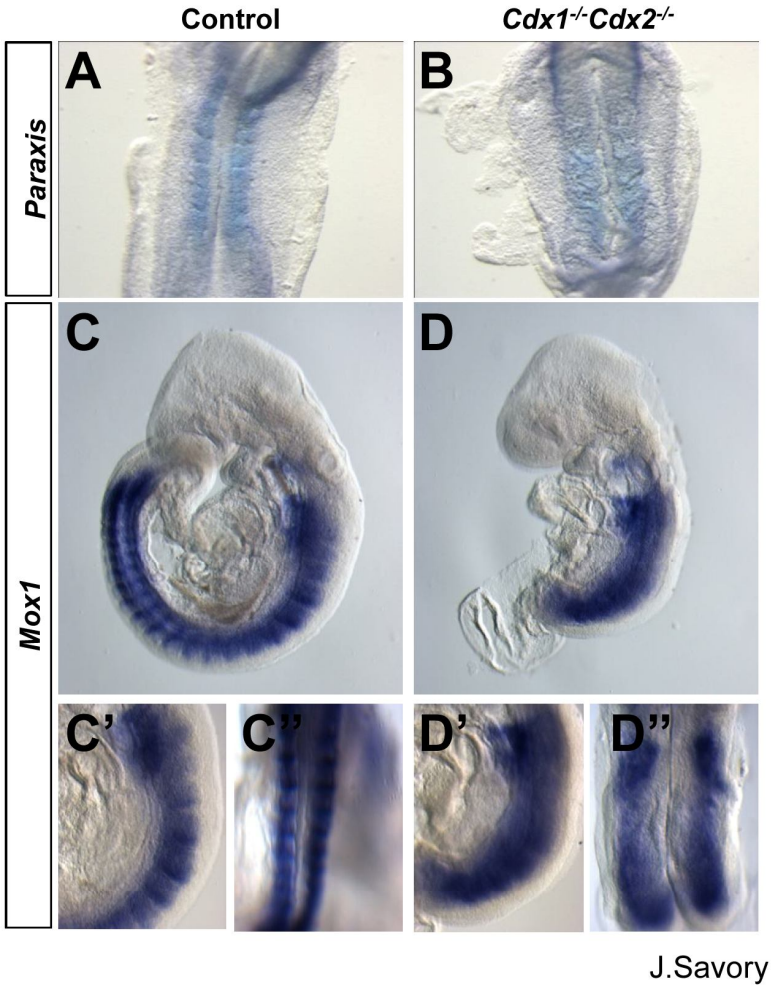

Figure 34: Loss of Cdx1 and Cdx2 results in abnormal somitogenesis. (A-D) Whole mount *in situ* hybridization of E8.5 (A and B) and E9.5 (C and D) *Cdx1*$^{-/-}$*Cdx2*$^{-/-}$ embryos (A and C) and littermate controls (B and D) stained with either *Paraxis (*A and B) or *Mox1* (C and D). (C' to D") Magnified lateral (C' and D') and dorsal (C" and D") views of the embryos shown in C and D. Note the abnormal somite segmentation in the *Cdx1*$^{-/-}$*Cdx2*$^{-/-}$ embryo.

embryo during relevant stages of somitogenesis (Beck et al., 1995; Bettenhausen et al., 1995; Meyer and Gruss, 1993), we examined *Cdx* mutant embryos for *Dll1* expression. As described previously (Bettenhausen et al., 1995), *Dll1* transcripts were detected in wild-type embryos around the mid-streak stage, at approximately E7.5, in the mesoderm and primitive streak region. Subsequent expression became confined to the tail bud and posterior mesoderm, with an anterior boundary just rostral to the node. *Dll1* was also detected in the caudal halves of condensed somites and was subsequently extinguished in the tail bud by E15.5 (data not shown). In *Cdx1-Cdx2* mutants, *Dll1* expression was indistinguishable from controls at E7.5 (Fig. 35). However, at E8.5, a reduction in *Dll1* transcripts, was observed in *Cdx1-Cdx2* mutants relative to controls (Fig. 36). These results suggest that Cdx1 and Cdx2 are essential for the maintenance of *Dll1* transcription in the paraxial mesoderm, consistent with the previously demonstrated functional overlap between these family members in the caudal embryo (Savory et al., 2011a; Savory et al., 2009b; van den Akker et al., 2002).

The *Dll1* promoter is occupied by Cdx members

The loss of *Dll1* expression in both the intestinal epithelium and the presomitic mesoderm of *Cdx* mutants, together with phenotypes consistent with perturbed Notch signaling, suggest that *Dll1* may be a direct Cdx target. Transcriptional Element Search System (TESS) analysis identified two potential Cdx response elements (CDREs), one at -3964 (relative to the transcriptional start site), composed of the canonical sequence TTTATG, and a more proximal element at -3404, which was the complement of this

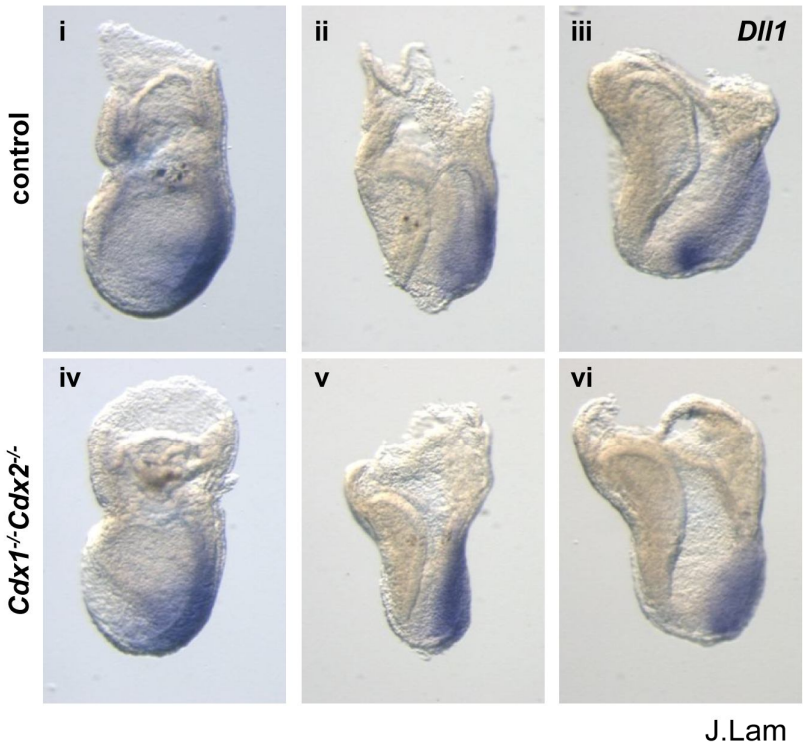

Figure 35: Loss of Cdx does not affect initiation of Dll1 expression. Whole mount *in situ* hybridization for *Dll1* in E6.5-E7.5 *Cdx1*$^{-/-}$*Cdx2*$^{-/-}$ (iv, v, vi) and littermate controls (i, ii, iii).

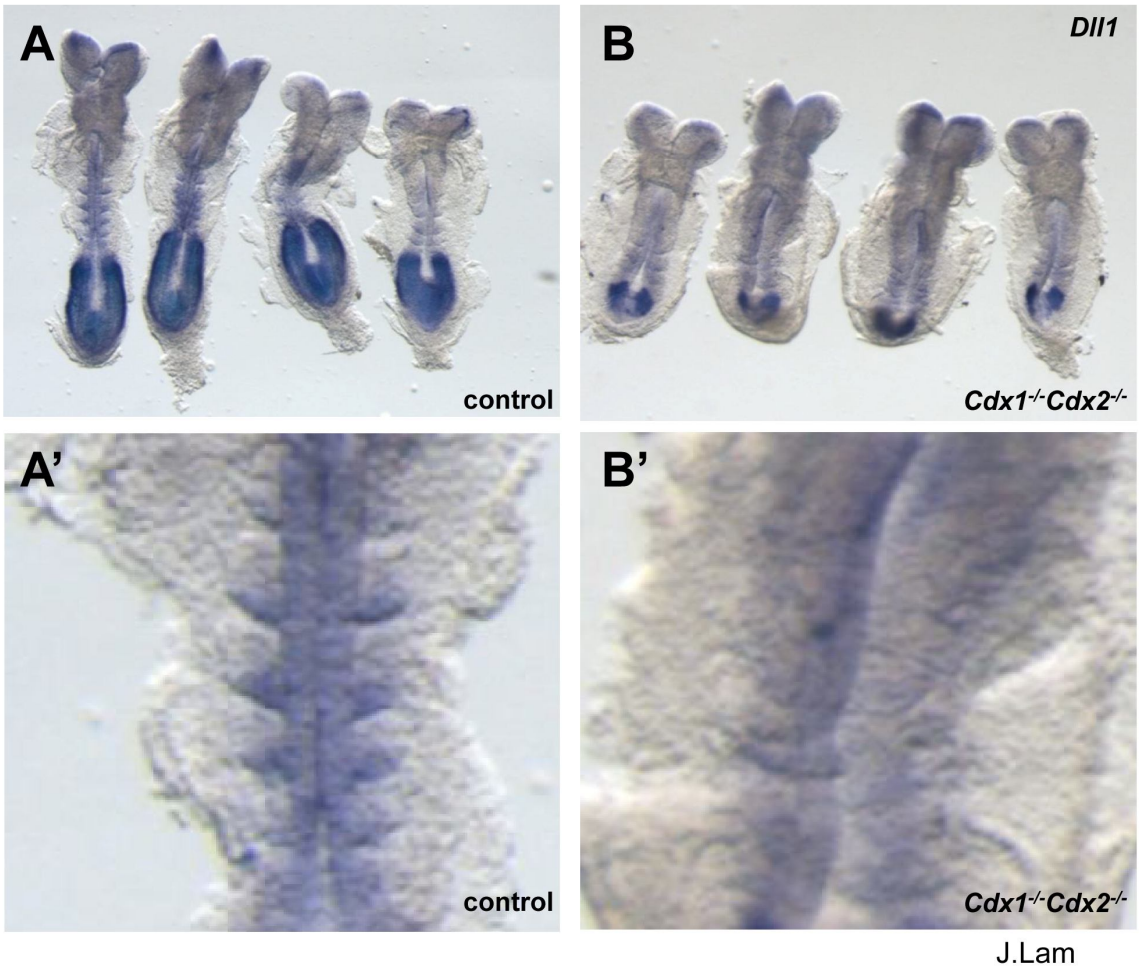

Figure 36: Loss of Cdx1-Cdx2 affects Dll1 expression. Whole mount *in situ* hybridization for *Dll1* in E8.5 *Cdx1*$^{-/-}$*Cdx2*$^{-/-}$ (ii, iv) and littermate controls (i, iii). Magnified views of the somites are shown in iii and iv.

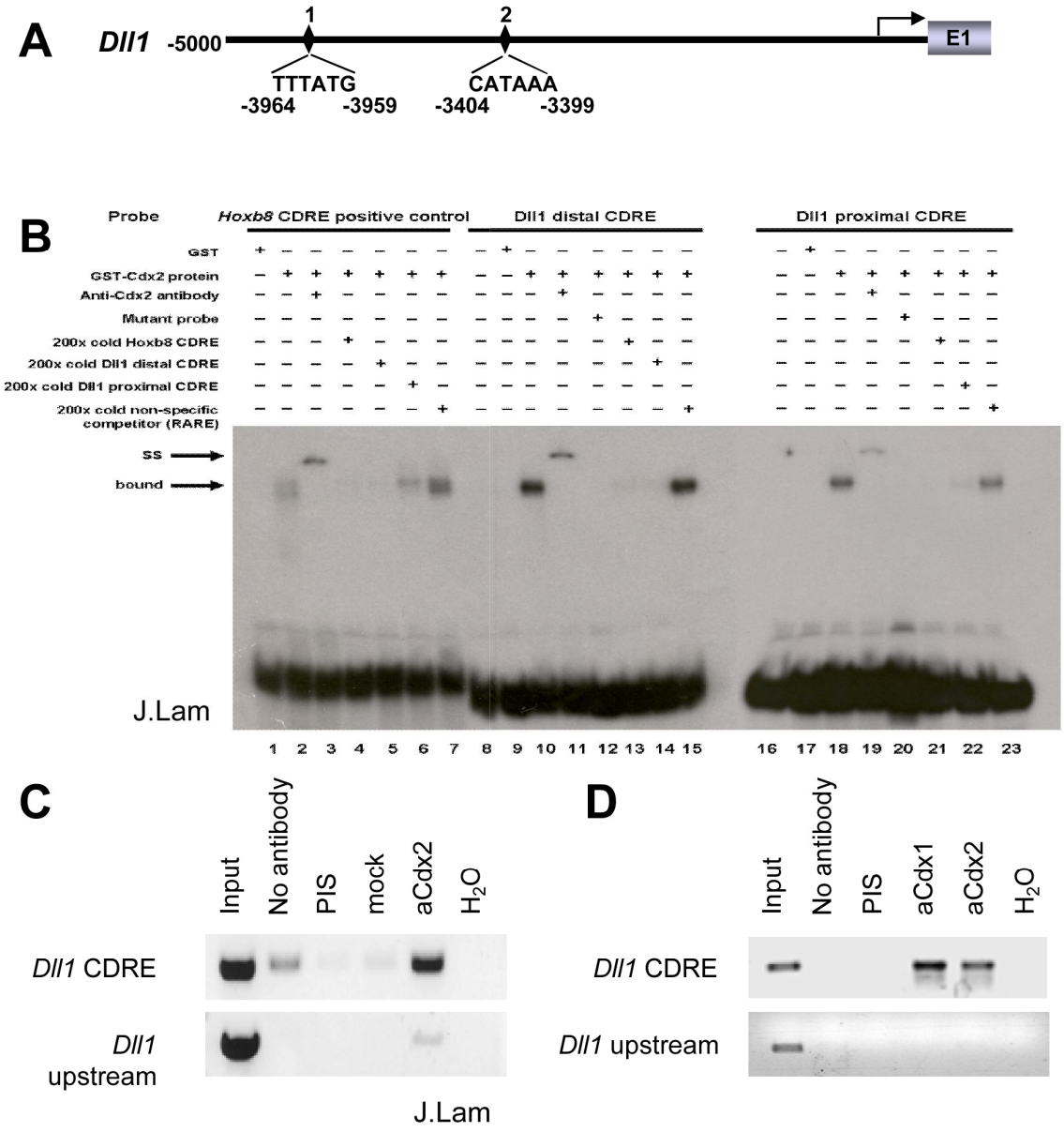

Figure 37: Cdx proteins bind to the Dll1 promoter *in vitro* and *in vivo.* (A) Schematic representation of the two CDREs found in the 5 kb region upstream of the *Dll1* transcriptional start site. (B) Electrophoretic mobility shift assay (EMSA) demonstrates that both of the CDREs can be bound *in vitro* by Cdx2. (C) Chromatin immunoprecipitation (ChIP) from a pool of wild-type E8.5 embryos. Pull-down for Cdx2 demonstrates that Cdx2 is bound to the *Dll1* promoter region, but does not bind to an upstream control *in vivo*. (D) ChIP of E18.5 wild-type intestinal tracts demonstrates that Cdx1 and Cdx2 are bound to the *Dll1* promoter, but do not bind to an upstream control *in vivo* in the intestinal tract.

sequence (Fig. 37A). Electrophoretic mobility shift assay (EMSA) demonstrated that Cdx2 could associate with either of these motifs in a manner comparable to the bona fide CDRE from the *Hoxb8* locus (Charite et al., 1998). This binding occurred in a specific manner as assessed by both supershift and competition analyses (Fig. 37B).

Since Cdx2 was able to bind the putative CDREs *in vitro*, we next sought to determine if Cdx members localized to the *Dll1* locus *in vivo* using chromatin immunoprecipitation (ChIP). ChIP analysis using Cdx1 or Cdx2 antibodies (Savory et al., 2009b) revealed that both Cdx1 and Cdx2 were enriched within the proximity of the putative CDREs using chromatin from either E8.5 embryos (Fig. 37C) or intestinal tracts from E18.5 fetuses (Fig. 37D). Cdx1 and Cdx2 were not enriched in regions encompassing a TTTATG sequence in the β-actin promoter (data not shown) or to regions approximately 10kb upstream of the *Dll1* transcriptional start site (Fig. 37B, C), suggesting that the interaction seen in the *Dll1* promoter region is specific.

Cdx1-Cdx2 and *Dll1* mutant alleles interact genetically

To assess if *Cdx* and *Dll1* operate in a common genetic pathway *in vivo*, we used a non-allelic non-complementation approach. This was accomplished through the generation of *Cdx1-Cdx2-Dll1* triple heterozygotes by crossing the $Dll1^{f/f}$ allele (Brooker et al., 2006) into the $Cdx1^{-/-}Cdx2^{f/f}$ *actin* Cre-ER^{T} or $Cdx1^{-/-}Cdx2^{f/f}$ *villin* Cre-ER^{T} backgrounds. Tamoxifen-mediated inactivation of the *Cdx2* and *Dll1* floxed alleles was elicited at either E5.5 or E13.5.

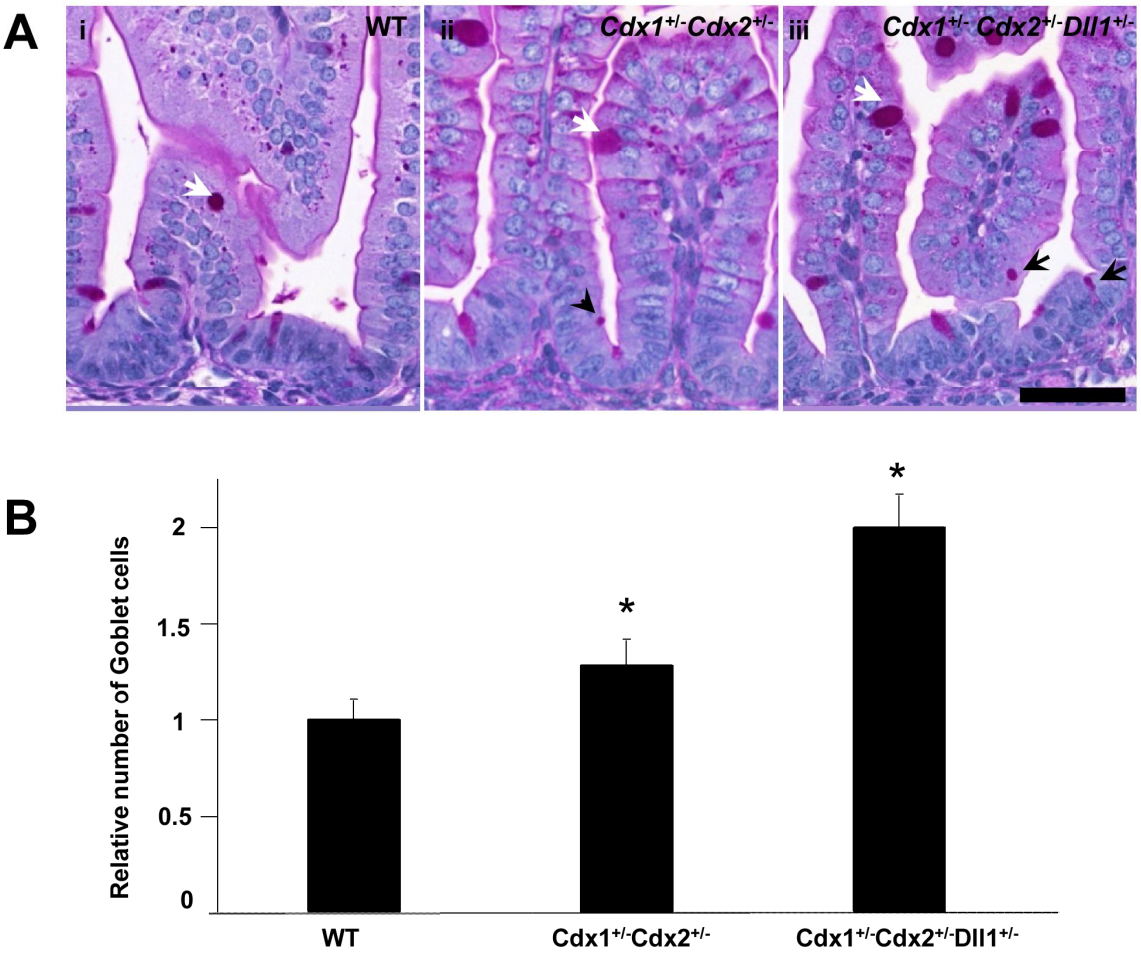

Figure 38: Cdx and Dll1 interact genetically in the intestine. (A) Representative PAS staining of proximal duodenum from E18.5 intestinal tracts from WT (i), *Cdx1*$^{+/-}$ *Cdx2*$^{+/-}$ (ii) and *Cdx1*$^{+/-}$*Cdx2*$^{+/-}$*Dll1*$^{+/-}$ (iii) mice. Scale bar represents 50 mm. (B) Quantification of the number of normal sized (white arrows) and smaller (black arrows) Goblet cells in A. Error bars represent s.d. from the mean of 5 independent fields. These results were consistent for n=3 animals from each genotype. * $p<0.05$ by Student's t-test.

To determine if interaction between *Cdx* and *Dll1* mutant alleles impacted on intestinal differentiation, Goblet cell differentiation in compound mutants was assessed by PAS and Alcian Blue staining in E18.5 intestinal tracts. Compared to wild-type and $Cdx1^{+/-}Cdx2^{+/-}$ controls, $Cdx1^{+/-}Cdx2^{+/-}Dll1^{+/-}$ compound heterozygotes exhibited an increased number of Goblet cells (Fig. 38). Notably, a number of smaller Goblet cells were observed which stained less robustly with Alcian Blue (data not shown); an identical outcome was seen with loss of Cdx2 (Fig. 31). $Cdx1^{+/-}Cdx2^{+/-}$ intestines also had a modest increase in PAS staining compared to wild-type littermates (Fig. 38).

To investigate the interplay between *Cdx* and *Dll1* mutant alleles in somitogenesis, embryos were collected at E9.5 and *in situ* hybridization was performed. As previously described (Savory et al., 2011a), $Cdx1^{+/-}Cdx2^{+/-}$ embryos had no apparent somite defects and exhibited wild-type *Mox1* and *Paraxis* staining (data not shown). $Cdx1^{+/-}$ littermate controls were also normal with regards to *Mox1* and *Paraxis* expression (Fig. 39, data not shown). Paraxial mesoderm was specified in $Cdx1^{+/-}Cdx2^{+/-}Dll1^{+/-}$ heterozygotes as assessed by *Paraxis* and *Mox1* expression. However, somite borders were indistinct and somite polarity also appeared to be impacted as evidenced by reduced expression of *Mox1* expression in condensed somites (Fig. 39, data not shown); a similar somitic phenotype is also seen in *Dll1* null mutants (Hrabe de Angelis et al., 1997). Taken together, these results are consistent with *Cdx* and *Dll1* operating in a common pathway during both somitogenesis and intestinal epithelial differentiation.

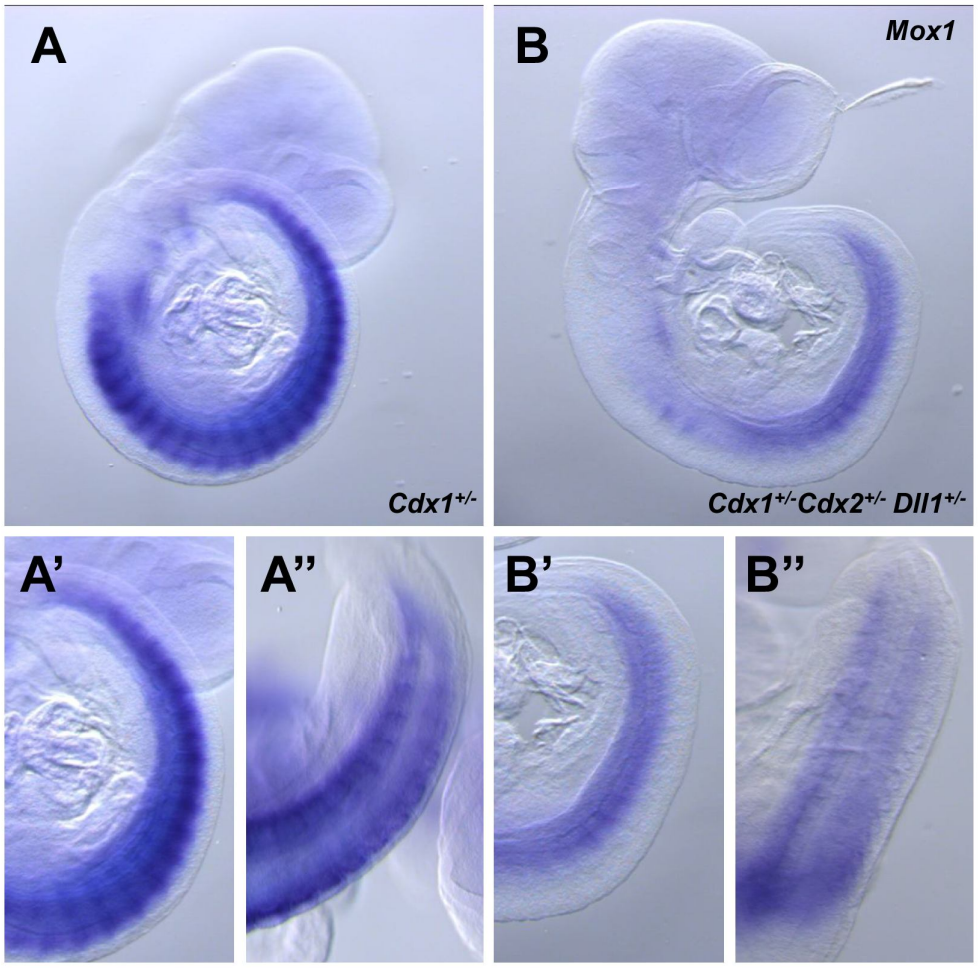

Figure 39: Cdx and Dll1 interact genetically during somitogenesis. Whole mount *in situ* hybridization of E9.5 *Cdx1*$^{+/-}$*Cdx2*$^{+/-}$*Dll1*$^{+/-}$ embryo (B) and littermate control (A) stained with *Mox1*. Magnified lateral (A' and B') and dorsal (A'' and B'') views of the embryos shown in A and B. Note the abnormal somites in the *Cdx1*$^{+/-}$*Cdx2*$^{+/-}$ *Dll1*$^{+/-}$ embryo.

DISCUSSION

Although Cdx members are known to play important roles in patterning of both the endoderm and mesoderm (Gao et al., 2009; Grainger et al., 2010; Savory et al., 2009a; Young et al., 2009), the targets mediating these outcomes are largely unknown. Moreover, the peri-implantation lethality inherent to *Cdx2* null mutants (Chawengsaksophak et al., 1997) and the functional overlap between Cdx members (Savory et al., 2009b; van den Akker et al., 2002; Young et al., 2009) has hampered our understanding of the roles of these transcription factors during development. To circumvent these limitations, we generated a conditional *Cdx2* mutant allele (Savory et al., 2009a) and derived *Cdx1-Cdx2* double mutants (Savory et al., 2011a) using ligand-activated Cre transgenes under the spatial control of either the *actin* or *villin* promoter to effect excision of *Cdx2* ubiquitously or in the intestinal epithelium, respectively. The impact of loss of function of Cdx2 or both Cdx1 and Cdx2 during somitogenesis or intestinal differentiation, together with promoter and genetic analyses, suggests that the Notch ligand *Dll1* is downstream of Cdx. This constitutes the first evidence for a role for Cdx in affecting the Notch signaling pathway, and that it does so in two distinct lineages.

Cdx in intestinal differentiation

Cdx2 plays a critical role in early intestinal patterning (Gao et al., 2009; Grainger et al., 2010). Studies in tissue culture models also suggest that Cdx2 plays important roles in terminal differentiation of the intestinal epithelium (Benoit et al., 2010; Suh et al., 1994; Suh and Traber, 1996), while more recent studies have supported a role for Cdx2 in

differentiation of Goblet cells (Crissey et al., 2011; Verzi et al., 2011), however, the molecular bases underlying this function are unknown.

Prior work illustrated that loss of Cdx2 at E13.5 results in a partial anterior transformation of the intestinal epithelium to a pyloric stomach phenotype (Grainger et al., 2010). Further analysis revealed an increase in the number of Goblet cells in the small intestine, as assessed by PAS staining, with less robust staining using Alcian Blue. This observation may be related to Goblet cell maturation, as Alcian Blue staining is associated with more mature acidic sialated mucins, while PAS staining is not specific to mature mucins (Ghaleb et al., 2011; Katz et al., 2002; Specian and Oliver, 1991). This finding is consistent with other studies describing an increase in Goblet cells following loss of Cdx2 in the mature intestine (Crissey et al., 2011; Verzi et al., 2011), suggesting that this relationship is maintained in the adult.

A number of pathways governing intestinal differentiation have been described (Jensen et al., 2000; Riccio et al., 2008a, b, c; Shroyer et al., 2005; Yang et al., 2001). RT-PCR analysis revealed that Cdx2 disruption resulted in a reduction of expression of genes indicative of enterocyte character with a concomitant increase in Goblet cell markers, as evidenced by a reduction in *IFABP* and a gain in *Math1* and *TFF3* levels, respectively. The Notch signaling pathway is central to intestinal differentiation, and we found that the Notch ligand *Dll1* was downregulated in both *Cdx2* and *Cdx1-Cdx2* mutant intestine, consistent with Cdx2 regulating intestinal differentiation through the Notch ligand *Dll1*. Of note, there was some residual *Dll1* expression after loss of Cdx2, which is likely related to regulation of *Dll1* by Cdx-independent mechanisms. This would appear to be specific, as other ligands (*Jag1* or *Jag2* and *Dll3* and *Dll4)* and the receptor *Notch1*

were unchanged. *Notch2* and *Notch3* were modestly upregulated (data not shown), consistent with the previously described feedback regulation between *Notch* and *Delta* (Sprinzak et al., 2010).

Loss of Cdx1 in the Cdx2 deficient intestine did not affect Goblet cell differentiation compared to loss of Cdx2 alone, consistent with the apparent lack of Cdx1 function in the small intestine, at least at this stage (Bonhomme et al., 2008; Grainger et al., 2010). However, $Cdx1^{+/-}Cdx2^{+/-}$ intestinal tracts exhibited an increase in the number of Goblet cells comparable to that seen in $Cdx2^{-/-}$ offspring. Since $Cdx1^{-/-}$ mice are wild-type in this respect, this finding may be related to cross- and auto-regulation of Cdx2 (Beland et al., 2004; Bonhomme et al., 2008; Chawengsaksophak et al., 2004; Crissey et al., 2008; Prinos et al., 2001; Savory et al., 2011a; Xu et al., 1999), rather than reflective of a role for Cdx1 in this process. In contrast, recent work suggests that Cdx1 and Cdx2 functionally overlap in the adult intestinal epithelium (Verzi et al., 2010b; Verzi et al., 2011), suggesting that Cdx1 does play a role in adult intestinal homeostasis.

Cdx and somitogenesis

Cdx1-Cdx2 double mutants, but neither single mutant, exhibit irregular and fused somites (Savory et al., 2011a). Although Cdx members are known to play fundamental roles in vertebral AP patterning and axial elongation through regulation of *Hox* and non-*Hox* targets (Davidson et al., 2003; Savory et al., 2009a; van Nes et al., 2006; Young et al., 2009), these associations fail to explain this phenotype. Notch signaling plays a prominent role in somitogenesis (Bessho et al., 2001a; Bessho et al., 2001b; Conlon et al., 1995; Dequeant et al., 2006; Hrabe de Angelis et al., 1997; Jouve et al., 2000). *Dll1*,

which is co-expressed with *Cdx* members in the caudal embryo, was markedly reduced in *Cdx1-Cdx2* mutants at E8.5 suggesting that Cdx functions upstream of *Dll1*. Despite concordant expression of *Cdx1* and *Cdx2* with *Dll1* at earlier stages, *Dll1* expression was indistinguishable between mutants and controls at E7.5 (Fig. 35). This suggests that Cdx may be involved in the maintenance rather than the initiation of *Dll1* activity.

Dll1 null mutants, like *Cdx1-Cdx2* mutants, exhibit aberrant somites with premature cessation of somitogenesis and compromised craniocaudal somite polarity (Hrabe de Angelis et al., 1997). This latter phenotype was also observed in $Cdx1^{+/-}Cdx2^{+/-}Dll1^{+/-}$ embryos, lending further evidence that Cdx and Dll1 operate in a common genetic pathway impacting somitogenesis. Also consistent with this is the finding that $Cdx1^{+/-}Cdx2^{+/-}Dll1^{+/-}$ offspring have modestly truncated tails, a finding which is expected based on the *Dll1* and *Cdx1-Cdx2* mutant phenotypes (Hrabe de Angelis et al., 1997; Savory et al., 2009a; Savory et al., 2011a). Furthermore, patterning defects in *Cdx1-Cdx2* mutant embryos are evident from the disruption of *Mox1* expression domains. This phenotype overlaps with *Dll1* mutants (Hrabe de Angelis et al., 1997), suggesting a role for Cdx members in somite rostral-caudal patterning of somites.

Cdx operates upstream of *Dll1*

The loss of *Dll1* expression in the fetal intestine and the caudal embryo, together with the overlapping phenotype between *Dll1* and *Cdx* null mutants, suggests that Cdx lies upstream of *Dll1*. Indeed, analysis of 5' proximal sequences revealed two candidate Cdx binding sites. Additionally, ChIP analyses indicated that Cdx1 and Cdx2 occupied the *Dll1* promoter *in vivo* in the region of the two putative CDREs, while EMSA revealed

that these motifs were capable of direct interaction with Cdx members. Together with the non-allelic non-complementation data, these findings are consistent with direct regulation of *Dll1* by Cdx.

Cdx members have been known to impart positional information during anterior-posterior patterning through regulation of *Hox* genes (Charite et al., 1998; Chawengsaksophak et al., 2004; Davidson et al., 2003; Deschamps et al., 1999; Lohnes, 2003). More recent evidence has suggested that Cdx members also function through non-*Hox* targets during both axis elongation and intestinal patterning (Gao et al., 2009; Savory et al., 2009a; Young et al., 2009). Furthermore, microarray and ChIP-seq studies have suggested that Cdx2 may regulate a plethora of other target genes, including *Dll1* (Boyd et al., 2010; Uesaka et al., 2004). Our present finding of *Dll1* as a Cdx regulated gene extends this paradigm. In addition, the data herein offers a mechanistic basis for several of the effects of Cdx loss-of-function in endoderm and mesoderm as well as novel evidence that some Cdx function can be mediated through the same target gene in two different lineages.

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CHAPTER 4: Cdx1 and Cdx2 have context dependent functional specificity in the intestine

Cdx1 and Cdx2 have context dependent functional specificity in the intestine

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Running title: Cdx-specific promoter regulation

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Stephanie Grainger designed and carried out experiments, wrote the manuscript, including figures.

Alexa Hryniuk assisted with generating experimental material.

David Lohnes helped to design experiments and to write the manuscript.

ABSTRACT

The caudal-related homeodomain transcription factors Cdx1 and Cdx2 are expressed in the developing endoderm with expression persisting into adulthood. *Cdx1*$^{-/-}$ mutants are viable and fertile and display no overt intestinal phenotype, while 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. In contrast, Cdx1 appears to be dispensable for development of the small intestine.

Cdx1 and Cdx2 are differentially expressed along the intestinal tract, 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, which house intestinal stem cells, and diminishing towards the differentiated cells in the villi. Cdx2 is expressed uniformly along this axis, but is differentially phosphorylated. The functional relevance of these different Cdx expression domains remains unknown. Herein, we use a gene substitution approach to begin to address this question, and found that *Cdx2* driven by *Cdx1* regulatory elements cannot functionally compensate for loss of endogenous Cdx2. This contrasts with the ability of Cdx2 to rescue Cdx1-dependent vertebral patterning, and provides novel evidence that Cdx1 and Cdx2 have context-dependent functional specificity in the intestinal tract.

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 (Kwon et al., 2008). The gut is subsequently patterned in an anterior to posterior order, which is later reflected by the distinct functionalities of the esophagus, stomach, small and large intestines and associated accessory organs (Wells and Melton, 1999). 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. Two distinct pools of stem cells are housed in the crypt region, either at the base of the crypt or four cells from the bottom (+4 cells) (Barker et al., 2007; Sangiorgi and Capecchi, 2008). Cells at the base of the crypts divide to produce highly proliferative transit-amplifying (TA) cells which subsequently differentiate into enterocytes, Goblet cells, and enteroendocrine cells, migrate towards the tip of the villus, and are shed 5-7 days later in the mouse (Tian et al., 2011). A fourth TA cell derivative, the Paneth cell, migrates towards the base of the crypt, where they reside with a lifespan of approximately 21 days. The colon lacks villi, which are replaced with a flattened epithelium which harbors mostly colonocytes and Goblet cells (Wells and Melton, 1999).

While the molecular mechanisms governing intestinal patterning along the anterior-posterior (AP) axis are incompletely understood, the Cdx gene products are known to play an important role in this process (Beck et al., 1999; Gao et al., 2009; Grainger et al., 2010; Verzi et al., 2011). 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

(Beck et al., 1995; Chawengsaksophak et al., 1997; Duprey et al., 1988). *Cdx1*$^{-/-}$ mutants are viable and fertile and exhibit vertebral homeotic transformations, but no overt intestinal phenotype (Subramanian et al., 1995). *Cdx2*$^{-/-}$ mutants are peri-implantation lethal (Chawengsaksophak et al., 2004; Chawengsaksophak et al., 1997); however, conditional deletion strategies have revealed key roles for Cdx2 in axial elongation and patterning of the mesoderm (Savory et al., 2009a) and in the definitive endoderm and intestinal epithelium (Gao et al., 2009; Grainger et al., 2010; Verzi et al., 2011).

Although poorly conserved outside of the homeodomain, considerable evidence from analysis of compound mutants suggests that the Cdx proteins functionally overlap in several developmental processes including neural tube closure and vertebral patterning (Gaunt et al., 2008; Savory et al., 2011a; van den Akker et al., 2002; van Nes et al., 2006). This is consistent with gene substitution approaches that have shown that Cdx2 can functionally substitute for Cdx1 in vertebral patterning (Savory et al., 2009b). However, functional overlap between Cdx1 and Cdx2 in the intestine has not been thoroughly investigated.

Cdx1 and Cdx2 are differentially expressed in the intestinal epithelium, with Cdx1 highest in the distal colon and Cdx2 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 (Beck et al., 1995; Guo et al., 2004). Although there does not appear to be a clear role for Cdx1 in the developing small intestine (Grainger et al., 2012; Grainger et al., 2010; Subramanian et al., 1995), Cdx1, together with Cdx2, may play a role in specification of the colon (Gao et al., 2009). Furthermore, in the adult, Cdx1 appears to

functionally overlap with Cdx2 in regulating intestinal homeostasis (Hryniuk et al., 2012; Verzi et al., 2011). In contrast, specific Cdx function in the intestine has been suggested by a number of studies, largely using tissue culture models. For example, the Cdx target *apical sodium-dependent bile acid transporter (ASBT)* is more responsive to Cdx2 than Cdx1 in transfection-based reporter assays (Ma et al., 2012). Furthermore, the calcium channel *MS4AI2* is responsive to Cdx2, but not Cdx1 (Koslowski et al., 2009), while the *intestinal alkaline phosphatase* gene is activated by Cdx1 and inhibited by Cdx2 (Alkhoury et al., 2005). Conversely, a number of intestinal genes respond similarly to Cdx members in tissue culture models, such as *SLC5A8* (Kakizaki et al., 2010). Functional overlap 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. Other intestinal genes, however, such as *Slc7a8* and *Alpi,* appear to exhibit Cdx-type specific regulation *in vivo* (Verzi et al., 2011).

The above observations suggest that Cdx1 and Cdx2 may be functionally distinct in certain contexts. To examine this further, we derived compound mutant mice from animals in which Cdx2 had been inserted in the Cdx1 locus ($Cdx1^{2ki/2ki}$) and disrupted the endogenous Cdx2 allele using the $Cdx2^{f/f}$ *villin* cre-ER^T conditional mutants. Herein, we demonstrate that Cdx2 driven by *Cdx1* regulatory regions cannot functionally compensate for loss of endogenous Cdx2. This is due, at least in part, to the inability of Cdx2 to function in an autoregulatory loop necessary for normal expression from the *Cdx1* locus. This study provides novel evidence that Cdx1 and Cdx2 exhibit context-dependent functional specificity *in vivo*.

RESULTS

Expression of *Cdx2* from the *Cdx1* locus does not support intestinal development

The differential expression of Cdx1 and Cdx2 in the intestine is suggestive of functionally distinct roles. We previously demonstrated that conditional loss of Cdx2 in the intestine at E13.5 leads to an anterior transformation of the intestinal epithelium to a partial glandular stomach identity. Cdx1 has no apparent role in this process (Grainger et al., 2010), nor do *Cdx1*$^{-/-}$ mutants exhibit any discernible intestinal phenotype (Subramanian et al., 1995).

To investigate whether *Cdx2* expressed under *Cdx1* regulatory sequences could support normal intestinal development and homeostasis, we combined the *Cdx2* conditional intestinal knockout line with a knock-in allele in which the *Cdx2* cDNA was inserted into the *Cdx1* locus (hereafter designated *Cdx1*$^{2ki/2ki}$) to create *Cdx1*$^{2ki/2ki}$*Cdx2*$^{f/f}$ *villin* Cre ER^{T} stud males. These were crossed with *Cdx1*$^{2ki/2ki}$ *Cdx2*$^{f/f}$ females and *Cdx2* inactivated at E13.5 or in the adult as previously described (Grainger et al., 2010; Hryniuk et al., 2012). The resultant Cre-positive offspring (referred to as *Cdx1*$^{2ki/2k}$*Cdx2*$^{-/-}$) were devoid of Cdx1 and exhibited Cdx2 in its place, while littermate controls (*Cdx1*$^{2ki/2ki}$) expressed *Cdx2* from both the endogenous and the knock-in alleles.

Upon gross examination, all offspring were of normal size and gastrointestinal accessory organs, including the liver, pancreas, spleen and lungs appeared normal (data not shown). The duodenum of *Cdx1*$^{2ki/2ki}$ *Cdx2*$^{-/-}$ mice was slightly distended, similar to *Cdx2*$^{-/-}$ mutants (Grainger et al., 2010), while *Cdx1*$^{2ki/2ki}$ mice appeared normal (data not shown). Histological analysis revealed no apparent intestinal abnormalities in *Cdx1*$^{2ki/2ki}$

animals (Fig. 40ii). In contrast, *Cdx1*$^{2ki/2ki}$ *Cdx2*$^{-/-}$ mice exhibited a disordered intestinal epithelium, with shortened villi and vacuolated cells (Fig. 40; compare iii to i), reminiscent of the outcome of intestinal Cdx2 loss at E13.5 (Grainger et al., 2010) (Fig. 40iv). As previously described, excision mediated by the *villin* Cre ER^{T} transgene in the large intestine was not sufficient to warrant further study (Grainger et al., 2010) (data not shown).

Cdx2$^{-/-}$ mice exhibit a partial transformation of the intestinal epithelium to a glandular stomach. The nature of the lesion in *Cdx1*$^{2ki/2ki}$*Cdx2*$^{-/-}$ mice was therefore further examined using differential staining. 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 epithelium and not the stomach (Culling et al., 1975). *Cdx2*$^{-/-}$ mice exhibit ectopic PAS staining along the apical edge of the aberrant villi, indicative of a transformation to pyloric stomach (Fig. 40xii), as well as supernumerary intestinal goblet cells due to aberrant Notch signaling (Grainger et al., 2012; Grainger et al., 2010). In contrast, neither the *Cdx1*$^{2ki/2ki}$ nor the *Cdx1*$^{2ki/2ki}$*Cdx2*$^{-/-}$ mice exhibited ectopic PAS or Alcian Blue staining, although *Cdx1*$^{2ki/2ki}$ *Cdx2*$^{-/-}$ mice appeared to have more Goblet cells (Fig. 40vi, vii, x, xi; data not shown). These results suggest that expression of *Cdx2* from the *Cdx1* locus results in a partial rescue of the Cdx2 null small intestinal phenotype.

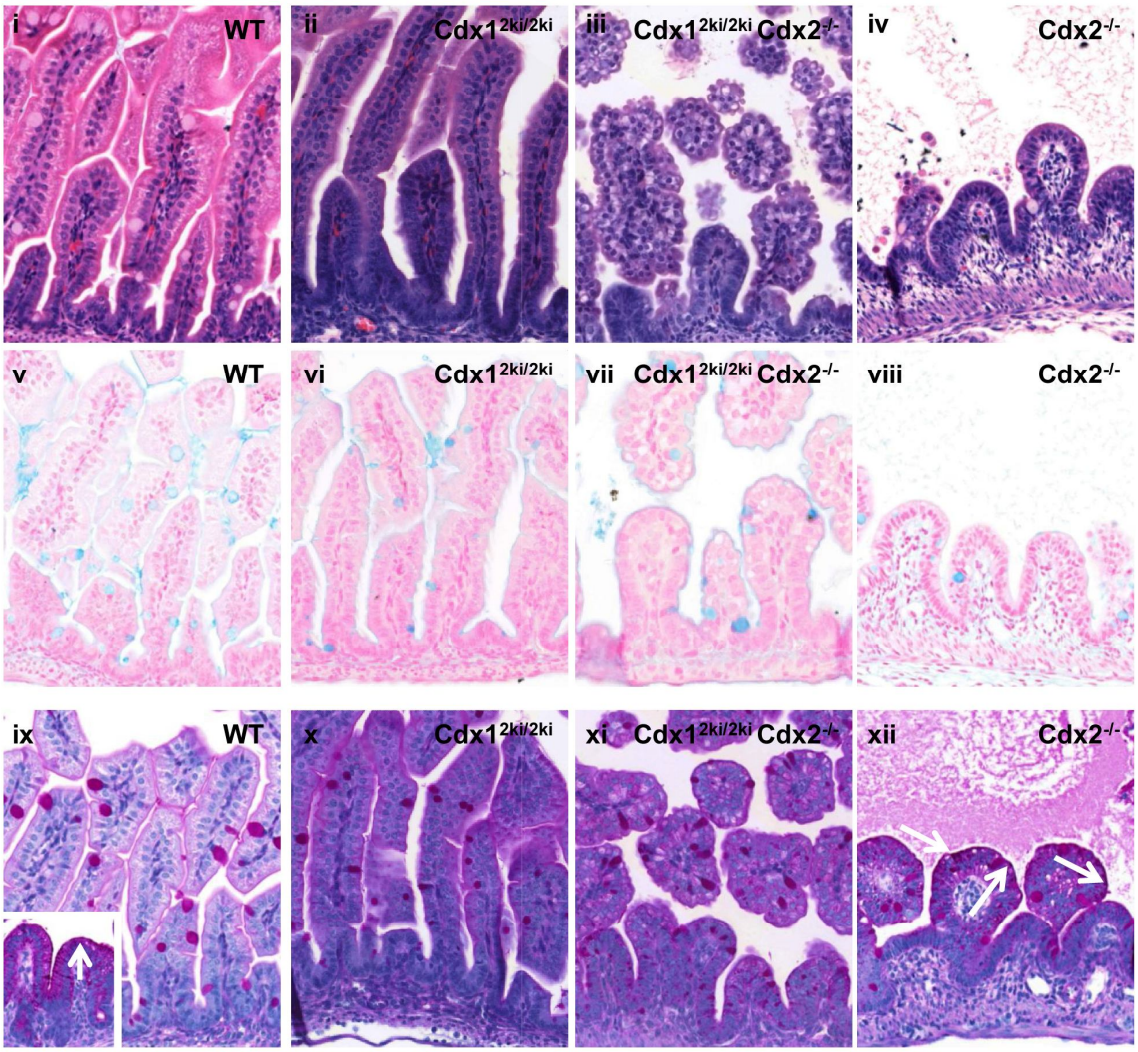

Figure 40: Cdx2 driven by *Cdx1* regulatory regions cannot complement loss of endogenous Cdx2 in the intestine. *Cdx1*$^{2ki/2ki}$-*Cdx2*$^{f/f}$ *villin* cre-ER^{T} mice or non-transgenic controls (designated wild type or WT) were treated with 5mg of tamoxifen in utero at embryonic day (E)13.5 and intestinal tracts harvested at E18.5, fixed, sectioned and stained with hematoxylin and eosin (i-iv), Alcian Blue (v-viii) or Periodic Acid Schiff (PAS) (ix-xii). Shown are small intestinal sections from *WT* (i, v, ix), *Cdx1*$^{2ki/2ki}$ (ii, vi, x), *Cdx1*$^{2ki/2ki}$*Cdx2*$^{-/-}$ (iii, vii, xi) and *Cdx2*$^{-/-}$ (iv, viii, xii) offspring. WT pyloric stomach is shown as an inset in ix. White arrows point to apical PAS staining, typical of pyloric stomach.

Cdx2 expressed from the *Cdx1* locus cannot support intestinal homeostasis in the adult

Cdx members are critical for normal differentiation and homeostasis of the entire adult intestinal tract (Hryniuk et al., 2012; Verzi et al., 2010b; Verzi et al., 2011). For example, chronic loss of Cdx2 in adults results in a transformation of the intestinal epithelium to a stomach character, similar to the effects of its loss during development (Hryniuk et al., 2012). To assess if Cdx2 expressed from *Cdx1* elements was able to rescue this phenotype, we used a suboptimal tamoxifen dosage regime in adult mice to elicit chimeric excision of Cdx2 as previously described (Hryniuk et al., 2012) and assessed the impact of loss of Cdx2 six weeks later. Consistent with the results from the developmental knockout above, $Cdx1^{2ki/2ki}Cdx2^{-/-}$ mice phenocopied chronic loss of *Cdx2* in the small intestine (data not shown).

Complete loss of Cdx2 or both Cdx1 and Cdx2 in the adult intestine results in rapid failure of the intestinal epithelium and death within a week (Hryniuk et al., 2012; Verzi et al., 2010b). $Cdx1^{2ki/2ki}Cdx2^{-/-}$ mice, generated by total ablation of the floxed *Cdx2* allele, exhibited a similar trajectory of wasting and lethality (data not shown). Taken together, these results indicate that Cdx2 driven from the *Cdx1* promoter cannot compensate for loss of endogenous Cdx2 in the small intestine, either in the adult or during development.

The large intestine consists of the cecum, the colon and the rectum, each with its own distinct morphology. $Cdx1^{-/-}$ mice have no reported intestinal phenotype, potentially due to functional compensation by Cdx2 (Subramanian et al., 1995). Loss of Cdx2 in the

small intestine from E13.5 onwards results in an anteriorization of the epithelium to a partial stomach fate. However, this phenotype is not further impacted by subsequent loss of Cdx1, suggesting that Cdx1 is dispensable for development or homeostasis of the small intestine (Grainger et al., 2010). In contrast, in the adult colon loss of Cdx2 results in an anteriorization of the distal colon to a cecal character, and this phenotype is exacerbated by concomitant loss of Cdx1 (Hryniuk et al., 2012). In this regard, it is notable that Cdx1 protein levels are maximal in the distal colon, while Cdx2 peaks in the proximal cecum and diminishes in either direction (Duprey et al., 1988; Guo et al., 2004; James et al., 1994; James and Kazenwadel, 1991; Subramanian et al., 1998). These findings are consistent with a role for Cdx1 in the distal colon that is revealed in the absence of Cdx2.

To test if Cdx2 under the control of *Cdx1* regulatory elements suffices for homeostasis of the large intestine, we examined the proximal and distal colon in $Cdx1^{2ki/2ki}Cdx2^{-/-}$ offspring six days after tamoxifen treatment, when $Cdx1^{-/-}Cdx2^{-/-}$ mice exhibit an anteriorization of the distal colon (Hryniuk et al., 2012). While $Cdx1^{2ki/2ki}$ colons appeared normal, $Cdx1^{2ki/2ki}Cdx2^{-/-}$ colons exhibited morphological alterations similar to those seen in $Cdx1^{-/-}Cdx2^{-/-}$ offspring (Hryniuk et al., 2012) (Fig. 41A).

Defensin5 and *TFF1* are enriched in the cecum compared to the distal colon (Birkenkamp-Demtroder et al., 2005), and this pattern of expression was conserved in both wild-type and $Cdx1^{2ki/2ki}$ mice (Fig. 41B). In contrast, both *Defensin5* and *TFF1* were markedly elevated in the proximal and distal colons of $Cdx1^{2ki/2ki}Cdx2^{-/-}$ mice relative to controls (Fig. 2B), consistent with an anterior transformation similar to that reported for $Cdx1^{-/-}Cdx2^{-/-}$ offspring (Hryniuk et al., 2012). These data suggest that

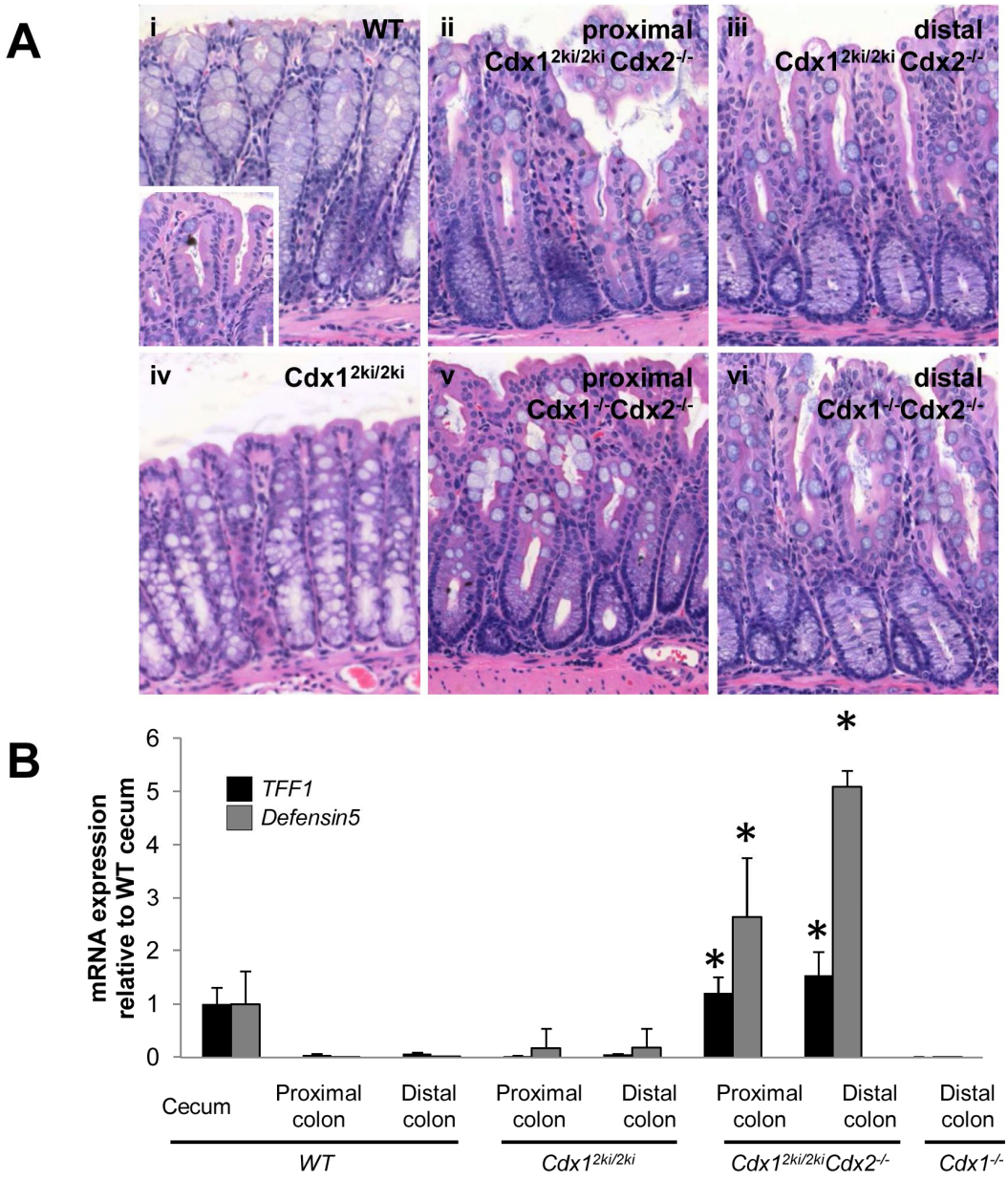

Figure 41: Cdx2 expressed from the *Cdx1* locus cannot complement loss of endogenous Cdx1 and Cdx2 in the adult distal colon. (A) Hematoxylin and eosin staining of adult proximal and distal colons from WT (i), *Cdx1*$^{2ki/2ki}$ (iv), *Cdx1*$^{2ki/2ki}$*Cdx2*$^{-/-}$ (ii, iii) and *Cdx1*$^{-/-}$*Cdx2*$^{-/-}$ (v, vi) mice. Note the semblance of the proximal and distal colon in *Cdx1*$^{2ki/2ki}$*Cdx2*$^{-/-}$ and *Cdx1*$^{-/-}$*Cdx2*$^{-/-}$ offspring to wild-type cecum (i, inset). (B) qPCR analysis for the cecal markers *TFF1* and *Defensin5* from proximal and distal colon of *Cdx1*$^{2ki/2ki}$ and *Cdx1*$^{2ki/2ki}$*Cdx2*$^{-/-}$ mice, relative to wild-type cecum. *P<0.05 by student's t-test compared to wild-type controls, n=3.

expression of Cdx2 under *Cdx1* regulatory domains cannot compensate for Cdx function in the adult distal colon.

Cdx2 cannot support *Cdx1* autoregulation

The phenocopy between *Cdx1*$^{2ki/2ki}$*Cdx2*$^{-/-}$ and *Cdx2*$^{-/-}$ offspring prompted us to examine Cdx protein expression. As expected, neither *Cdx1*$^{2ki/2ki}$*Cdx2*$^{-/-}$ nor *Cdx1*$^{2ki/2ki}$ mice exhibited Cdx1 expression (Fig. 42A i-iii), consistent with prior work (Savory et al., 2009b). *Cdx1*$^{2ki/2ki}$ mice displayed robust Cdx2 expression along the entire crypt-villus axis, comparable to wild type offspring (Fig. 42A; compare v to iv). In marked contrast, *Cdx1*$^{2ki/2ki}$ *Cdx2*$^{-/-}$ offspring had very low levels of Cdx2, with more robust staining at the base of the crypts and tapering off towards to villus tip, similar to the pattern of distribution of Cdx1 (Fig. 42A vi) (Duprey et al., 1988; Subramanian et al., 1998). Western blot analysis also showed markedly decreased expression of Cdx2 in *Cdx1*$^{2ki/2ki}$ *Cdx2*$^{-/-}$ animals compared to littermate controls (Fig. 42C).

The above data suggest that *Cdx2* is not effectively expressed from the *Cdx1* promoter in the small intestinal epithelium. To determine the basis for this observation, we examined transcripts produced from the *Cdx1* promoter by qPCR. In the small intestine of *Cdx1*$^{2ki/2ki}$ mice, there was a very strong reduction in *Cdx1*-derived transcripts compared to wild-type controls (Fig. 42B), suggesting a failure in transcriptional activity at this promoter. It is also worthy to note that further deletion of Cdx2 in *Cdx1*$^{2ki/2ki}$*Cdx2*$^{-/-}$ mice did not affect expression from the *Cdx1* promoter (Fig. 42B). This is consistent with the finding that loss of Cdx2 does not affect Cdx1 protein levels (Fig. 42D), suggesting

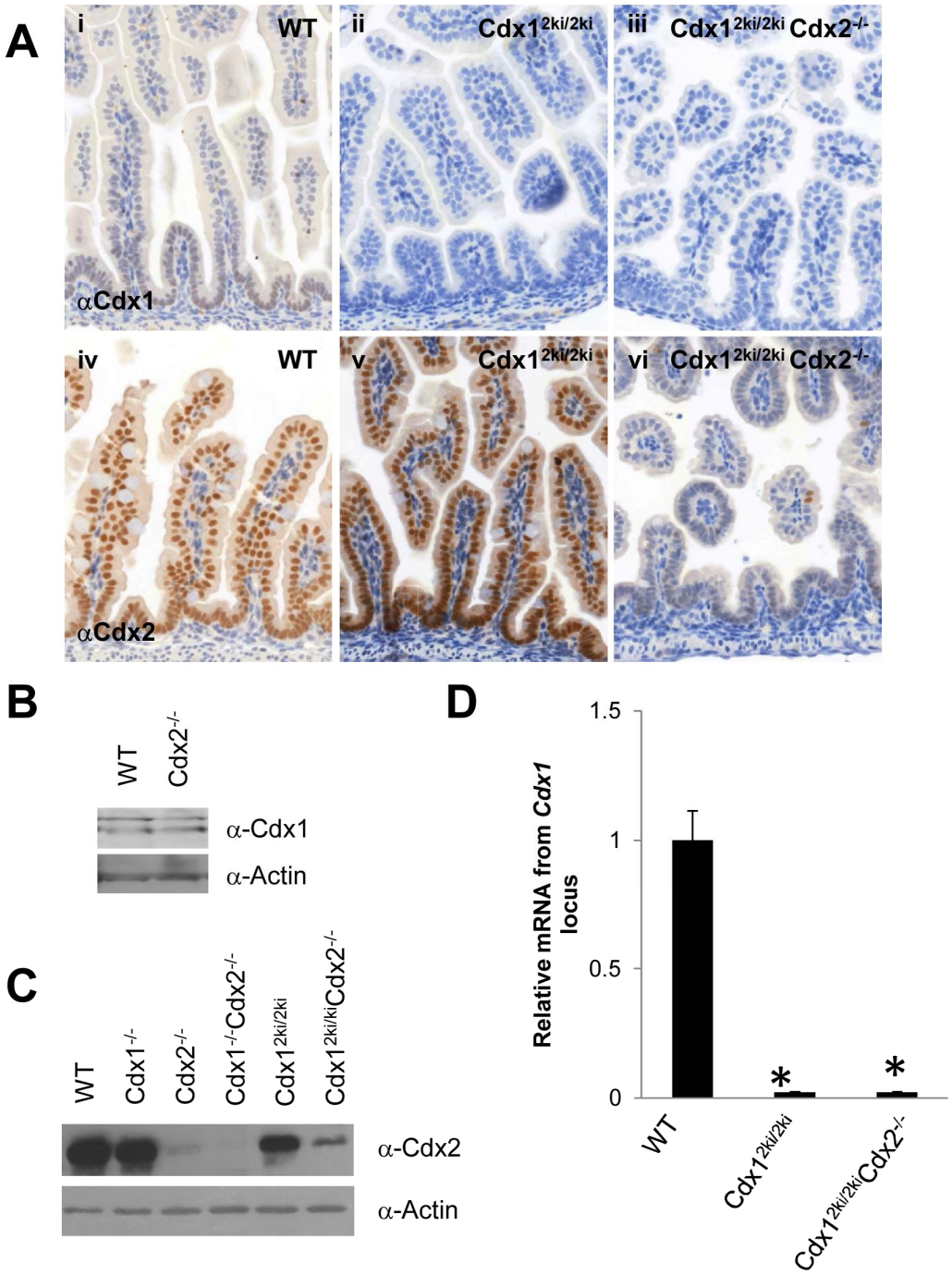

Figure 42: Cdx2 does not support transcription from the *Cdx1* locus. (A) Immunohistochemistry for Cdx1 (i-iii) or Cdx2 (iv-vi) in E18.5 small intestinal sections of *WT* (i, iv), *Cdx1*$^{2ki/2ki}$ (ii, v), *Cdx1*$^{2ki/2ki}$*Cdx2*$^{-/-}$ (iii, vi). (B) Western blot analysis for Cdx2 (C) or Cdx1 (B) and actin loading controls from small intestine. (D) qPCR analysis for transcripts from the *Cdx1* locus relative to controls. *P<0.05 by student's t-test, n=3.

that Cdx1, but not Cdx2, is essential for expression of *Cdx1* in the intestine. There appeared to be a similar relationship in the adult colon, as assessed by both immunohistochemistry (Fig. 43A) and western blot analysis (Fig. 43B), although there appeared to be a modest increase in Cdx2 levels in $Cdx1^{2ki/2ki}$ mice compared to wild-type, suggesting potential compensatory mechanisms in the adult colon.

Cdx1 is regulated by an auto-regulatory loop comprised of Cdx1 and LEF1 functioning through a LEF/TCF response element in the proximal Cdx1 promoter (Beland et al., 2004). The reduction of Cdx2 in $Cdx1^{2ki/2ki}Cdx2^{-/-}$ offspring suggested that Cdx2 could not support this autoregulatory pathway. To assess this, we used transfection assays to monitor the ability of Cdx members to elicit expression from the *Cdx1* promoter in combination with LEF/β catenin, and found that compared to Cdx1, Cdx2 was compromised in its ability to transactivate from either the proximal *Cdx1* promoter or a larger 3kb fragment from the *Cdx1* locus (Fig. 44A and data not shown). This outcome was not due to differences in Cdx protein levels (Fig. 44B, C). Moreover, both Cdx1 and Cdx2, in combination with LEF/β-catenin, induced expression comparably from a reporter derived from *Dll1*, a Wnt/Cdx target, as well as a synthetic Cdx response element (Dearolf et al., 1989; Grainger et al., 2012) (Fig. 44A). These findings suggest that Cdx1 and Cdx2 differ in their transcriptional competency in a context-specific manner.

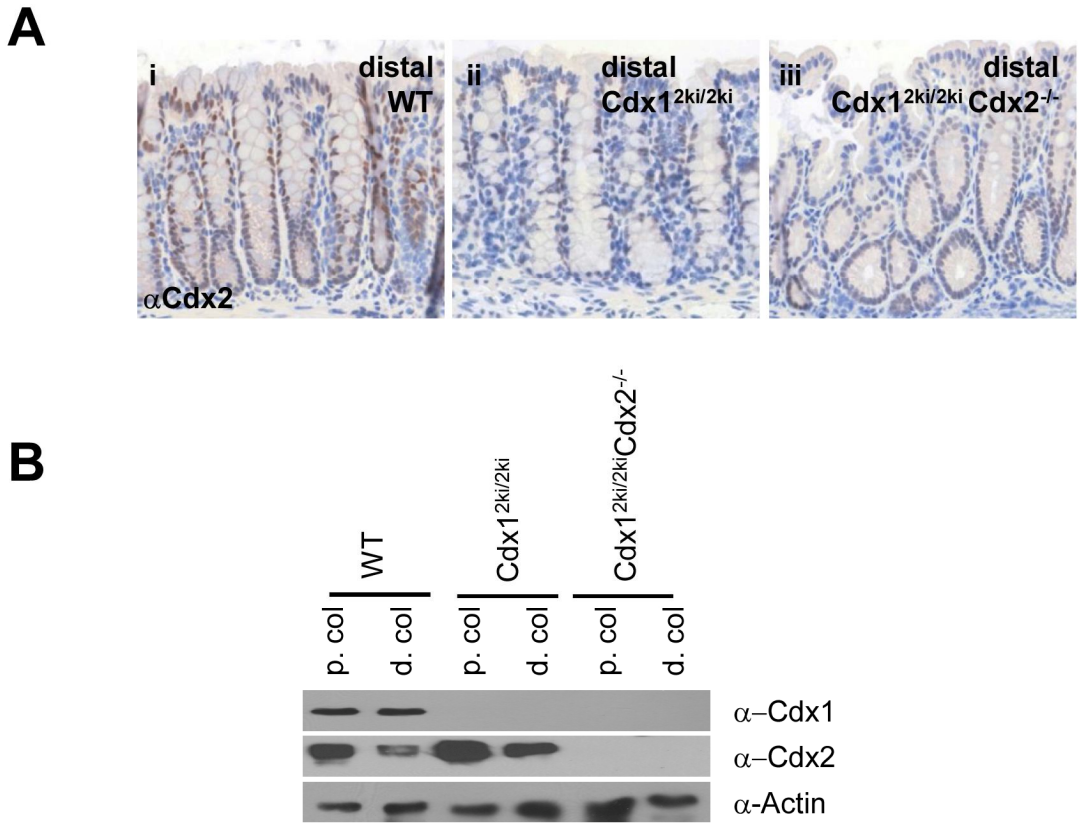

Figure 43: Cdx2 is poorly expressed from the *Cdx1* promoter in the adult distal colon. (A) Cdx2 immunohistochemistry for distal colon from WT (i), *Cdx1*$^{2ki/2ki}$ (ii) and *Cdx1*$^{2ki/2ki}$*Cdx2*$^{-/-}$ (iii) mice. (B) Western blot analysis of Cdx2 in proximal and distal colon from WT, *Cdx1*$^{2ki/2ki}$ and *Cdx1*$^{2ki/2ki}$*Cdx2*$^{-/-}$ mice.

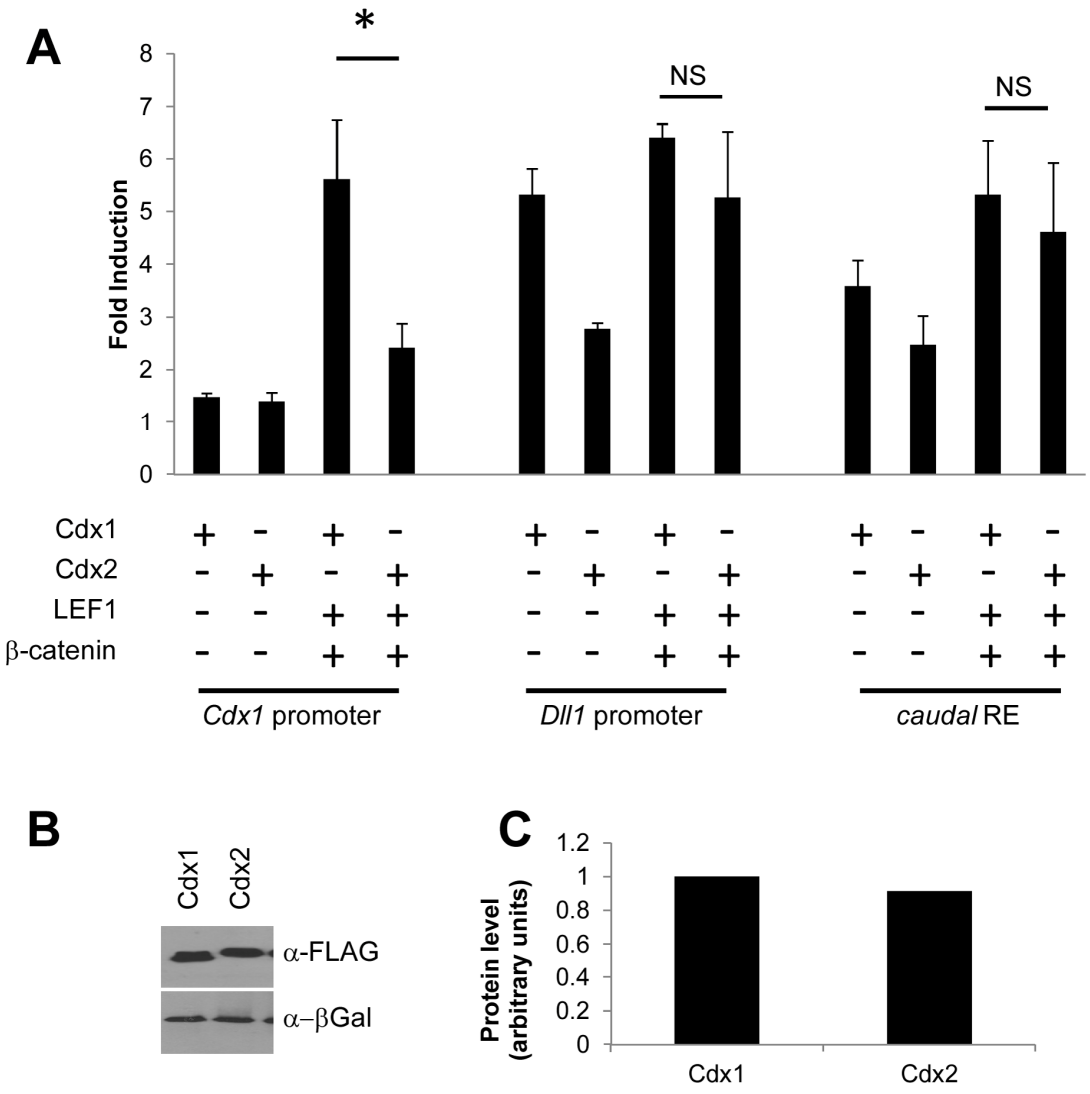

Figure 44: The *Cdx1* promoter is differently regulated by Cdx1 and Cdx2. (A) Luciferase reporter assay in P19 embryonal carcinoma cells with reporters derived from *Cdx1* or *Dll1* promoters or a synthetic Cdx response element (caudal RE). Fold induction is shown relative to reporter vector in response 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 compared to Cdx1 with LEF1 and β-catenin in each experiment, n=6.

Cdx1 N-terminal sequences confer specific transcriptional activity

The *Cdx1* autoregulatory loop is thought to be governed by Cdx1 interaction with LEF/TCF members, with only the latter directly associated with DNA regulatory motifs (Beland et al., 2004; Lickert et al., 2000). To determine if Cdx1 and Cdx2 differentially interacted with LEF/TCF members, we compared association between Cdx1 or Cdx2 and LEF1 or TCF7l2, the dominant LEF/TCF in the intestine (Faro et al., 2009; Gregorieff et al., 2004; Muncan et al., 2007). We found that both Cdx1 and Cdx2 interacted comparably with either LEF1 or TCF7l2 using proteins generated *in vitro* or from COS7 cell lysates (Fig. 45, data not shown). These results suggested that differential association between Cdx and LEF/TCF proteins does not underlie the different transactivation competency between Cdx1 and Cdx2 on the *Cdx1* promoter.

To assess further the basis for the differential regulation between Cdx1 and Cdx2, chimeric proteins were generated in which the N-terminal sequences of either Cdx1 or Cdx2 was fused to the homeodomain containing C-terminal region of the other family member. Assessment of these chimeric proteins for transactivation from the Cdx1 promoter revealed that Cdx1, but not Cdx2, N-terminal sequences induced transcription (Fig. 46A). 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. 47A). These differences were not due to variance in protein levels (Fig. 46B, C, 47B, C), but rather suggest that the basis for the functional differences between Cdx1 and Cdx2 on the *Cdx1* promoter lies in their N-terminal transactivation domains.

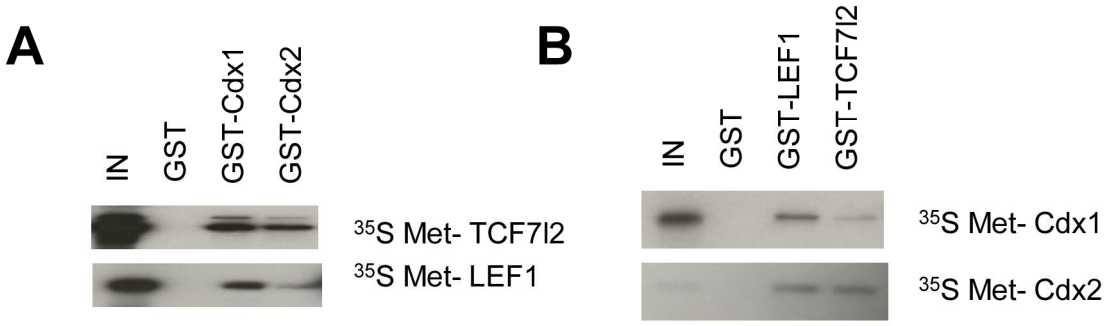

Figure 45: Cdx1 and Cdx2 can both bind TCF7l2 and LEF1 directly *in vitro*. Cdx1 and Cdx2 (A) or TCF7l2 and LEF1 (B) were transcribed and translated in vitro in the presence of ^{35}S-methionine and pulled down with GST-Cdx1 and GST-Cdx2 (B) or GST-TCF7l2 and GST-LEF1 (A). Inputs represent 5% in all cases. Note that Cdx1 and Cdx2 are both able to bind to TCF7l2 and LEF1 directly.

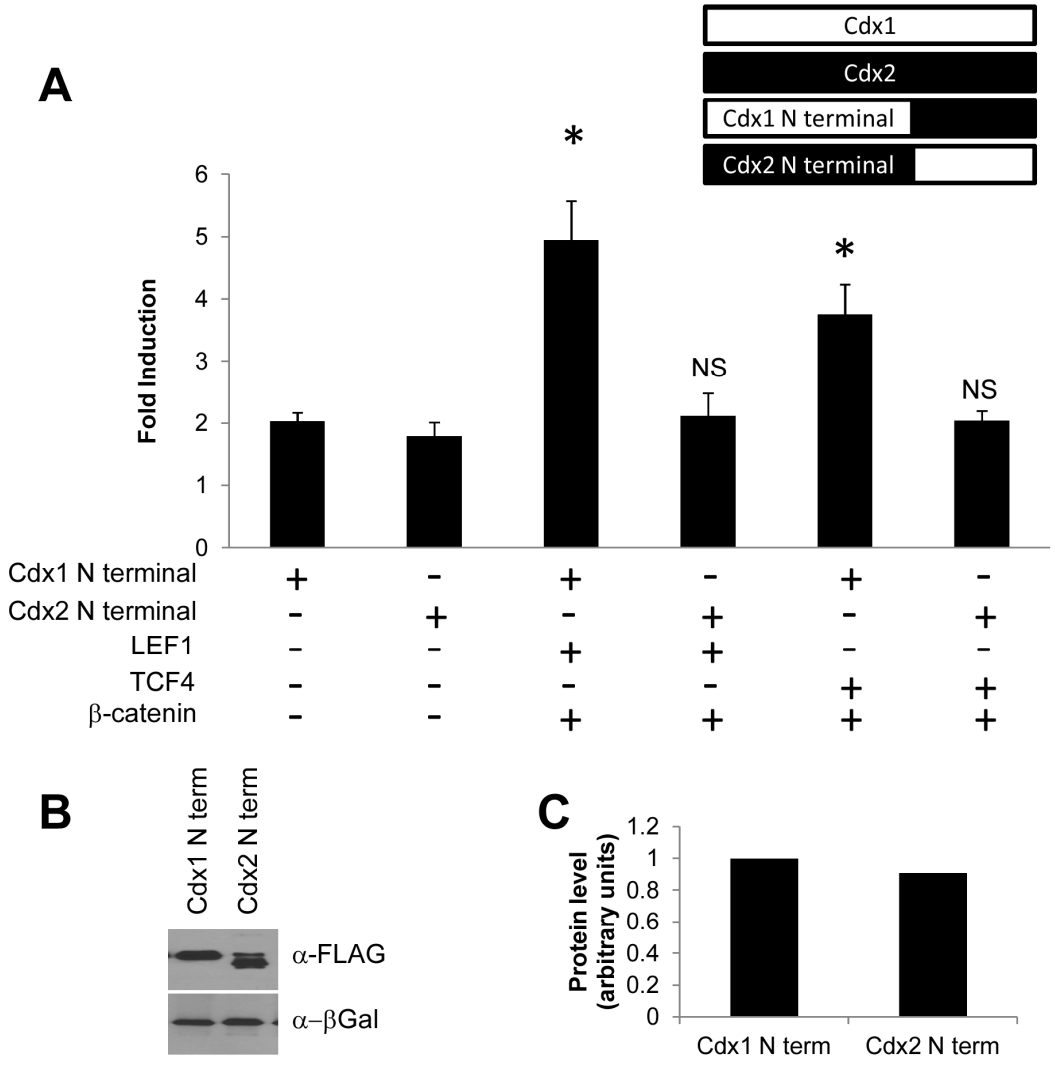

Figure 46: Differential regulation by N-terminal Cdx transcriptional activation sequences. (A) Regulation of a *Cdx1* promoter reporter vector in P19 embryonal carcinoma cells. Fold induction is shown relative to reporter vector alone in response to fusion proteins harboring the Cdx1 N terminal and Cdx2 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. Cdx1 N terminal groups were statistically different by one-way ANOVA ($F(3.7, 69.6)$, $P=2.58 \times 10^{-8}$) *$P<0.05$ compared to Cdx1 N terminal by Tukey's post-test, n=6. Cdx2 N terminal groups were not statistically different by one-way ANOVA ($F(3.68, 3.17)$, $P=0.07$)

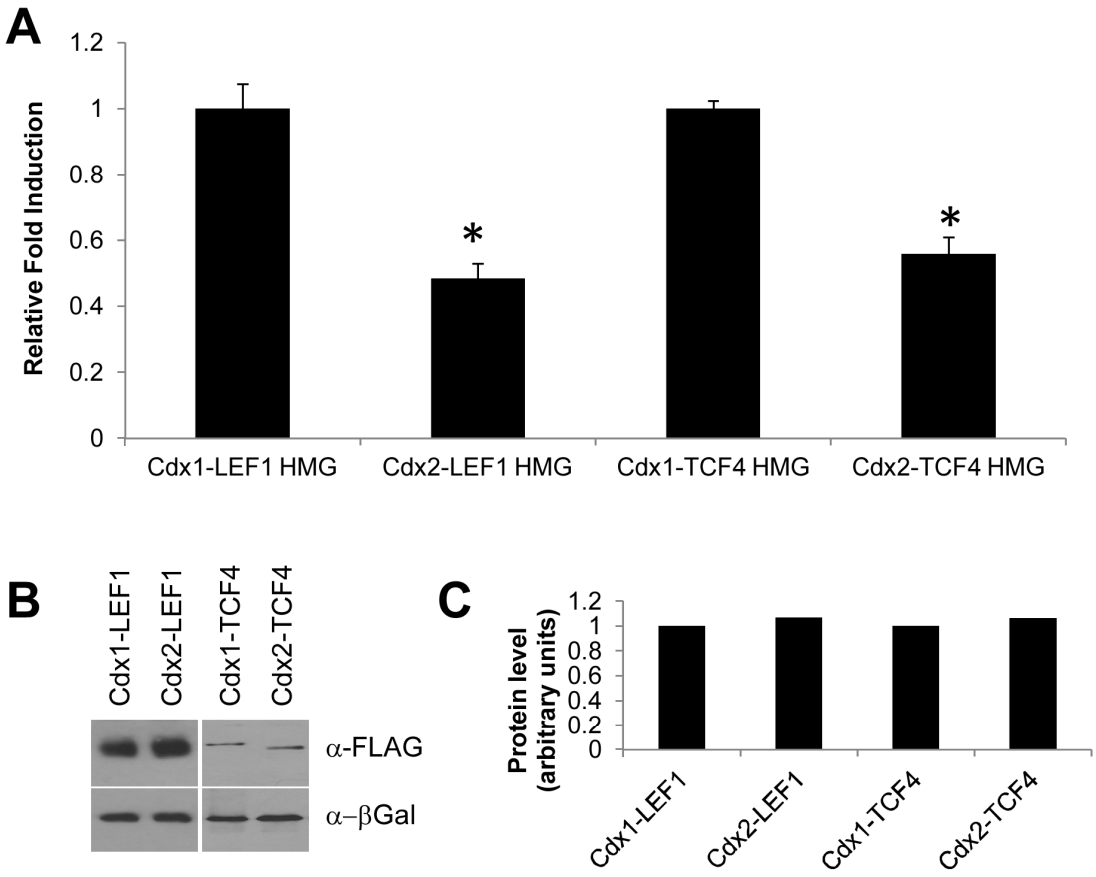

Figure 47: Cdx1 and Cdx2 differ in functionality of their N-terminal activation domains. (A) Cdx1 promoter regulation in P19 embryonal carcinoma cells. Fold induction is shown relative to Cdx1 fusion protein reporter activity, in response to chimeric proteins harboring the Cdx1 N terminus fused to LEF1 or TCF4 DNA binding domains (Cdx1-LEF1 HMG, Cdx1-TCF4 HMG, respectively) or analogous constructs using the Cdx2 N terminus (Cdx2-LEF1 HMG, Cdx2-TCF4 HMG). Western blot (B) and quantification (C) of Cdx1 and Cdx2 fusion proteins using β-galactosidase as a loading control. *P<0.05 by student's t-test compared to Cdx1 fusion proteins in each experiment, n=6.

DISCUSSION

Recent studies have revealed critical functions for Cdx2 in patterning the endoderm (Gao et al., 2009; Grainger et al., 2010; Hryniuk et al., 2012; Verzi et al., 2011), intestinal differentiation (Gao et al., 2009; Grainger et al., 2010; Verzi et al., 2010b) and axial elongation (Savory et al., 2009a; Savory et al., 2011a; van den Akker et al., 2002). Cdx2 is able to functionally replace Cdx1 in the context of vertebral patterning (Savory et al., 2009b), but the functional relatedness of Cdx members is less clear as regards the intestinal tract. To this end, we sought to determine if Cdx2 expressed in the *Cdx1* spatio-temporal domain could complement Cdx2 loss-of-function in the intestine. We found that Cdx2 was not expressed from the Cdx1 locus at levels that suffice to fully support Cdx-dependent roles in the gastrointestinal tract. The basis for this appears to reside in the inability of Cdx2 to direct auto-regulation of the *Cdx1* promoter; this represents the first *in vivo* demonstration of functional specificity between these family members.

Cdx1 promoter regulation

Cdx1$^{2ki/2ki}$*Cdx2*$^{-/-}$ mice have an intestinal phenotype consistent with insufficient levels of Cdx2. This effect is seen both in fetal and in adult small intestine, as well as the colon, suggesting that this relationship is maintained throughout the lifespan of the mice and throughout the intestinal tract. The loss of expression from the *Cdx1* promoter is evidenced by qPCR, immunohistochemistry and western blot analyses of *Cdx1*$^{2ki/2ki}$ offspring, suggesting that Cdx2 is not able to drive expression from this promoter. These

findings are consistent with reporter assays demonstrating that Cdx2 is less efficient at transactivation from the *Cdx1* promoter relative to Cdx1. The intestinal phenotype of *Cdx1*$^{2ki/2ki}$*Cdx2*$^{-/-}$ mice is, however, hypomorphic relative to Cdx1-Cdx2 conditional null mutants, likely due to low, but functionally significant, levels of Cdx2.

Basis for differential Cdx function

Cdx1 and Cdx2 are well conserved in their C terminal regions, including their homeodomains, but have divergent N terminal sequences which harbor transactivation domains (Guo et al., 2004; Rings et al., 2001; Taylor et al., 1997). Consistent with previous work (Beland et al., 2004; Lickert et al., 2000), we found that the N terminal of Cdx1 could direct transcription from its own promoter in concert with LEF/TCF, but that this effect was not recapitulated by Cdx2. This suggests that the N terminus of Cdx1 differentially interacts with a transcriptional co-regulator(s). Although auto-regulation of the *Cdx1* promoter proceeds through association with a LEF/TCF member bound to a LEF response element in the proximal promoter region (Beland et al., 2004), differential association of LEF/TCF and Cdx proteins did not appear to underline the differential *Cdx1* promoter activity. In this regard, recent work has suggested that Cdx2 is capable of association with a number of intestinal transcription factors and that this association varies in a manner that reflects the differentiation state of the cell (Verzi et al., 2010a). It is therefore tempting to speculate that one or more of these co-regulators may be implicated in Cdx1-specific transcriptional regulation. It will prove interesting to see

what co-regulator(s) is/are differentially recruited between Cdx1 and Cdx2 in this context.

The paradigm of Cdx specificity may extend to other target genes. For example, we have found that the Wnt target gene *Dll1* is a presumed direct Cdx target gene (Grainger et al., 2012). However, while Cdx1 and Cdx2 seem to co-regulate *Dll1* in the presomitic mesoderm, Cdx1 does not appear to impact on *Dll1* expression in the intestine (Grainger et al., 2012). In this regard, in the present study, we found that Cdx1 and Cdx2 exhibit different levels of transcriptional potency on the *Dll1* promoter in P19 embryocarcinoma cells, but similar levels of induction in conjunction with Wnt signaling (Fig. 32A). This is again consistent with modulation of Cdx activity by binding partners such as TCF/LEF. Finally, as Cdx members are critical regulators of intestinal differentiation and axial elongation (Gao et al., 2009; Savory et al., 2009a), it follows that they likely act via different combinatorial means in these two lineages.

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 in regards to vertebral patterning, axial elongation and neural tube closure (Chawengsaksophak et al., 2004; Savory et al., 2009a; Savory et al., 2011a; Subramanian et al., 1995; van Nes et al., 2006). This is consistent with gene substitution approaches which have shown that Cdx2 can fully compensate for Cdx1 loss in vertebral patterning (Savory et al., 2009b). This latter observation also used the $Cdx1^{2ki/2ki}$ line,

which exhibits eventual failure of *Cdx1* autoregulation in the paraxial mesoderm lineage (our unpublished observations). This suggests either that a minimal level of Cdx protein is needed to support Cdx-dependent mesodermal patterning, or that *Cdx1* autoregulation fails after this requirement has been met.

Several groups have investigated functional specificity of Cdx members in various contexts. In tissue culture models, there is contrasting evidence regarding specificity of Cdx1 and Cdx2 on the regulation of intestinal genes (Alkhoury et al., 2005; Ericsson et al., 2006; Gautier-Stein et al., 2003; Kakizaki et al., 2010; Koslowski et al., 2009; Ma et al., 2012). In the embryo, Cdx1 does not appear to play any role in patterning (Grainger et al., 2010) or differentiation (Grainger et al., 2012) of the small intestine in the absence of Cdx2. In contrast, functional overlap between Cdx1 and Cdx2 has been suggested in the adult, where deletion of Cdx1 results in an exacerbation of intestinal failure associated with Cdx2 loss (Hryniuk et al., 2012; Verzi et al., 2010b). However, gene profiling of $Cdx2^{-/-}$ and $Cdx1^{-/-}Cdx2^{-/-}$ mutants suggests that not all intestinal genes are comparably affected (Verzi et al., 2011), indicative of context specific Cdx target gene regulation. The functional outcomes of Cdx-specific function is also evidenced in the context of intestinal cancers, where Cdx1 has been reported to act as an oncogene and Cdx2 as a tumour suppressor (Beck et al., 2003; Beck et al., 1999; Bonhomme et al., 2003; Chawengsaksophak et al., 1997; Domon-Dell et al., 2003). Further characterization of different Cdx1 and Cdx2 target genes and binding partners will be important in elucidating molecular mechanisms governing development and cancer.

EXPERIMENTAL PROCEDURES

Mice

Cdx1$^{-/-}$, *Cdx2*$^{f/f}$, *Cdx1*$^{2ki/2ki}$ and *villin*-Cre ER^{T} mice have been previously described (el Marjou et al., 2004; Santagati et al., 2005; Savory et al., 2009a; Savory et al., 2009b; Subramanian et al., 1995). *Villin*-Cre ER^{T}-mediated deletion of Cdx2 was initiated by Tamoxifen (Tam) administration at embryonic (E)13.5 or at 6 weeks of age as previously described (Grainger et al.; Hryniuk et al., 2012). Gastrointestinal tracts were harvested at E18.5 or 24 hours to 6 weeks (for chimeric excision) post-treatment in adults. Tam-treated non-transgenic littermates were used as controls in all instances.

Histology and western blot analysis

E18.5 intestinal tracts were processed for histological and immunohistochemical staining as previously described (Grainger et al., 2010). Slides were mounted using Permount (Fisher) and images captured using a Zeiss Mirax Midi Scanner (Zeiss). Protein was harvested using 500µL of solubilizer buffer (8M urea, 4% CHAPS, 2mM DTT, protease inhibitor cocktail (Chemicon)). Lysates were sonicated for 30s at 30% output using a Branson sonifier 450, lysates cleared by centrifugation at 14,000g for 20 minutes at 4^{o}C and proteins quantified using the Bradford method (Bradford, 1976). Western blots were performed as previously described (Grainger et al., 2010).

Plasmid constructs

The glutathione S-transferase (GST)-Cdx1 and GST-Cdx2 fusion proteins have been described previously (Beland et al., 2004). GST-LEF1 and GST-TCF7l2 contstructs were derived by subcloning appropriate open reading frames into pGEX4T-1. A FLAG-tagged TCF7l2 expression vector was generated using plasmid number 11031 (p043 mTCF-4B) from Addgene (Lee et al., 1999). The LEF1-HA, Cdx1 and Cdx2 expression vectors and *Cdx1*-luciferase reporter vectors have been previously described (Beland et al., 2004).

GST fusion protein purification

BL-21 bacteria were transformed with either empty GST expression plasmid, GST-Cdx1, GST-Cdx2, GST-LEF1 or GST-TCF7l2 fusion constructs. Cultures were grown to an OD_{600} 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 10,000Xg. Binding and subsequent washing of glutathione-agarose beads (BD Biosciences) was carried out as per the manufacturer's recommendations. Beads were analyzed for effective binding and equal loading by Coomassie staining prior to use.

Tissue culture and transfection

COS7, SW480 and P19 cells were grown under the recommended standard conditions (ATCC). Transfections were carried out using the calcium phosphate precipitation method. Briefly, for generation of proteins, 10cm plates of COS7 cells were transfected with 10μg of each expression vector construct, for a total of 20ug of DNA per plate. Empty expression vectors were used as negative controls. Cells were harvested 36 h post-transfection and lysed on ice in 300 μL lysis buffer (20 mM Tris [pH 8.0], 25 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 1% Triton X-100, 10% glycerol, 1mM DTT, and protease inhibitors) for 30 min, followed by sonication.

For promoter analysis, P19 or SW480 cells in 6 well plates were transfected with one μg reporter vector, varying amounts of expression vectors and 200ng of β-gal expression vector, to a total of 3μg of DNA per well. Cells were harvested 48 hours post-transfection and the lysates processed and analyzed using the Promega Luciferase Assay System according to the manufacturer's instructions. β-galactosidase activity was assayed using the chlorophenolred-ß-D-galactopyranoside (CPRG) assay system (Calbiochem) and used to correct for transfection efficiency.

Protein-protein interaction assays

In vitro protein-protein interaction assays were conducted as previously described (Beland et al., 2004) using the Quick-coupled transcription and translation kit (Promega) according to manufacturer's recommendations. For each assay, 5μg of GST fusion protein and 5μL of translation reaction were used.

To monitor interaction in a cellular environment, LEF1-HA or TCF7l2-FLAG expression vectors were co-transfected with Cdx expression vectors in COS7 cells as described above, and lysates precipitated using anti-Cdx1 or Cdx2 antibodies (Savory et al., 2009b). Precipitates were assayed using an anti-FLAG antibody (Sigma) by western blot as previously described (Beland et al., 2004). Inputs shown represent 5% of the total protein used for immunoprecipitation in all cases.

Quantitative reverse-transcriptase polymerase chain reaction (qPCR)

RNA was extracted from E18.5 small intestine using Trizol reagent (Invitrogen) and used to generate cDNA by standard procedures. cDNA was subsequently amplified by qPCR using oligonucleotides specific for the *Cdx2* replacement allele, wild-type *Cdx1* or *β-actin.* qPCR was performed using the MX3005P cycler (Agilent Technologies) with SsoFast EvaGreen Supermix (qPCR, BioRad), according to the manufacturer's recommendations. Results were analyzed using the $2^{-\Delta\Delta Ct}$ method (Schefe et al., 2006), normalized for *β-actin,* with the dissociation curve considered for the specificity of each amplicon. Results reflect the mean of 3 independent biological samples. Primer sequences are available upon request.

ACKNOWLEDGEMENTS

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CHAPTER 5: GENERAL DISCUSSION

Questions of Cdx function

There are several outstanding questions in Cdx biology which were addressed in this study. To begin, both *Cdx1* and *Cdx2* are expressed in the developing endoderm and persist throughout adulthood in the intestinal epithelium (Beck et al., 1995; Silberg et al., 2000; Subramanian et al., 1998), although their functions in this lineage remained elusive. Although *Cdx1*$^{-/-}$ mice have no discernible intestinal phenotype, this could be due to functional compensation by Cdx2 (Subramanian et al., 1995). Although the peri-implantation lethality of *Cdx2* null mutants (Chawengsaksophak et al., 1997) has made it difficult to assess this using germ-line knockout models, studies of heterozygous offspring suggest that Cdx2 may regulate development and homeostasis of the intestinal tract (Beck, 2004; Beck et al., 2003; Beck et al., 1999; Beck and Stringer, 2010; Beck et al., 2000; Beck and Pmid, 2004; Bonhomme et al., 2003; Chawengsaksophak et al., 1997). However, given the lack of appropriate mutant models, the extent and nature of these involvements are poorly understood. Secondly, Cdx members are expressed in specific spatio-temporal domains during development in all germ layers (Beck et al., 1995; Gamer and Wright, 1993; Gaunt et al., 2005; Hierholzer and Kemler, 2009; Meyer and Gruss, 1993), yet whether they operate on common targets in these distinct lineages was unknown. Finally, Cdx1 and Cdx2 expression considerably overlaps in the intestinal epithelium, along both the anterior-posterior and crypt-villus axes (Beck, 2004; Beck et al., 1995; Beck and Stringer, 2010; Silberg et al., 1997; Silberg et al., 2000), and Cdx2 can fully complement Cdx1 loss of function during vertebral patterning (Savory et al., 2009b); despite this, it remained to be seen if Cdx members are functionally equivalent in the intestine. The main findings of these three studies are briefly summarized below.

Cdx2 regulates patterning and differentiation of the intestinal epithelium

Using a ligand-activated Cre under the control of the *villin* promoter, I was able to effect deletion of Cdx2 in the intestinal epithelium at E13.5. In the small intestine, but not the large intestine, Cdx2 protein was essentially abolished, with no evidence for a truncated protein that may have theoretically arisen from our approach. Differential staining and expression of molecular markers demonstrated that loss of Cdx2 at this stage resulted in a partial anterior transformation of the small intestinal epithelium to glandular stomach. Interestingly, Cdx1 does not appear to play a role in this process, since *Cdx1*$^{-/-}$ *Cdx2*$^{-/-}$ double mutants were identical to *Cdx2*$^{-/-}$ mutants. These results are in agreement with a concurrent study where Cdx2 was deleted in the definitive endoderm commencing at E9.0; however, this latter knockout resulted in a more pronounced transformation of the small intestine to an esophageal character. Taken together, these results indicate that Cdx2 regulates patterning of the intestinal epithelium, and that the plasticity inherent to the small intestine is progressively altered in a time-dependant manner.

Cdx regulates *Dll1* in multiple lineages

Patterning of both the endoderm and mesoderm are regulated in part by Cdx (Gao et al., 2009; Grainger et al., 2010; Savory et al., 2009a). Although our understanding of Cdx function has improved, the molecular mechanisms governing patterning in the gut are poorly understood. In addition to the role for Cdx2 in patterning the intestinal epithelium, I was also able to show that Cdx2 regulates cell fate decisions, as *Cdx2*$^{-/-}$ mutants were found to have more Goblet cells than littermate controls; again Cdx1 does not appear to play a role in this process. We were able to demonstrate that Cdx operates

through the same Notch pathway ligand, *Dll1*, in endoderm and mesoderm. This was the first demonstration that Cdx can act through the same molecular target *in vivo* in two different contexts, as well as the first demonstration of regulation of Notch signaling by Cdx.

Cdx1 and Cdx2 have context-dependent functional specificity in the intestine

The different expression domains of Cdx1 and Cdx2 in the intestinal epithelium, as well as their poor sequence conservation at the protein level, suggest functional specificity; however, studies in both mouse and tissue culture models have provided contradictory evidence in this regard (Alkhoury et al., 2005; Calon et al., 2007; Gautier-Stein et al., 2003; Hryniuk et al., 2012; Savory et al., 2011a; Savory et al., 2011b; Savory et al., 2009b; Silberg et al., 2000; van den Akker et al., 2002; Verzi et al., 2010b; Verzi et al., 2011). We chose to address the question of Cdx specificity in the intestine by crossing a previously generated *Cdx1*$^{2ki/2ki}$ line with our *Cdx2*$^{f/f}$ *villin* cre-ER^{T} line. These offspring had Cdx2 driven by *Cdx1* regulatory regions with Cre-mediated deletion of endogenous Cdx2 in the intestinal epithelium elicited by tamoxifen. This revealed that Cdx2 driven in *Cdx1* expression domains is not able to complement loss of endogenous Cdx2, likely due to the inability of Cdx2 to function in the Cdx1 autoregulatory loop. This provides novel *in vivo* evidence that Cdx1 and Cdx2 are functionally specific in at least some contexts.

Cdx2 is a critical regulator of intestinal identity

During development, concerted activation of gene expression is required to initiate programs simultaneously across tissues. As such, tight regulation of transcriptional programs is achieved in different ways. For example, chromatin opening can be initiated by "pioneer factors" such as *Foxa1* (also called HNF3α), which encodes a forkhead transcription factor expressed at the earliest stages of endoderm development (Lai et al., 1991). Prior to differentiation, HNF-3 binding sites are occupied in the endoderm, despite transcription being inactive. As hepatic differentiation proceeds, HNF-3 recruits machinery necessary for the chromatin opening and subsequent recruitment of other transcription factors to initiate lineage-specific target gene transcription (Cirillo et al., 1998; Gualdi et al., 1996).

Another way to achieve synchronous gene activation is through "master regulators"; for example, MyoD is thought to be the master regulator of skeletal myogenesis by virtue of its ability to induce a muscle phenotype in diverse non-muscle cell types (Choi et al., 1990; Weintraub et al., 1989); however, it is not necessary for skeletal myogenesis, owing to functional overlap with Myf-5 (Rudnicki et al., 1992; Rudnicki et al., 1993). Furthermore, *in vivo*, the sufficiency of MyoD to induce myogenesis is less clear (Faerman et al., 1993; Hopwood and Gurdon, 1990), perhaps related to the complexity of *in vivo* muscle development.

Similar to MyoD, overexpression of Cdx2 is sufficient to cause intestinal differentiation *in vitro* in several intestinal cell lines (Lorentz et al., 1997; Suh and Traber, 1996). Furthermore, transgenic expression of either Cdx1 or Cdx2 in the stomach

epithelium is able to induce transdifferentiation to intestinal epithelium *in vivo* (Mutoh et al., 2002; Mutoh et al., 2004). However, overexpression of Cdx2 in ES cells causes differentiation to the trophectoderm lineage, and not endoderm (Ralston et al., 2010). Taken together, these results suggest that Cdx1 or Cdx2 is sufficient to induce intestinal differentiation *in vivo* in endodermal-related lineages. However, during normal development, Cdx members are also expressed in non-endoderm lineages, including trophectoderm, mesoderm and ectoderm, which do not give rise to intestine (Beck et al., 1995; Gamer and Wright, 1993; Hierholzer and Kemler, 2009; Meyer and Gruss, 1993). These results suggest that Cdx-mediated induction of intestinal identity is dependent on other factors and that Cdx is a critical, but not master regulator of intestinal identity *per se*.

Several studies have now examined the effects of loss of Cdx in the developing and adult intestinal tract (Beck et al., 2003; Gao and Kaestner, 2010; Gao et al., 2009; Hryniuk et al., 2012; Stringer et al., 2012; Subramanian et al., 1995; Verzi et al., 2010b; Verzi et al., 2011). While *Cdx1*$^{-/-}$ mutants have no intestinal phenotype, it is clear that Cdx2 is required for initiation and maintenance of intestinal identity, as conditional mutants lose characteristics of the intestinal epithelium to varying degrees, and acquire characteristics of more anterior fates such as the stomach and esophagus (Gao et al., 2009; Grainger et al., 2010). These results suggest that the default state for definitive endoderm is anterior (esophagus) in nature, and that Cdx2 imposes more posterior character. Taken all together, these studies indicate a prominent role for Cdx2 as a critical regulator of intestinal identity.

Although Cdx1 and Cdx2 have shown functional overlap in other lineages (Savory et al., 2009a; Savory et al., 2011a; Savory et al., 2009b), Cdx1 does not appear to overlap with Cdx2 during intestinal development (Gao et al., 2009; Grainger et al., 2010). In contrast, a role for Cdx1, and overlap with Cdx2, is seen in the adult colon since combined loss of Cdx1 and Cdx2 results in a transformation of colon to a cecum nature, a defect not seen in Cdx2 mutants. Furthermore, these Cdx double mutants die sooner after tamoxifen treatment than Cdx2 single mutants, and exhibit some differences in gene expression profiles, suggesting at least some level of functional overlap (Hryniuk et al., 2012; Verzi et al., 2010b; Verzi et al., 2011).

There are several reasons for these apparent differences in Cdx function in the adult and developing intestinal epithelium. The incomplete deletion of Cdx2 by *villin* cre-ER^T in the large intestine may mask a function for Cdx1 (Figure 22). It is also conceivable that Cdx1 plays a role during or after crypt development, which is completed at least two weeks post partum. The gradient of Cdx1 expression along the crypt-villus axis offers circumstantial evidence that it may play a role in crypt morphogenesis that is simply not evident at the stages examined. However, all present data suggests a limited role for Cdx1 in the intestine compared to Cdx2, although there is some contribution in the distal intestinal tract.

Cdx targets

Until recently, Cdx members were thought to convey much of their activity through regulation of *Hox* gene expression (Charite et al., 1998; Davidson and Zon,

2006; Deschamps et al., 1999; Gaunt et al., 2004; Isaacs et al., 1998; Tabaries et al., 2005; Young et al., 2009). However, it is now apparent that Cdx plays broad roles in various developmental processes. For example, Cdx also regulates *Ptk7* and planar cell polarity (Savory et al., 2011a) as well as *T* and *Cyp26A1* in the caudal region of the embryo (Savory et al., 2009a).

The demonstration of *Dll1* as a target of Cdx in both endoderm and mesoderm raises the question of whether all Cdx function operates through conserved pathways in different lineages. Although this may be the case for *Dll1*, this seems unlikely for all target genes. For instance, *Hox* genes are largely unaffected by Cdx2 loss in the developing intestine (Gao et al., 2009). However, in addition to *Dll1* and Notch signalling, it is conceivable that there are some other Cdx functions that operate in multiple lineages. As an example, Wnt signaling is strongly down regulated in the absence of Cdx2, due at least in part to loss of *Wnt3a* in the developing embryo (Savory et al., 2009a). Perturbations in Wnt signaling such as in $Tcf1^{-/-}Tcf4^{-/-}$ mutants result in anteriorization of the intestinal epithelium, as evidenced by ectopic expression of the stomach marker *Sox2* (Gregorieff et al., 2004). This is a partial phenocopy of $Cdx2^{-/-}$ mutants, suggesting a correlation between the two in the intestinal tract. Consistent with this, preliminary data indicate that Cdx does regulate Wnt signaling in the intestine (Appendix Figure 48), suggesting that Cdx functions through Wnt in the intestine and the caudal mesoderm. In addition and consistent with its function as a critical regulator in the intestine, Cdx2 impacts on many pathways affecting intestinal development and homeostasis. For example, deletion of Cdx2 results in aberrant Notch (Figure 33) and expression of TGFβ signaling components (Appendix Figure 49) in the intestine. These

are consistent with Cdx playing a role in differentiation, homeostasis and patterning of the intestinal tract. It remains to be seen whether Cdx mediates its effects in other contexts through common pathways such as these.

In addition to regulatory hierarchies, we have also found synergistic induction by Cdx and Wnt on several promoters, including *Dll1* (Figure 44 and our own unpublished data). This is consistent with an emerging body of work which shows that Cdx and Wnt co-regulate a number of target genes (Beland et al., 2004; Mankertz et al., 2004; Pilon et al., 2006; Savory et al., 2011b; Verzi et al., 2010a), a phenomenon that we have also observed in the adult intestinal stem cell niche (Lohnes laboratory, unpublished data). Since Cdx members physically interact with members of the LEF/TCF family (Beland et al., 2004), it seems possible that this interaction occurs at the protein-protein level. There are several mechanisms through which this could occur. For instance, LEF/TCF members are thought to be resident at target promoters, along with transcriptional repressors. Upon activation of the Wnt signal and translocation of β-catenin to the nucleus, co-repressors are replaced by co-activators and transcription initiates (Barker, 2008); it is possible that Cdx and its associated regulators may serve as one such co-regulator. It will be interesting to see in upcoming studies if this is a general paradigm of Cdx function.

Cdx2 and intestinal plasticity

Developmental plasticity is a phenomenon where a developing tissue changes in its response to a given stimulus. For example, in the developing nervous system, learning and signaling cues result in changes in neural connections, which are thought to be necessary for the variety of brain functions throughout development and adulthood (Kral

and Sharma, 2012). This plasticity appears to be progressively restricted. The auditory cortex can be trained to respond to different stimuli (Kilgard and Merzenich, 1998b), and is remodeled to receive important stimuli and ignore irrelevant stimuli (Kilgard and Merzenich, 1998a) in adult rats, but to a much lesser extent during development (Nakahara et al., 2004; Zhang et al., 2002). This is an important mechanism of adaptation used, for example, after cochlear implant (Kral and Sharma, 2012). Plasticity is also seen in the endoderm (Afelik et al., 2006; Fukuda-Taira, 1981; Wells and Melton, 2000), but is relatively unexplored.

Comparison of the impact of Cdx2 deletion at different times provides some novel information regarding the plasticity of the intestinal epithelium. Using tamoxigen to inactivate *Cdx2* at E13.5 revealed a transformation of the small intestine towards a distal stomach phenotype (Grainger and Lohnes, 2009); however, inactivating Cdx2 around E9.0 results in a more anterior transformation to an esophageal-like nature (Gao et al., 2009). Although *villin* Cre-ER^{T} was ineffective at eliciting sufficient Cdx2 deletion at these earlier stages, we have observed a similar transformation using an *actin* Cre-ER^{T} line with tamoxifen administration at E8.0 (appendix Figure 50). Taken together, these results indicate a progressive restriction in plasticity of the developing intestine with respect to Cdx2 function.

Acute ablation of Cdx2 in the adult intestinal epithelium results in death due to intestinal insufficiency within one week (Hryniuk et al., 2012). To circumvent this limitation, we effected a chimeric Cdx2 deletion using a sub-optimal dose of tamoxifen. Interestingly, we were able to show that loss of Cdx2 function in the adult small intestine also results in acquisition of stomach-like characteristics. These lesions never progress to

include regions of keratinized epithelium, unlike those found in *Cdx2*$^{+/-}$ mice (Beck et al., 2003; Beck et al., 1999), and again that plasticity becomes restricted early in development. *Cdx1*$^{-/-}$*Cdx2*$^{-/-}$ mutants, but not *Cdx2*$^{-/-}$ mutants also have a transformation of the epithelium of the colon towards a cecum-like nature, indicative of functional compensation of Cdx1 for Cdx2 in the adult colon, as well as plasticity (Hryniuk et al., 2012). This data is analgous to Barrett's esophagus, a condition arising in adult humans, where esophageal epithelium takes on characteristics of the small intestine as a result of excessive exposure to stomach acid. Furthermore, Cdx2 expression increases in Barrett's esophagus and transgenic expression of Cdx2 in the esophagus can transform to a Barrett's esophagus-like phenotype (Colleypriest et al., 2010; Eda et al., 2003; Hu et al., 2007). These findings suggest that even at adult stages, the intestinal epithelium retains some level of plasticity, the degree of which still remains unclear.

Endoderm-mesoderm interactions

Previous studies have shown that both the endoderm and mesoderm play roles in establishing the mature intestinal tract (Duluc et al., 1994; Wells and Melton, 2000). In this regard, it is thought that interactions between the endoderm and underlying mesoderm are essential for establishment and maintenance of the intestine. More recent evidence, however, suggests that this may not be true, or may be confined to specific developmental windows. For example, localized loss of Cdx2 in the intestinal epithelium of *Cdx2*$^{+/-}$ mice had been associated with ectopic expression of the stomach mesenchyme marker *BarX1*, which is thought to reflect the repatterning of the underlying mesenchyme in response to Cdx2 loss (Beck et al., 2003). This was not, however, seen in conditional

Cdx2 or Cdx1-Cdx2 mutants (Figure 28) (Grainger et al., 2010; Hryniuk et al., 2012). This could be simply due to the timing of Cdx2 loss; however, earlier knockout of Cdx2 similarly lacks mesenchymal transformation (Gao et al., 2009). Taken together, these findings suggest that transformation of the intestinal epithelium to a more anteriorized fate is not sufficient to yield a mesenchymal transformation and that such a transformation must occur during a restricted developmental window.

Although intestinal stem cells are classically thought to grow in combination with mesenchymal niche cells, in the adult mouse, *Lgr5*$^{+}$ stem cells are able to produce crypt-villus organoid units *in vitro* with only support from Paneth cells. In these studies, the authors demonstrate that *Lgr5*$^{+}$ stem cells require a Wnt signal, EGF and Noggin, all of which are produced by Paneth cells, in combination with laminin-rich Matrigel. In fact, Paneth cells can be substituted for by exogenous WNT3A (Sato et al., 2011b; Sato et al., 2009). This does not preclude other essential contributions from the tissue culture media, however, which may be able to recapitulate some mesenchymal factors. Nevertheless, this does suggest that the adult intestinal epithelium can undergo normal differentiation without apparent positional cues from the mesenchyme, suggesting that the mesenchyme plays a permissive, rather than an instructive, role in supporting the epithelial crypt-villus axis.

In the developing intestinal tract, *in vitro* culture systems for stem cells have not yet been established, making studies of isolated stem cells difficult. However, studies using manually isolated endoderm and mesoderm populations from developing rodents have suggested that mesoderm cells respond to the developing endoderm and that this instructive potential is reduced as the intestinal tract develops (Wells and Melton, 2000).

This also consistent with findings from Cdx2 mutant studies, where transformation of the mesoderm is progressively restricted with timing of Cdx2 loss of function (Gao et al., 2009; Grainger et al., 2010; Hryniuk et al., 2012). Taken together, these studies suggest that although earlier in development (i.e. prior to E9.0), mesoderm is patterned by endoderm, this potential is restricted at later stages.

Context-dependent differences in Cdx1 and Cdx2 function

There are many studies that illustrate functional overlap between Cdx members in several settings, such as neural tube closure, vertebral pattering, placental development and axial elongation (Savory et al., 2009a; Savory et al., 2011a; van den Akker et al., 2002; van Nes et al., 2006; Young et al., 2009). In the adult intestinal tract, Cdx1 and Cdx2 also have some functional overlap (Hryniuk et al., 2012; Verzi et al., 2011), but Cdx1 does not appear to play a role in the developing small intestine (Grainger et al., 2010). These results suggest that Cdx1 and Cdx2 functionally overlap in many circumstances. We found, however, that Cdx2 cannot function in Cdx1 autoregulation in the intestinal tract, representing the first *in vivo* demonstration of functional specificity between these two family members. This is not unexpected, since the transactivation domains of these transcription factors are poorly conserved (Lynch et al., 2003; Taylor et al., 1997; Trinh et al., 1999). Nevertheless, this specificity is particularly interesting because Cdx2 can fulfill Cdx1 autoregulation in mesoderm (appendix Figure 51). These results therefore suggest that Cdx2 is able to drive *Cdx1* autoregulation in early mesoderm but this regulatory loop fails in the endoderm. These differences likely lie in

tissue specific expression of transcriptional co-factors which differentially bind Cdx1 and Cdx2, the nature of which remain to be elucidated.

Cdx and intestinal stem cells

Cdx members have been proposed to function as developmental switches to promote differentiation from stem cells. For example, ectopic expression of Cdx4 and Hoxb4 in ES cells forces differentiation to hematopoietic stem cells (Lengerke et al., 2007), while Cdx2, HNF1α and GATA4 have been proposed to act in concert to initiate differentiation in human intestinal cells (Benoit et al., 2010). The precise mechanisms involved in driving stem cells to transit-amplifying cells in the intestine are poorly understood, but Wnt signaling is known to be important in maintenance and differentiation of the stem cell niche in the intestine (van der Flier and Clevers, 2009). In this regard, as discussed above, considerable data suggests that *Cdx1* and *Cdx2* are Wnt targets (Beland et al., 2004; Lickert et al., 2000; Pilon et al., 2006; Prinos et al., 2001; Shimizu et al., 2005), leading to the possibility that Cdx function may mediate some aspects of the Wnt signal. Alternatively, Cdx function has been shown to directly regulate *Wnt3a* (Savory et al., 2009a), suggesting that Cdx lies both upstream and downstream of the Wnt signal; this may also be the case in the intestinal tract. Also, TA cells have been suggested to revert back to a stem cell fate upon injury (Cheng and Leblond, 1974; Marshman et al., 2002; Munoz et al., 2012). It is therefore possible that upon loss of Cdx2, TA cells are programmed to revert back to a stem cell-like fate. Consistent with this hypothesis, the stem cell niche of $Cdx1^{-/-}Cdx2^{-/-}$ adult mice showed a marked decrease in the number of proliferating cells by 3 days after treatment (appendix Figure

52) (Hryniuk et al., 2012), suggesting a reduction in the number of TA cells. This effect is not likely due to apoptosis, since TUNEL assays did not reveal a difference in apoptotic levels (data not shown), but, may be due to loss of Cdx2 resulting in a block in the differentiation from stem cell to transit amplifying cell. This was supported by an upregulation of intestinal stem cell marker *Lgr5* (Barker et al., 2007) (appendix Figure 53); however, further studies are necessary to determine if this observation is significant.

Genome-wide mapping studies comparing ES cells, neural progenitors and differentiated fibroblasts revealed association of specific post translational modifications of histones with actively transcribed or repressed chromatin (Bernstein et al., 2005; Mikkelsen, 2007). For example, trimethylation of lysine 4 on histone 3 (^{3}Me-H3K4) is typically associated with activated chromatin, while ^{3}Me-H3K27 is associated with inactive genomic regions (Bernstein, 2006; Guenther et al., 2007). To this end, in stem cells, DNA is "poised" to be either activated or repressed by a combination of activating and repressive chromatin marks (Bernstein et al., 2006). Therefore, compared to a differentiated population, stem cells have an enrichment of both the activating H3K4 and the repressive H3K27 trimethylation marks. Consistent with an increase in stem cells, there was a modest increase in ^{3}Me-H3K27 and ^{3}Me-H3K4 levels in both Cdx2 single and Cdx1-2 double mutants compared to controls, as assessed by western blots (appendix Figure 54). This was also confirmed through ChIP analysis targeting regions encompassing the TSS of several intestinal promoters after immunoprecipitation with the in ^{3}Me-H3K27 and ^{3}Me-H3K4 antibodies (appendix Figure 55). The methylation status of histones is established by a balance between Polycomb (PcG) and Trithorax (trxG) methyltransferase-containing complexes, which lead to chromatin in an closed or open

format, respectively (Fritsch et al., 1999; Lee, 2007; Mujtaba, 2008; Pasini, 2008; Srinivasan, 2005; Srinivasan et al., 2008); demethylation of histones is thought to be mediated by Kdm6a and Kdm6b (Klose, 2006; Whetstine, 2006). Notably, the increase in methylation in Cdx mutants was not due to increase expression of demethylases *Kdm6a* and *Kdm6b* (data not shown). Taken together, these results suggest an increase in stem cell-like cells in the Cdx mutant background, indicating a role for Cdx in regulating the transition from stem cell to TA cell in the small intestine.

Future directions

There still remain many outstanding questions to be addressed in Cdx biology. For example, Cdx1 and Cdx2 are both expressed in the early endoderm, but their roles at this stage have not been clearly elucidated. Although we now know that Cdx members play roles in patterning the intestinal epithelium and maintenance of intestinal identity, the manner in which they convey these functions have yet to be determined. Furthermore, Cdx1 and Cdx2 are emerging as important regulators in colorectal cancer, and further studies of Cdx function may aid in prognosis or in developing novel therapeutic strategies. Finally, Cdx members are expressed early in development, through to adulthood, in different germ layer derivatives. The manner in which Cdx elicits function in these different tissues is currently unknown.

To determine Cdx mechanisms of function in different tissues, several approaches could be considered. To determine protein interacting partners for Cdx2, immunoprecipitation followed by mass spectrometry can be employed using SILAC approaches to identify putative interacting partners (reviewed in Trinkle-Mulcahy, 2012).

Considering the differences in function between Cdx1 and Cdx2 in different tissues, it would be interesting to consider an *in vivo* protein pulldown approach comparing Cdx1 and Cdx2 interacting proteins in identical and different tissues. To achieve this, we could use *WT*, *Cdx1*$^{-/-}$, *Cdx2*$^{-/-}$ and *Cdx1*$^{-/-}$*Cdx2*$^{-/-}$ mice fed different series of SILAC diets to differentially label each mouse population (Sury et al., 2010). As an example, intestinal epithelial cells could be harvested from each population and mass spectroscopy used to identify proteins that interact differentially with Cdx1 and Cdx2 in the intestine. This analysis could potentially be expanded to include endoderm and tail bud to determine the differential protein binding partners involved in these processes. Cdx tissues from different sources can be isolated by fluorescence activated cell sorting (FACS) using a tagged Cdx allele or an antibody can be used to isolate cells of interest. In this regard, Epithelial Cell Adhesion Marker (epCAM) can be used to isolate endoderm from WT, and Cdx mutant embryos to capture early stage endoderm cells. Likewise, tail buds and epithelium from older stage fetuses and adults can be isolated either manually or with FACS methods.

In addition to examining protein-protein interactions, resultant RNA could then be analyzed by microarray to determine targets for Cdx2 in relevant tissues, including developing endoderm, tail bud and/or adult intestine. This can be paired with ChIP-seq data to determine sites where Cdx2 is bound in the genome as an indication of direct target genes. These target genes can then be verified with a combination of directed ChIP, EMSA and luciferase assays. To verify the biological functionality, putative targets can be examined *in vivo* through transient transgenesis to observe the impact of specific

CDRE deletion on gene expression, or through homologous recombination to determine a functional effect of such regulatory elements.

Conclusion

Determination of Cdx function and elucidation of the mechanisms governing its regulation of patterning and differentiation may ultimately be important for therapeutic strategies in intestinal cancers. Through this study, I have shown that during intestinal development, Cdx2, but not Cdx1, regulates patterning and differentiation of the intestinal epithelium. I have further demonstrated that at least some of the differentiation effects arise through Cdx regulation of *Dll1*, a mechanism that is conserved in mesoderm as well as endoderm derivatives. Finally, I have demonstrated context dependent specificity of Cdx1 and Cdx2 in the intestine for the first time *in vivo*. I look forward to learning about mechanisms of Cdx function in the future.

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APPENDICES

Table 1: Chapter 3 PCR oligos

Gene (number of cycles)	**Primer sequence**
Dll1 RT-PCR *(33 cycles)*	5’ - ATGGGCCGTCGGAGCGCGCTA - 3’ (sense)
	5’ - TGTGCGGCCGCTACTGTGAAGG - 3’ (antisense)
β-actin RT-PCR *(23 cycles)*	5’ - AGCCATGTACGTAGCCATCC - 3’ (sense)
	5’ - CTCTCAGCTGTGGTGGTGAA - 3’ (antisense)
Dll1 CDREs for ChIP	5’ - TTGGCTAGGCCAACATTCAGGC - 3’ (sense)
	5’ - TTCCTCCTCCTCATCCTCCTGT - 3’ (antisense)
Dll1 upstream negative control for ChIP	5’ - GGTAGGGGATGGTTGGGTTCC - 3’ (sense)
	5’ - GGTCTGCCTACCTCCACAGTC - 3’ (antisense)
β-actin CDRE for ChIP	5’ - GGCTGAGGACACCCAGACGA - 3’ (sense)
	5’ - GCGTGGGGGGGATTTCTCTTG - 3’ (antisense)
Dll1 promoter outer primers	5’ - TATACCTGAGTCTGCCCTATCCACC - 3’ (sense)
	5’ - TATACACAGCAGGGCAGAGACCAC - 3’ (antisense)
Dll1 promoter inner primers	5’ – TATAGTCGACTTATTCAGATTGGGGCTGGG - 3’ (sense)
	5’ - TATAAGATCTGGTACCGCTGGACGCC - 3’ (antisense)

Dll1 promoter CDRE1 mutagenesis	5’ - GGTGAAGGTGAGGAGGATCCGTGTGTGGGGAGGGG - 3’ (sense)
	5’ - CCCCTCCCCACACACGGATCCTCCTCACCTTCACC - 3’ (antisense).
Dll1 promoter CDRE2 mutagenesis	5’ - GCCCTATCCACCCATTGCAATTATTCAGATTGGGG - 3’ (sense)
	5’ - GCATTCATTCTTTTCCAACTTAAGGATCCCCACACCATTCTGTATTGG - 3’ (antisense)
	5’ - CCAATACAGAATGGTGTGGGGATCCTTAAGTTGGAAAAGAATGAATGC - 3’ (sense)
	5’ - GGAATTTCTGCACTTCACGTCGTCATCGCCAACTC - 3’ (antisense)
TFF3 RT-PCR	5’ - ATGGAGACCAGAGCCCTCTGGC - 3’ (sense)
(28 cycles)	5’ - CAAAATGTGCATTCTGTCTCCTGCAGAG - 3’ (antisense)
IFABP RT-PCR	5’ - GATCATGGCATTCGACGGCA - 3’ (sense)
(26 cycles)	5’ - GCTTTTACTTCTTTAGCTTTGAC - 3’ (antisense)

Math1 RT-PCR	5’ - ATGTCCCGCCTGCTGCATGCAG - 3’ (sense)
(37 cycles)	5’ - GCGGCGGTTGCTCTCCGAC - 3’ (antisense)
Dll1 qPCR	5’ - AACCATGAACAACCTAGCCAATT- 3’ (sense)
	5’ - CATGGTCCCCGTGAAAGTC- 3’ (antisense)
Math1 qPCR	5’ - ATGTCCCGCCTGCTGCATGC- 3’ (sense)
	5’ - GGCGCGTGGGTCGGTGC- 3’ (antisense)
IFABP qPCR	5’ - CCGAGAGGTTTCTGGTAATGAACTAATC- 3’ (sense)
	5’ - CTTTTACTTCTTTAGCTTTGACAAGGCTGG- 3’ (antisense)
TFF3 qPCR	5’ - ATGGAGACCAGAGCCCTCTGG- 3’ (sense)
	5’ - GGGCACATTTGGGATACTGGAGTC- 3’ (antisense)
β-Actin qPCR	5’ - GCTATGAGCTGCCTGACGGC- 3’ (sense)
	5’ - GGATGTCAACGTCACACTTCATGATGG- 3’ (antisense)

All RT-PCR amplifications were performed with a melting temperature of 95℃ and an annealing temperature of 58℃.

All qPCR amplifications were performed with a melting temperature of 95℃ and an annealing temperature of 61℃.

Table 2: EMSA oligos

WT CDRE proximal	5’-AGGTGAGGATTTATGGTGTGTGGG-3’
WT CDRE distal	5’-ATGGTGTGGCATAAATTAAGTTGG-3’
Mutated CDRE proximal	5’-ATGGTGTGGTCCGCCCTAAGTTGG-3’
Mutated CDRE distal	5’-AGGTGAGGCCCGCCTGTGTGTGGG-3’
Hoxb8 positive control	5’-GCTATAAAAGTTTATAGGGTATAAATT-3’
RARE negative control	5’-AGAGGTCACCGAAAGGTCACT-3’

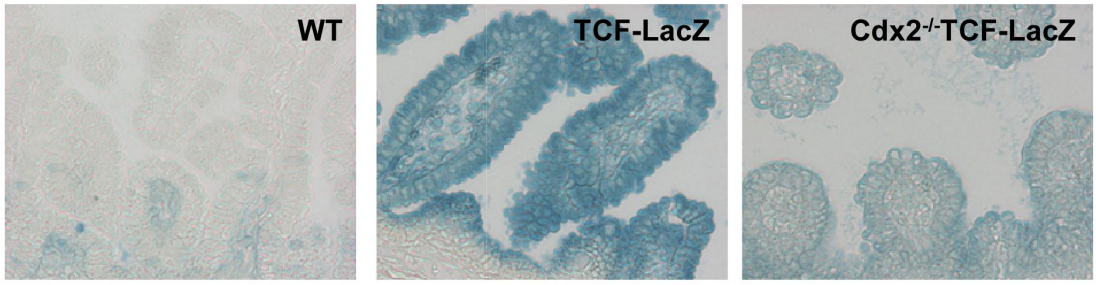

Figure 48: Loss of Cdx2 affects canonical Wnt signaling. LacZ staining of wild-type and *Cdx2*$^{-/-}$ small intestine in an E18.5 Wnt reporter (upper panels). Note loss of reporter expression in mutant small intestine.

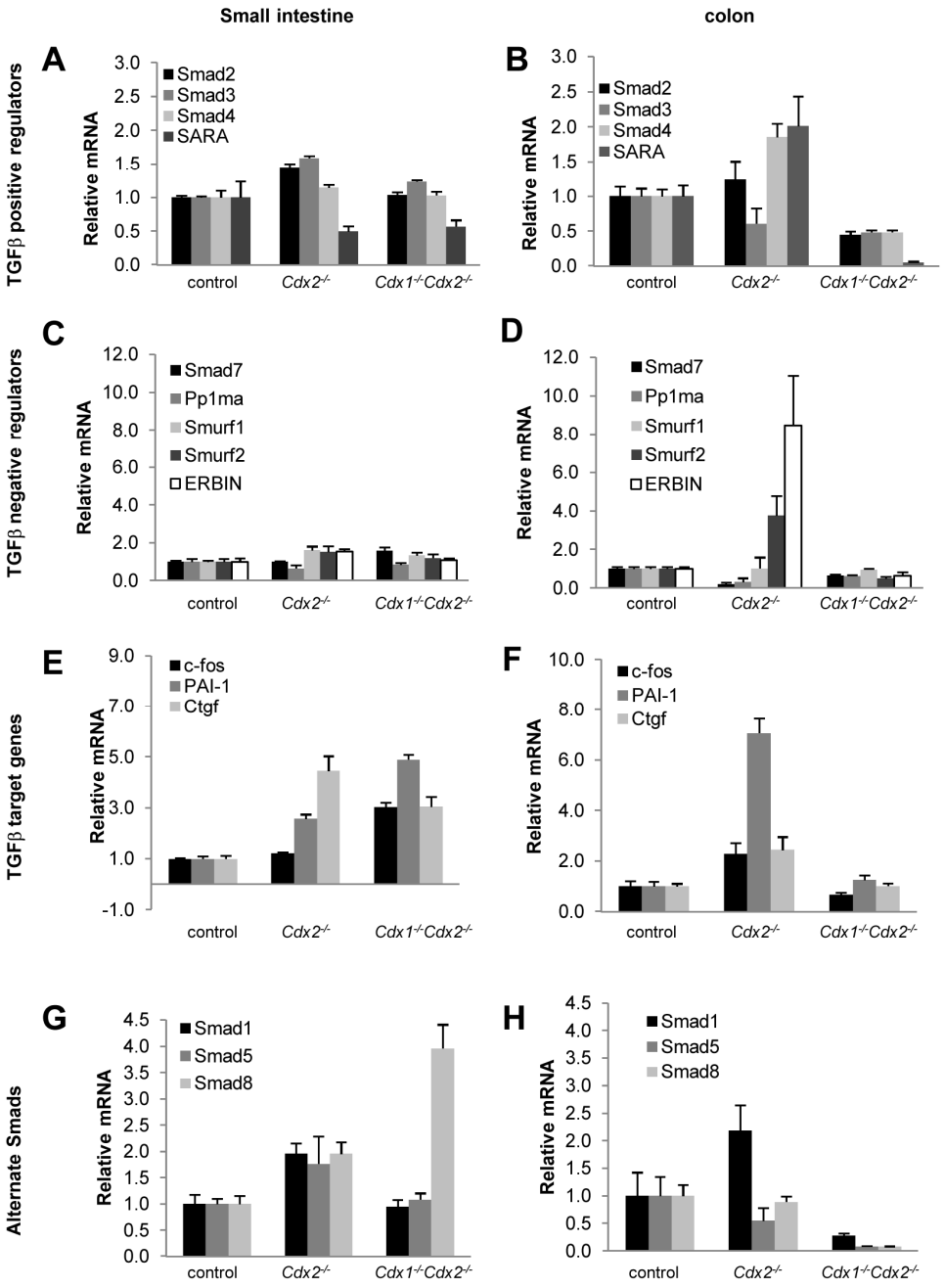

Figure 49: Cdx impacts on TGFβ signaling in the small and large intestine. qPCR of TGFβ postive (A,B) and negative (C,D) regulators, target genes (E.F) and alternate Smads in adult control, *Cdx2*$^{-/-}$ and *Cdx1*$^{-/-}$*Cdx2*$^{-/-}$ conditional mutants treated with 5mg tamoxifen for 48 hours.

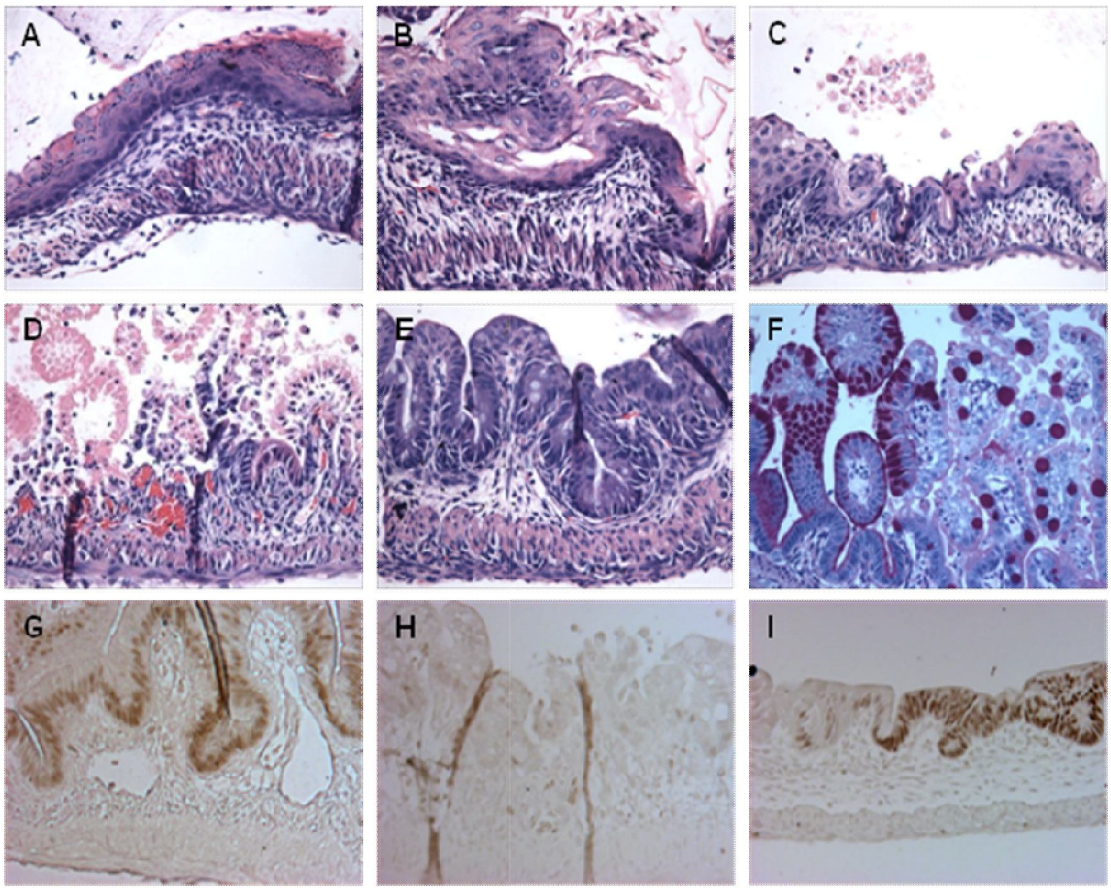

Figure 50: Removal of Cdx2 at E8.5. A *Cdx2$^{f/f}$ actin* cre-ER^{T} male was crossed with a *Cdx2$^{f/f}$* female and presence of a vaginal plug marked embryonic day (E) 0.5 at noon of the next day. Pregnant dams were dosed with 5mg of tamoxifen at E8.5, embryos harvested by caesarian section at E18.5. Hematoxylin and eosin staining of *Cdx2$^{-/-}$* small intestines are shown in A-E. F shows Periodic Acid-Schiff staining of a *Cdx2$^{-/-}$* small intestine roughly at the level of the proximal ileum. Pdx1 staining in *Cdx2$^{-/-}$* mice in the proximal small intestine (G), distal small intestine (H) and colon (I).

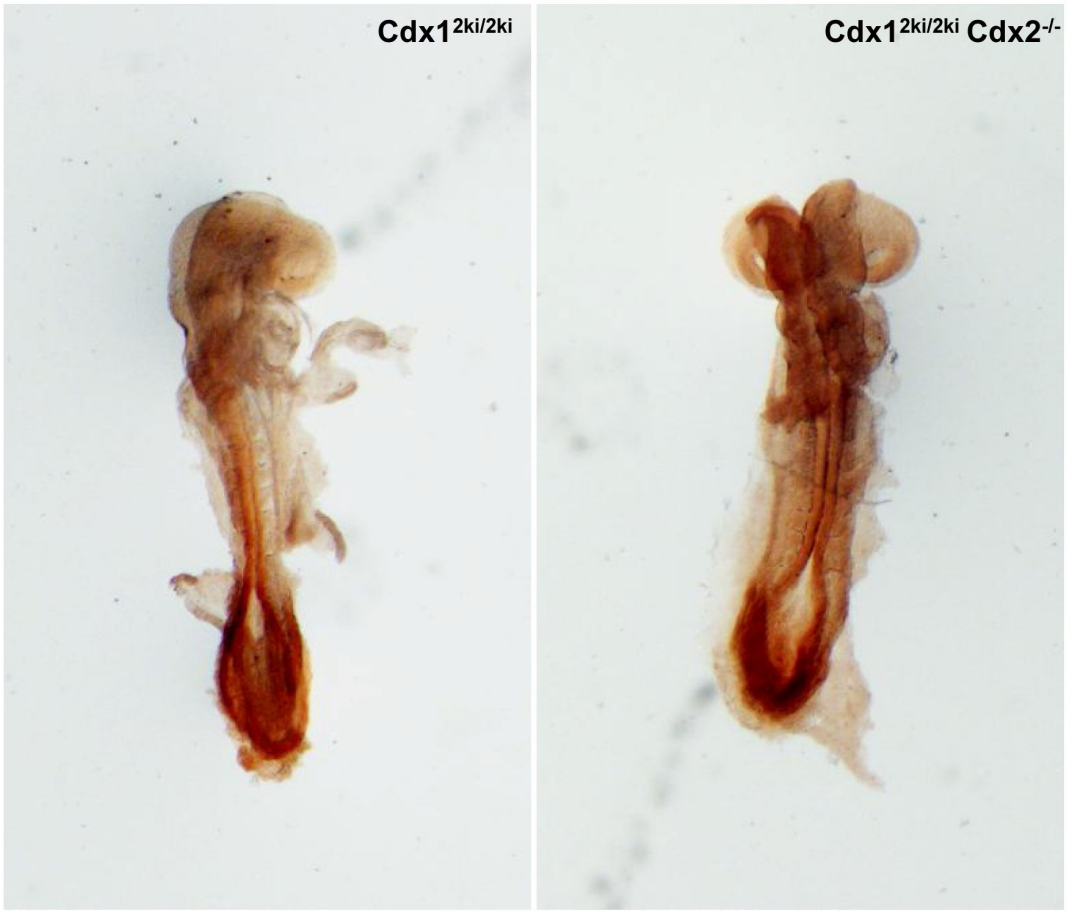

Figure 51: Cdx1 expression does not fail in presomitic mesoderm at E8.5. Immunohistochemistry for Cdx2 in $Cdx1^{2ki/2ki}$ and $Cdx1^{2ki/2ki}$ $Cdx2^{-/-}$ E8.5 embryos. Note that expression of Cdx2 from the *Cdx1* locus is not lost at E8.5, as represented here with Cdx2 immunostaining in the absence of endogenous Cdx2 (right).

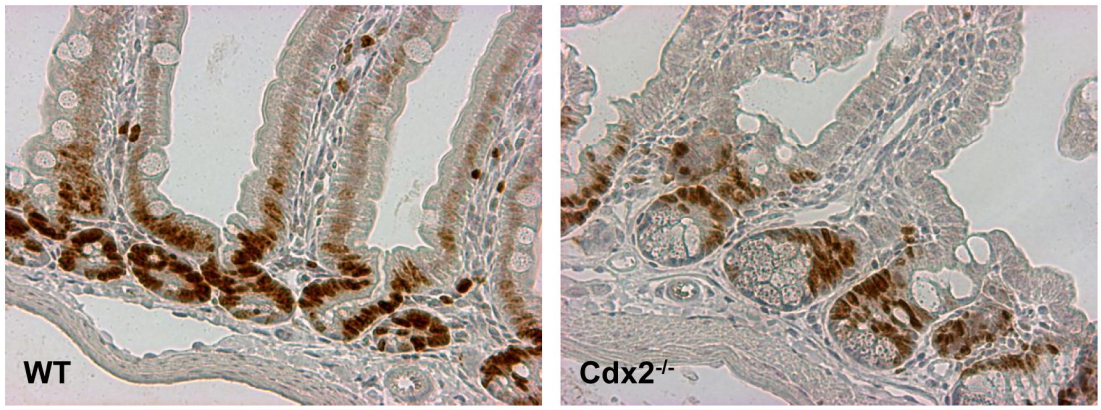

Figure 52: Loss of Cdx affects the transit amplifying cell population. Immunohistochemistry for ki67 in WT and *Cdx2*$^{-/-}$ adult small intestine 72 hours after tamoxifen treatment.

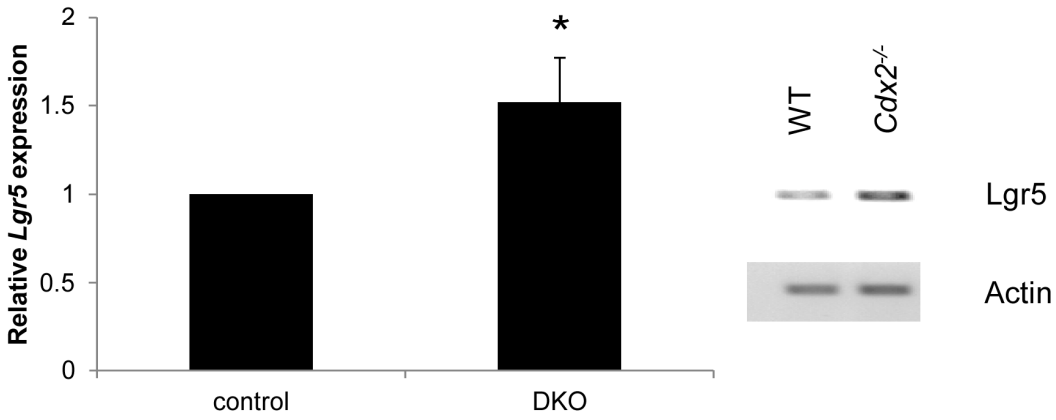

Figure 53: Loss of Cdx affects stem cells of the intestine. Semi-quantitative RT-PCR for stem cell maker *Lgr5*. RT-PCR was performed on triplicate *Cdx2*$^{-/-}$ small intestines and littermate controls. *P<0.05 by student's t-test

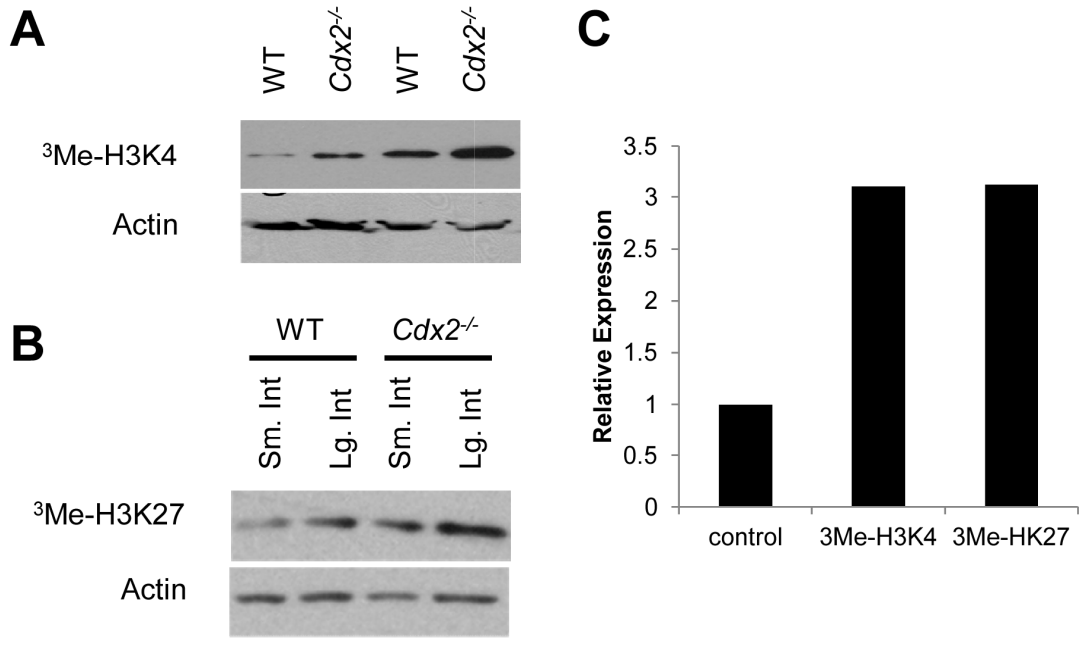

Figure 54: Loss of Cdx2 affects methylation status. (A) Western blot for trimethyl-H3K4 on *Cdx2*$^{-/-}$ E18.5 small intestines and littermate controls. (B) Western blot for trimethyl-H3K27 of *Cdx2*$^{-/-}$ E18.5 small and large intestines and littermate controls. (C) Quantification of A and B

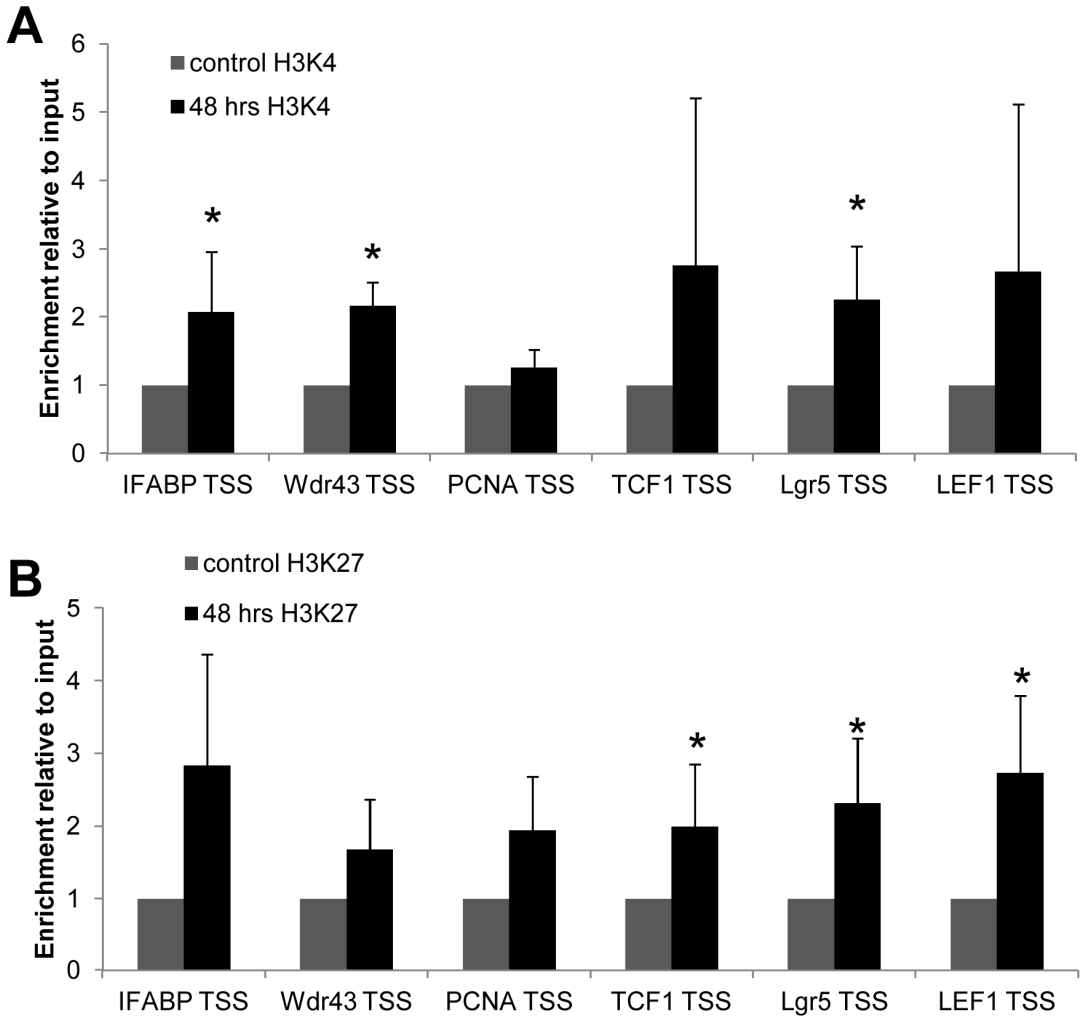

Figure 55: *Cdx2*$^{-/-}$ crypt cells have increased methylation at intestine specific promoters. Crypts from small intestines of WT and Cdx2-/- adults were harvested 48 hours after tamoxifen treatment and subjected to chromatin immunoprecipitation using antibodies targeted to H3K4-Me^3 (A) and H3K27-Me^3 (B). PCR was performed targeting transcriptional start sites of intestinal genes as shown. Note the increase in bivalent chromatin marks, indicative of stem cell-like characteristics. Error bars represent standard deviation from the mean of triplicate samples. *P<0.05 by student's t-test.