The role of Cdx transcription factors in the adult intestine

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ABSTRACT

The homeodomain transcription factor family of Cdx genes, Cdx1, Cdx2 and Cdx4, are known to play essential roles in many developmental processes including neural tube closure, axial elongation, hematopoiesis and gastrointestinal patterning. In the adult, Cdx1 and Cdx2 are both expressed strictly in the adult intestinal epithelium, but their functions and mechanisms of action at this stage are poorly understood. Cdx transcription factors have also been reported to be lost in intestinal cancers. To circumvent early lethality, a conditional loss of function strategy was used to inactivate Cdx2 in the adult intestinal epithelium. These conditional mutants were crossed to $Cdx1^{-/-}$ mice to examine potential functional compensation between these family members as well as into $APC^{(min/+)}$ mice to study their role in tumorigenesis. Using these models, I have found that Cdx2 regulates adult intestinal homeostasis and differentiation in the small intestinal epithelium, while both Cdx1 and Cdx2 contribute to colon homeostasis. Furthermore, Cdx transcription factors are tumor suppressors in the development of Wnt-induced colorectal cancer, and impact several pathways including TGF-β and Eph-ephrin signaling. Finally, Cdx2 regulates Eph-ephrin signaling through direct activation of the Notch pathway. Altogether, this study underscores critical roles and mechanisms of action for Cdx members in the adult intestine and in intestinal tumorigenesis.

RÉSUMÉ

La famille des facteurs de transcription des gènes Cdx, Cdx1, Cdx2 et Cdx4, sont connus pour jouer un rôle essentiel dans de nombreux processus de développement, notamment la fermeture du tube neural, l'allongement axial, hématopoïèse et le développement de l'intestin. Chez l'adulte, Cdx1 et Cdx2, mais pas Cdx4, sont exprimés strictement dans l'épithélium intestinal, mais leurs fonctions et mécanismes d'action sont mal connus. De plus, chez les cancers de l'intestin, l'expression des Cdxs est réprimée. Nous avons utilisé un modèle de <<knock-out>> conditionnel afin d'inactiver Cdx2 de l'épithélium intestinal adulte. On a croisé ces mutants conditionnels avec les souris Cdx1-/- pour examiner la compensation fonctionnelle potentielle entre ces membres de famille, ainsi qu'avec les souris APC(min / +) pour étudier leur rôle dans la tumorigenèse. Les conclusions principales de cette étude sont que Cdx2 régule l'homéostasie intestinale des adultes et la différenciation de l'épithélium du petit intestin, tandis que Cdx1 et Cdx2 contribue à l'homéostasie du côlon. En outre, les facteurs de transcription Cdx sont des suppresseurs de tumeurs dans le développement du cancer colorectal, affectant plusieurs voies de signalisation telle que TGF-β et eph-éphrine. Finalement, Cdx2 régule la signalisation eph-éphrine par l'activation directe de Notch. En somme, cette étude révèle les rôles et les mécanismes d'action des membres Cdx dans l'intestin des adultes et dans la tumorigenèse intestinale.

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LIST OF ABBREVIATIONS

α	alpha
AB	Alcian Blue
AIP	anterior intestinal portal
Akt	serine/threonine-specific protein kinase B
A-P	anterior-posterior
APC	adenomatous polyposis coli
ASBT	apical sodium-dependent bile acid transporter
β	beta
bHLH	basic helix-loop-helix
Bmi	B lymphoma Mo-MLV insertion region 1 homolog
BSA	bovine serum albumin
ca	caecum
Caco	cancer of the colon
cad	caudal
Cas	CRISPR associated protein
CBC	crypt based columnar
cDNA	complimentary DNA
CDRE	Cdx response element
Cdx	Caudal related homeobox
ChIP	chromatin immunoprecipitation
ChIP-seq	ChIP sequencing
CIP	caudal intestinal portal

co	colon
Co-Smad	comediator Smad
CRC	colorectal cancer
Cre	Cre recombinase
CRISPR	clustered regularly interspaced short palindromic repeats
CSL	CBF-1-Su(H)-Lag-1
DAPT	N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester
Def	Defensin
Dkk	Dickkopf
DIG	digoxigenin
Dll	delta like ligand
DNA	deoxyribonucleic acid
DREF	DNA replication-related element binding factor
DSB	double stranded break
DTT	dithiothreitol
Dvl	dishevelled
E	Embryonic day
e-cells	epithelial cells
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetra acetic acid
ELF	E47-like factor
EMSA	electron mobility shift assay
EMT	epithelial-mesenchymal transition

EphA	ephrin type-A receptor
EphB	ephrin type-B receptor
ER	estrogen receptor
FAP	familial adenomatous polyposis
FBS	fetal bovine serum
flox	flanked by loxP
Fz	frizzled
γ	gamma
Gfi	growth factor independent
Ghrl	ghrelin
Gkn	gastrokine
GI	gastrointestinal
GSK	glycogen synthase kinase
h	hours
HD	homeodomain
HDR	homology-directed repair
Hes	Hairy/enhancer/split
Hey	Hairy/enhancer-of-split related with YRPW motif protein
Hox	homeobox protein
HRP	horseradish peroxidase
Hsp	heat shock protein
IAP	intestinal alkaline phosphatase
IFABP	intestinal fatty acid binding protein

IgG	immunoglobulin Type G
indel	insertion/deletion
I-Smad	inhibitory Smad
Jag	jagged
κ	kappa
kb	kilo bases
Klf	kruppel like factor
Kras	Kirsten rat sarcoma viral oncogene homolog
Krt	keratin
LacZ	gene encoding β-galactosidase
LEF	lymphoid enhancing factor
Lgr	Leucine-rich repeat-containing G-protein coupled receptor
LMP	low melting point
LOH	loss of heterozygosity
μ	micro
Math	mouse atonal homologue
Min	multiple intestinal neoplasia
Mmp	matrix metallopeptidase
mRNA	messenger RNA
MSI	microsatellite instability
mTOR	mammalian target of rapamycin
Muc	Mucin
NF	neurofibromin

Ngn	neurogenin
NHEJ	non-homologous end joining
NICD	notch intracellular domain
Olfm	olfactomedin
PAI	plasminogen activator inhibitor
PAGE	polyacrylamide gel electrophoresis
PAS	periodic acid-Schiff
PBS	phosphate buffered saline
PCR	polymerase chain reaction
Pdgf	platelet derived growth factor
PFA	paraformaldehyde
PI3K	phosphoinositide 3-kinase
PI	propridium iodide
pSmad	phosphorylated Smad
PVDF	polyvinylidene flyoride
qPCR	quantitative PCR
RB	retinoblastoma protein
RBPJ	recombining binding protein suppressor of hairless
r-cells	round cells
RNA	ribonucleic acid
RNA-seq	RNA sequencing
RT-PCR	reverse transcriptase PCR
R-Smad	receptor Smad

SARA	Smad anchor for receptor activation
Scnn	sodium channel non-voltage gated
s.d.	standard deviation
SDS	sodium dodecyl sulphate
sh-RNA	short hairpin RNA
SI	sucrase isomaltase
si	small intestine
SLC	solute carrier family
Smad	sma/mothers against decapentaplegic
Smoc	SPARC related modular calcium binding
Sox	Sry-related HMG box
T	tamoxifen
TA	transit-amplifying
TBR	TGF-beta receptor
TCF	T-cell factor
TESS	transcriptional element search system
TGF	Transforming Growth Factor
Tff	trefoil factor
VPA	valproic acid
Wnt	wingless

DEDICATION

To my little “sprout”, my next adventure in life.

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CHAPTER 1

INTRODUCTION

In Canada there are roughly 20 million people currently suffering from some form of intestinal disease, which contributes to 4% of total deaths in Canada per year. The digestive system is a specialized system that has developed for the intake of nutrition and the excretion of waste. Several transcription factors, one family being the Caudal-related homeobox (Cdx) transcription factors, has been shown to be involved in the development of this system and its maintenance throughout adulthood. Moreover, Cdx members have been implicated in intestinal cancer as well as other cancers of the digestive tract. By studying the role of Cdx transcription factors in the adult intestine we hope to gain insight into their function in intestinal development and homeostasis and their role in intestinal cancers.

Intestinal development and homeostasis

Development of the gastrointestinal tract

The three embryonic germ layers, the endoderm, ectoderm and mesoderm, form during gastrulation. The mammalian gut is composed of all three of these germ layers – endoderm which forms the gastrointestinal epithelium, mesoderm which forms the submucosa and smooth muscle layers, and ectoderm which includes the most anterior and posterior luminal digestive structure and the enteric nervous system(de Santa Barbara et al., 2003). Of the three germ layers, the development of mesoderm and ectoderm has been the most extensively studied. Until recently, it had been presumed that endodermal development was driven by the same signals as those of the ectoderm and mesoderm; however the endoderm is now being

regarded as a potential source of morphogenetic activity and of signals for cell differentiation in the development of the intestinal tract (Lewis and Tam, 2006).

It has been suggested that endoderm specification is reliant on cues that are imparted immediately prior to exit from the primitive streak, although this concept is presently not well defined. In the mouse, the formation of the endoderm begins eight to ten hours after the beginning of gastrulation by the recruitment of epiblast cells through the anterior portion of the primitive streak (Figure 1). Endoderm is regionalized in an anterior to posterior order as it exits the primitive streak, with cells exiting first being recruited to the foregut region and those exiting last forming the hindgut region.

After gastrulation, the endoderm is a pseudostratified epithelium. Subsequent formation of the primitive gut then begins with the development of two intestinal pockets (or portals) at the anterior and posterior regions of the embryo (Figure 1). The foregut section deepens anteriorly as the pocket moves towards the posterior end of the embryo. Six to eight hours later, a similar event will proceed with the formation of the hindgut intestinal portal. The hindgut pocket will move anteriorly to meet that of the foregut to complete the primitive gut tube by embryonic day 9.0 (E9.0). The subsequent inversion of the germ-layer arrangement leads to the ectoderm enveloping the embryo, while the endoderm rolls inwards. By this stage, the endoderm has already received patterning instructions and as a result is beginning cell fate decisions (Figure 1) (Lewis and Tam, 2006; Wells and Melton, 1999).

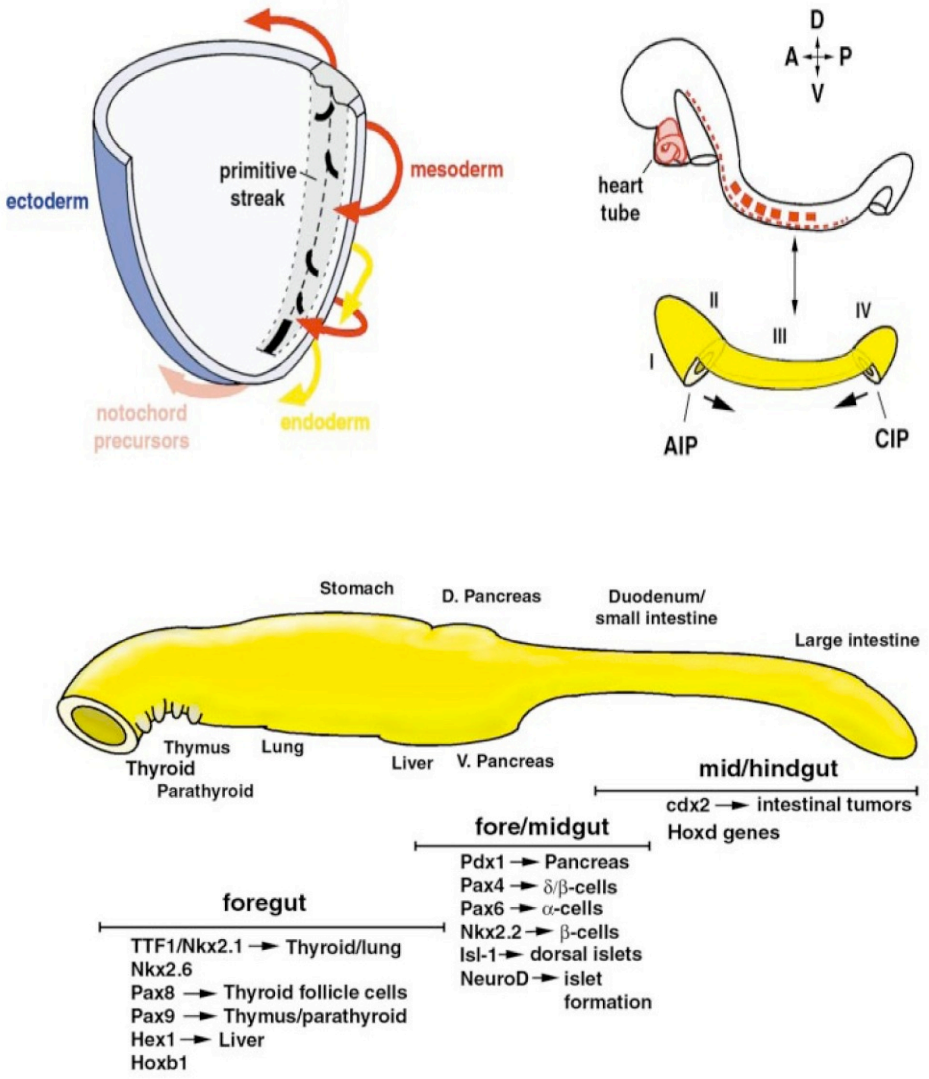

Figure 1: Schematic of the process of mouse intestinal development.
The upper left panel shows a gastrulating embryo at E7.5, giving rise to the three primary germ layers. The upper right panel shows a diagram of the embryo at E8.5, showing the developing anterior and posterior pockets (anterior intestinal portal (AIP) and the caudal intestinal portal (CIP)). The lower panel shows the discrete expression domains of transcription factors along the A-P axis of the early gut tube (E9.5). At this stage, the development of several organs are visible as pockets along the gut tube. Transcription factors are grouped according to their A-P expression at the time of development. Figure and legend adapted with permission from Wells and Melton, 1999. (Wells and Melton, 1999)

The development of the gut continues with the elongation and turning of the gut tube. In the foregut, the esophageal region narrows while the stomach dilates at E10.5. At this stage, the midgut is continuing to grow and differentiate. In the hindgut, the cloacal membrane differentiates at E10.5 and the anal sphincter has developed by E14.5, around the same time that the endoderm differentiates from a pseudistratified epithelium into a simple columnar intestinal epithelium. By E15.5, the caecum is first evident. Invaginations which house the dividing cells begin forming at E14.5 in the intestinal epithelium, beginning with the most anterior portion of the small intestine (Figure 2) (Wells and Melton, 1999). As these cells divide, the villi are formed. These morphogenetic changes continue along the length of the intestinal tract until the embryo is fully developed around E18.5. It is not until a few weeks after birth that the intestinal invaginations fully develop into mature crypts. As the crypts mature, they become deeper, especially in the region of the large intestine, where a flat epithelial surface is found instead of the protruding villi characteristic of the small intestine.

Gastrointestinal histology and structure

The gastrointestinal tract is composed of a variety of organs with their own specialized epithelial lining (Figure 3). For example in mice, the esophagus is lined by a keratinized epithelium surrounded by a thick muscular layer. Posterior to the esophagus is the stomach which consist of three regions of distinct epithelial lining. The proximal cardiac region is lined by keratinized squamous epithelium, the distal pyloric region is lined with a simple glandular epithelium and the middle body region has aspects of both proximal keratinized and distal glandular epithelium.

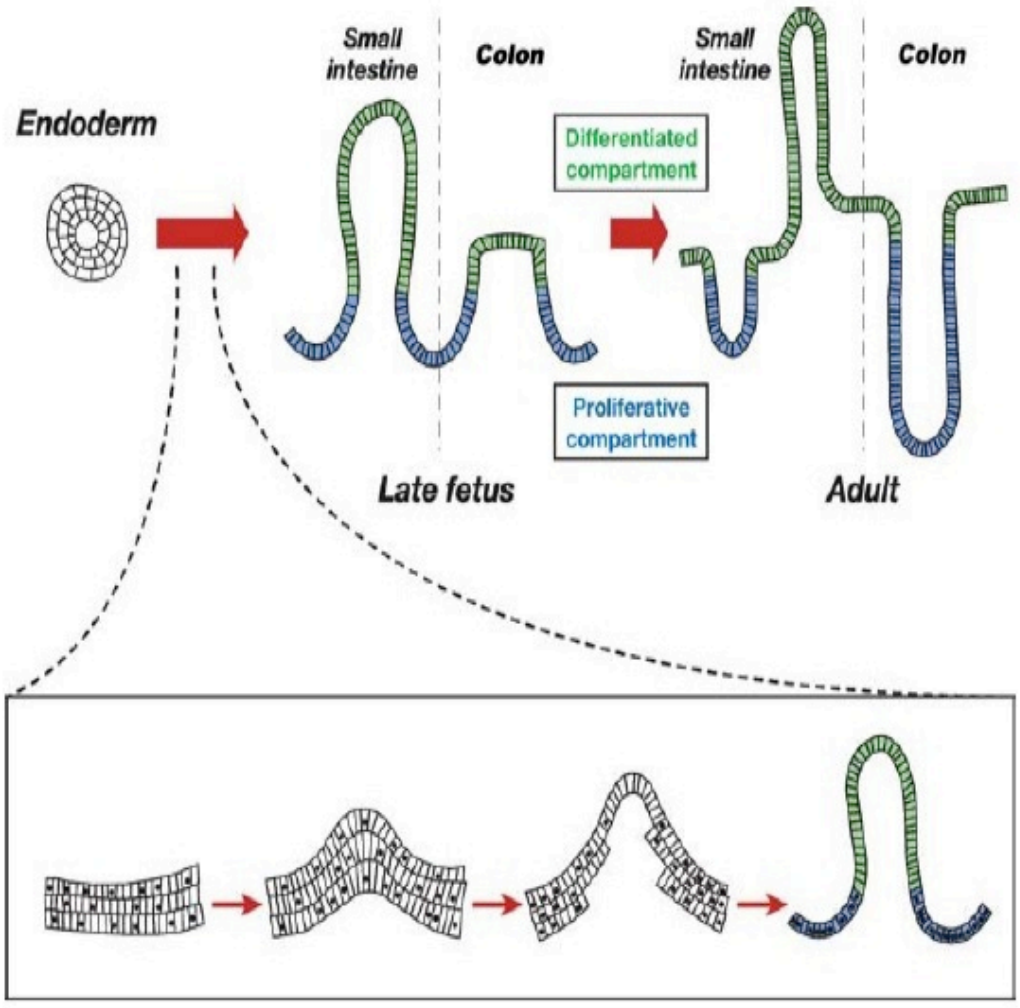

Figure 2: Schematic of the development from endoderm to mature intestinal epithelium.
During the late stages of the fetus (E14-E17 in mouse), the multilayered endoderm differentiates to form simple columnar epithelium. Note that the crypts deepen and villi elongate in the adult mouse. Figure and legend adapted with permission from Sancho, et al, 2004. (Sancho et al., 2004)

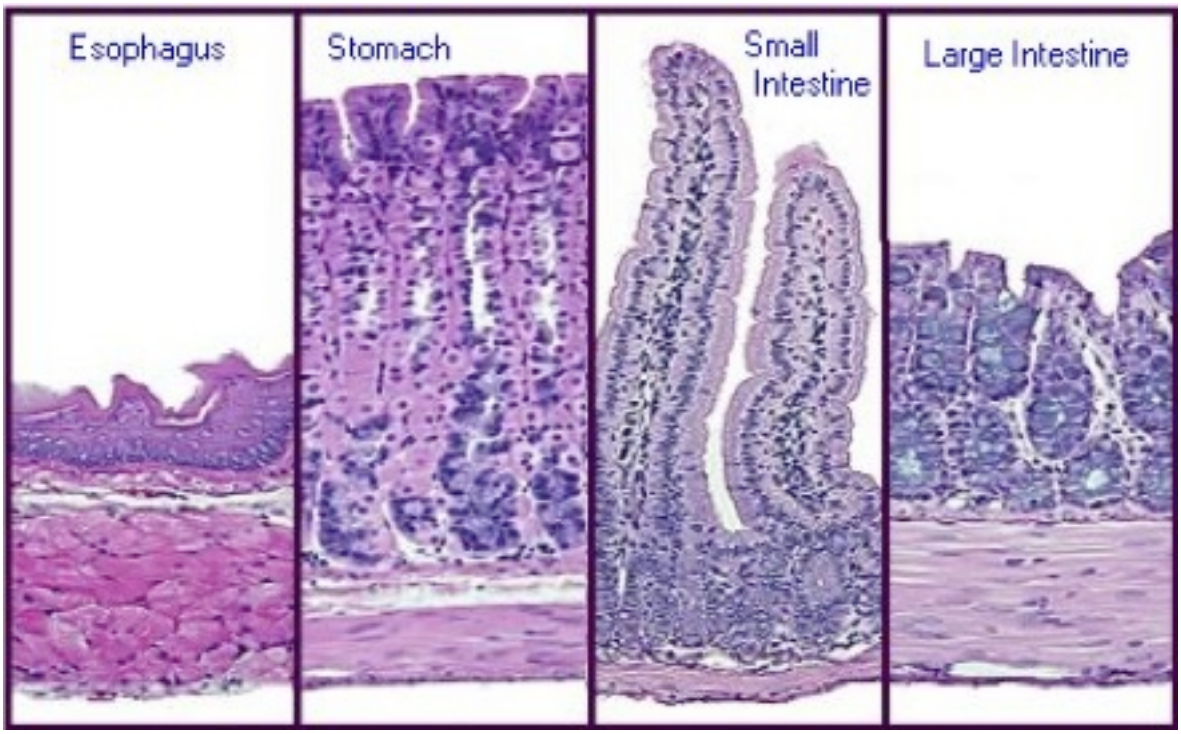

Figure 3: Epithelium of the organs of the mammalian digestive tract.
Epithelium of the esophagus, stomach, small and large intestines. Figure and legend adapted with permission from Bowen, 2004. (Bowen, 2004).

Specialized cells of the stomach include mucous cells that produce a protective mucous layer, parietal cells that secrete hydrochloric acid and chief cells that secrete the proteolytic precursor, pepsinogen. At the distal pyloric region, the stomach connects to the duodenum, the first segment of the small intestine (Ross, 1989).

The small intestine is comprised of the duodenum, jejunum and ileum, all of which are lined with a columnar epithelium that protrudes into the lumen along with the finger-like villi and invaginations demarcating the intestinal crypts. The intestinal epithelium has the fastest cell turnover in the body and therefore needs to be constantly renewed for proper function. Under normal circumstances the epithelium of the small intestine is renewed approximately every 5-6 days by proliferation of cells in the crypts and the upward migration of these cells into the villi as they terminally differentiate (except for the Paneth cells that migrate downward into the crypts). Intestinal stem cells are housed in the crypts and are able to repopulate all the cells in the intestinal epithelium (Figure 4).

Pluripotent stem cells are particularly important for the intestine due to its rapid rate of turnover. Intestinal stem cells divide to produce stem cells and/or transit-amplifying (TA) daughter cells. The daughter cells go on to divide and differentiate into the specific cells of the intestine(Lopez-Garcia et al., 2010). It is believed that each crypt contains approximately six independent stem cells. To identify these potential stem cell populations label retention studies were initially performed. Two different populations of stem cells were proposed using this method: (i) the stem cells that reside at the +4 position relative to the bottom of the crypt and (ii) the crypt based columnar (CBC) cells, which are small undifferentiated

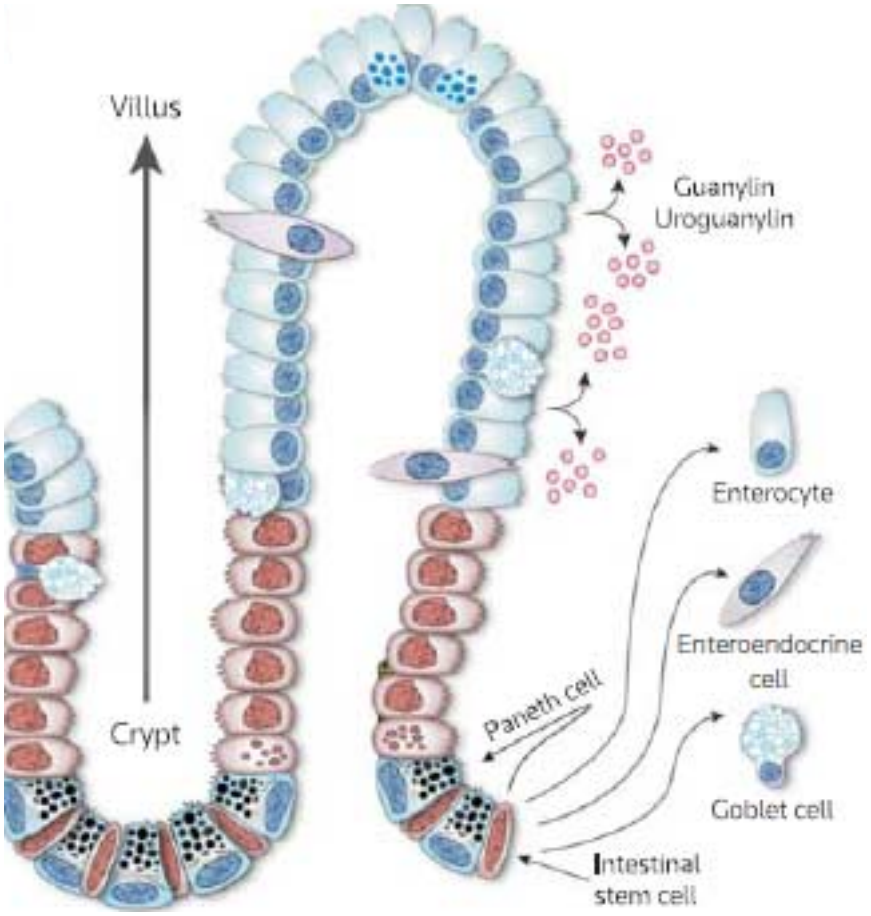

Figure 4: Schematic of a crypt-villous axis in the small intestine.
Depicting the types of cells found in the epithelium of the intestines: enterocytes, Goblet cells, Paneth cells, enteroendocrine cells and stem cells and their location along the crypt villous axis. Figure and legend adapted with permission from Li, et al, 2009. (Li et al., 2009)

cells located between Paneth cells (Cheng and Leblond, 1974; Potten et al., 1974). Both groups of proposed stem cells were able to retain DNA-labelling in the small intestine. However, until recently, definitive identification of intestinal stem cells was not possible due to lack of appropriate markers. The G-protein coupled orphan receptor *Lgr5,* a Wnt target gene, was found to be expressed in the CBC cells. Subsequent labelling and lineage tracing experiments revealed that Lgr5-positive CBC cells are able to give rise to all of the differentiated cell types of the intestine (Barker et al., 2007; Barker et al., 2008). Furthermore, *in vitro* culturing of a single Lgr5 positive cell was able to produce an organoid that recapitulated a crypt-villus axis (Sato et al., 2009; Sato et al., 2011; Sato et al., 2011). Another marker, Bmi1, expressed in the +4 population of proposed stem cells, was suggested to mark a second distinct population of stem cells in the intestine (Sangiorgi and Capecchi, 2008). Further studies have revealed that Lgr5 cells produce the *Bmi1* cells (Munoz et al., 2012), suggesting that Lgr5 CBC cells are the actual stem cells of the intestine.

The differentiated intestinal epithelium is mainly composed of four different cell types: adsorptive enterocytes (which are the predominant cell type) and the three secretory cell types (goblet, enteroendocrine and Paneth cells; Figure 4). Enterocytes make up more then 80% of all intestinal epithelial cells. These cells are highly polarized with an apical brush border that is responsible for the absorption and transportation of nutrients across the epithelium (Ross, 1989). Goblet cells secrete protective mucins and tre-foil proteins that are necessary for the movement and excretion of the gut contents and provide protection against damage. The proportion of goblet cells increases along the A-P axis of the intestines from 4% in

the duodenum to 16% in the distal colon (Karam, 1999). Markers of goblet cells include *Muc2*, *TFF3, Gfi1* and *Klf4* (Katz et al., 2002; Podolsky et al., 1993; Shroyer et al., 2005). Enteroendocrine cells are a rare cell type comprising approximately 1% of the intestinal epithelium(Schonhoff et al., 2004). These cells aid in gut function through secretion of specific peptide hormones, and are marked by Neurogenin3 (Ngn3) (Jenny et al., 2002). Paneth cells, unlike their other terminally differentiated counterparts, reside at the base of the crypts and their life expectancy is approximately three weeks (Bjerknes and Cheng, 1981). Paneth cells have a function in innate immunity and contain large secretory granules that house specialized proteins including lysozymes, antimicrobials and defensins. Sox9 plays an important role for the differentiation of Paneth cells in the intestinal epithelium (Bastide et al., 2007; Mori-Akiyama et al., 2007). Due to their proximity to stem cells, Paneth cells appear to be an important part of the *Lgr5* stem cell niche (Sato et al., 2011), though they are not absolutely required (Kim et al., 2012).

The most distal portion of the intestine consists of the cecum, the colon and the rectum. Compared to the small intestine, the colon has deeper crypt invaginations and a flat epithelial surface with no villi. The most abundant cell type are the absorptive cells, termed colonocytes in the colon, which mainly function to absorb water. As indicated earlier, the percentage of goblet cells increase distally, with a role in secreting lubricating mucous, as well as establishing a chemical barrier against pathogens (Ross, 1989). Conversely, Paneth cells decrease in number in the proximal colon and have been reported distally only in pathological states (Tanaka et al., 2001).

Signaling pathways important for intestinal differentiation

Wnt signaling

One of the pathways implicated in intestinal morphogenesis and differentiation is the Wnt (wingless) signaling cascade. Membrane Frizzled receptors are bound by Wnts, which are secreted lipid-modified glycoproteins, ultimately impacting on a variety of cellular events including cell fate determination and migration (Westendorf *et al.*, 2004). The canonical Wnt signaling cascade regulates gene expression through the stabilisation and subsequent nuclear localisation of β-catenin. In the absence of a Wnt ligand, β-catenin is phosphorylated on specific serine residues by a destruction complex comprised of the scaffolding protein Axin, the tumor suppressor gene Adenomatous Polyposis Coli (APC) and Glycogen Synthase Kinase 3-β (GSK3-β), leading to its ubiquitination and degradation. In this state, T-cell factor/Lymphoid enhancing factor (TCF/LEF) nuclear complexes recruit co-repressors to Wnt target gene promoters. However when a Wnt ligand binds to Frizzled, a signal is transduced resulting in inhibition of the destructor complex and stabilization of β-catenin which subsequently translocates to the nucleus where it associates with TCF/LEF members, displacing repressors and leading to transcriptional activation of Wnt target genes (Figure 5).

In the intestine Wnt signaling plays important roles in maintaining cellular proliferation in the crypts, differentiation of Paneth cells, and in directing the migration of epithelial cells along the crypt-villus axis. Wnt activity in the intestine is expressed as a gradient being maximal at the base of the crypts. A number of studies have examined the effects of deleting or inactivating downstream effectors

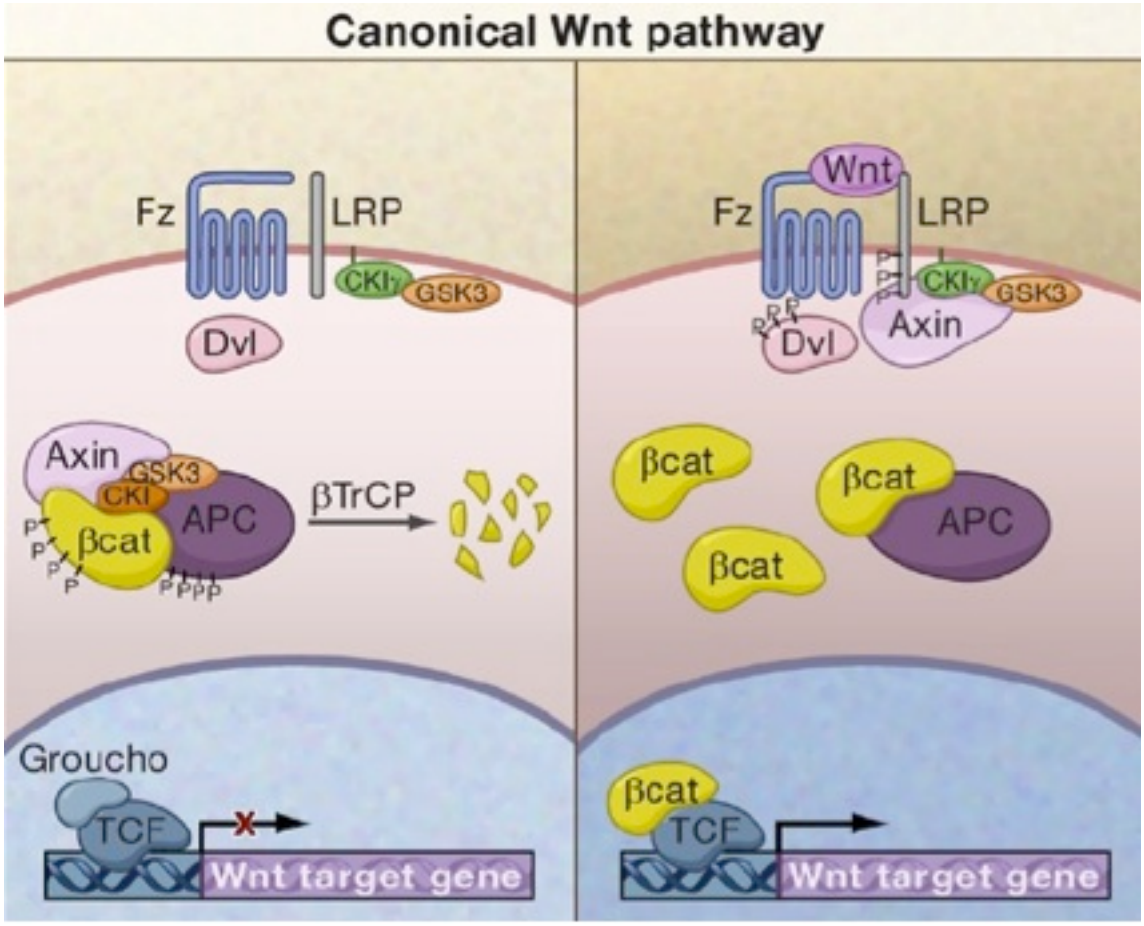

Figure 5: Canonical Wnt signaling pathway.
In the absence of ligand, β-catenin is sequestered by the APC complex and targeted for degradation by phosphorylation. In the nucleus, repressors of the Groucho family are associated with the TCF/LEF transcription factors and inhibit transcription. In the presence of Wnt ligand, the Frizzled receptor signals through Dishevelled (Dvl), resulting in lack of β-catenin phosphorylation, cytoplasmic accumulation and translocation of β-catenin to the nucleus. β-catenin can then associate with TCF/LEF members resulting in transcription of Wnt target genes. Figure and legend adapted with permission from Clevers et al, 2006. (Clevers, 2006)

or targets of the Wnt pathway in the intestine. Deletion of TCF4 results in the loss of the proliferative compartment in the crypts accompanied by death shorty after birth in mice (Korinek et al., 1998). Conditional deletion of β-catenin in the intestinal epithelium also resulted in the loss of the proliferating crypt cells and death from intestinal failure (Fevr et al., 2007; Kuhnert et al., 2004). Paneth cell differentiation is incomplete in the absence of Frizzled 5 (Fz5) (van Es et al., 2005), while deletion of Sox9, a Wnt target gene, results in a complete lack of Paneth cells and a reduction in goblet cells (Bastide et al., 2007).

Upregulation of the Wnt pathway by inactivating mutations to APC or its deletion causes an expansion of the proliferating compartment and villi shortening. Long-term loss eventually leads to development of adenomatous polyps, which are the precursors of colorectal cancer in humans (Andreu et al., 2005; Sansom et al., 2004). The Min ($APC^{min/+}$) mice harbors inactivating APC mutations and are common experimental models for colorectal cancer. Additionally, overexpression of Dickkopf-1 (Dkk1), a specific negative regulator of the Wnt pathway, caused a reduction in crypt size, villi length as well as a decrease in cells of the secretory lineage (Kuhnert et al., 2004; Pinto et al., 2003). Taken together, these observations suggest that Wnt signaling has a major role in the maintenance of the intestinal crypts and proliferation as well as in differentiation of the secretory cell lineages.

Notch signaling

The Notch pathway affects spatial patterning and cell fate decisions of neighbouring cells. Activation of the signaling cascade requires a Notch ligand (Jagged 1 and 2, and Delta-like 1, 3, and 4) to bind to a transmembrane receptor

(Notch 1-4) between two adjacent cells. Ligand-receptor binding initiates proteolytic cleavage of the receptor by γ-secretase releasing the Notch intracellular domain (NICD) from the membrane. The NICD then translocates to the nucleus where it binds with RBP-Jκ on Notch target gene promoters and increases their transcription (Scoville et al., 2008)(Figure 6). The best-characterized transcriptional targets of Notch include the *Hes* and *Hey* family genes. Both the *Hes* and *Hey* families function as transcriptional repressors with a basic helix-loop-helix (bHLH) domain, regulating progenitor cell fate in multiple tissues. Several intestinal Notch targets, including *Hes1, Hes5, Hes6, Sox9*, *Dkk2* and *Olfm4*, are also critically involved in stem cell renewal or lineage decision.

In the intestine Notch ligands and receptors are almost exclusively expressed in the adult intestinal crypt and TA cells, suggesting a role in maintaining the stem cell niche, proliferation and cell fate decisions. Continuous Notch activation in the intestine of the developing embryo results in an increase in the population of proliferative progenitor cells (Fre et al., 2005). Inhibition of Notch specifically in the crypt based columnar cells shows loss of proliferative ability (VanDussen et al., 2012). In contrast, disruption of Notch signaling shows the importance of Notch for intestinal cell fate decisions. For example, conditional ablation of RBP-Jκ results in a dramatic increase in the number of secretory cells with a concomitant decrease in absorptive cells (van Es et al., 2005). Similarly, deletion of Dll1, a Notch ligand, also shows a shift in goblet cell numbers (Pellegrinet et al., 2011). Inhibition of Notch cleavage by a γ-secretase inhibitor results in the conversion of the proliferative crypt cells into post-mitotic goblet cells (van Es et al., 2005). All of these

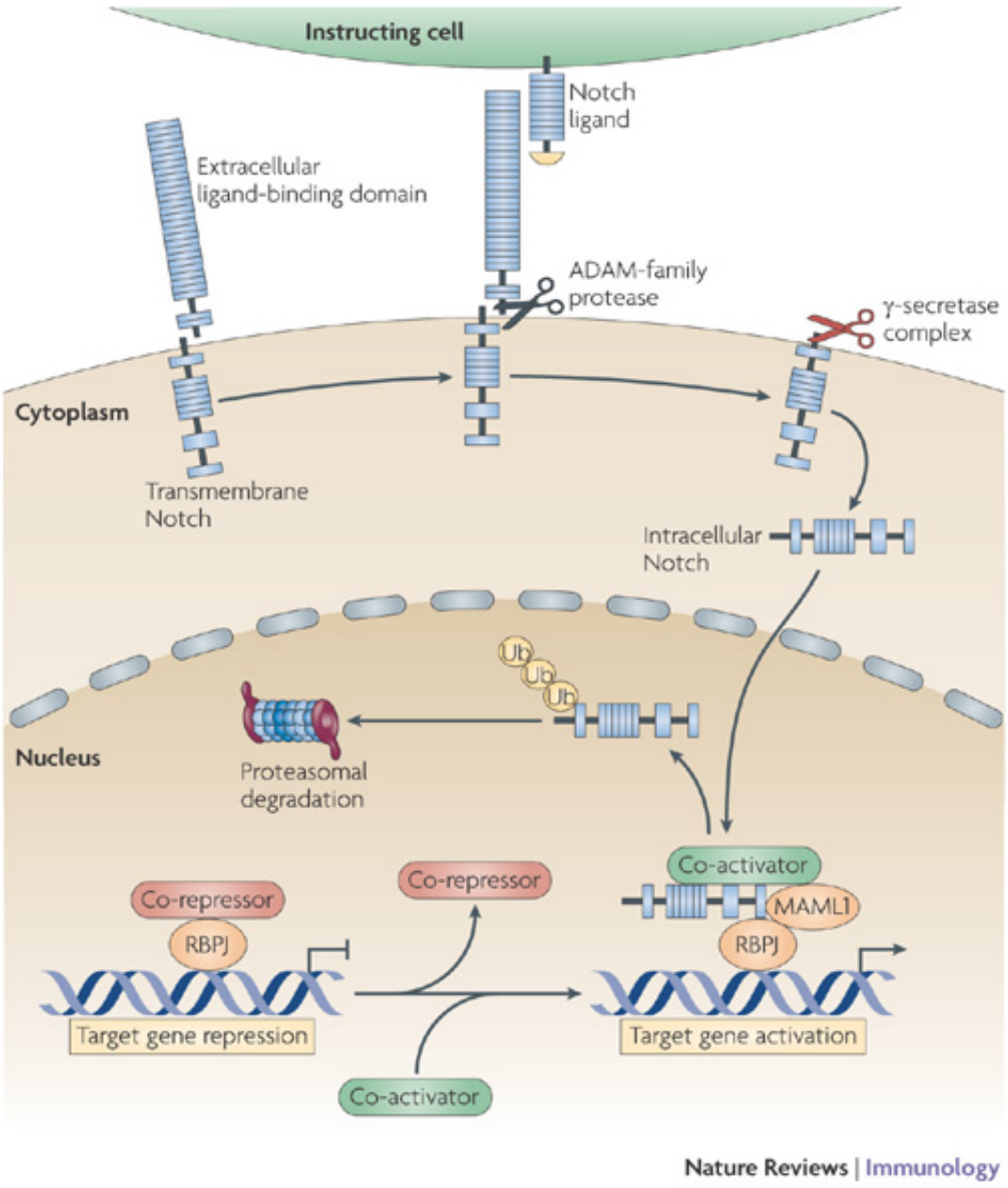

Figure 6: The Notch Signaling Pathway
Signaling is initiated by the interaction of Notch with Delta-like ligands (DLLs) or Jagged ligands on the surface of instructing cells. Following ligand binding, sequential cleavages occur in the transmembrane region of Notch. The final cleavage results in the release of the Notch intracellular domain (NICD) from the plasma membrane and its translocation to the nucleus, where it binds to recombination-signal-binding protein for immunoglobulin- J region (RBPJ) transcription factors that are bound to the promoters of Notch target genes. Figure and legend adapted with permission from Amsen et al, 2009. (Amsen et al., 2009)

observations suggest that Notch signaling is important for the homeostasis of the intestinal epithelium.

Eph-ephrin signaling

Similar to Notch, Eph-ephrin signaling is propagated by bidirectional signaling between neighbouring cells, one expressing an Eph receptor and the other cell expressing an ephrin ligand. The ephrin ligands as well as their receptors are grouped into two subclasses: type A and B ephrin ligands and EphA and EphB receptors. Usually, EphA receptors bind ephrinA ligands and EphB receptors to ephrinB ligands, however, promiscuity between the two groups has also been observed. For example, EphA4 binds both classes of ligands and ephrin-A5 binds both EphA and EphB2 receptors (Himanen et al., 2004; Pasquale, 2004).

In the intestine, Eph receptors and ephrin ligands are expressed in an opposite gradient along the crypt villus axis (Figure 7). EphB2 is expressed by the Lgr5+ stem cells as well as the proliferating TA cells (but not in the Paneth cells) with expression decreasing towards the top of the crypt. EphrinB1 and ephrinB2 are expressed in the terminally differentiated cells of the intestine, with expression being the highest at the crypt-villus junction. In the intestine, eph-ephrin interactions work via a repulsive mechanism to guide cells up the villi away from the crypts as they differentiate. The exception is the Paneth cells that express EphB3, and which are cued to remain in the base of the crypt. Deletion of EphB2 in adult mice causes a mislocalization of progenitor cells in the crypts in the small intestine and a reduced proliferation in the colon (Holmberg et al., 2006). Loss of EphB3 prevents Paneth cells from migrating downwards causing them to be found

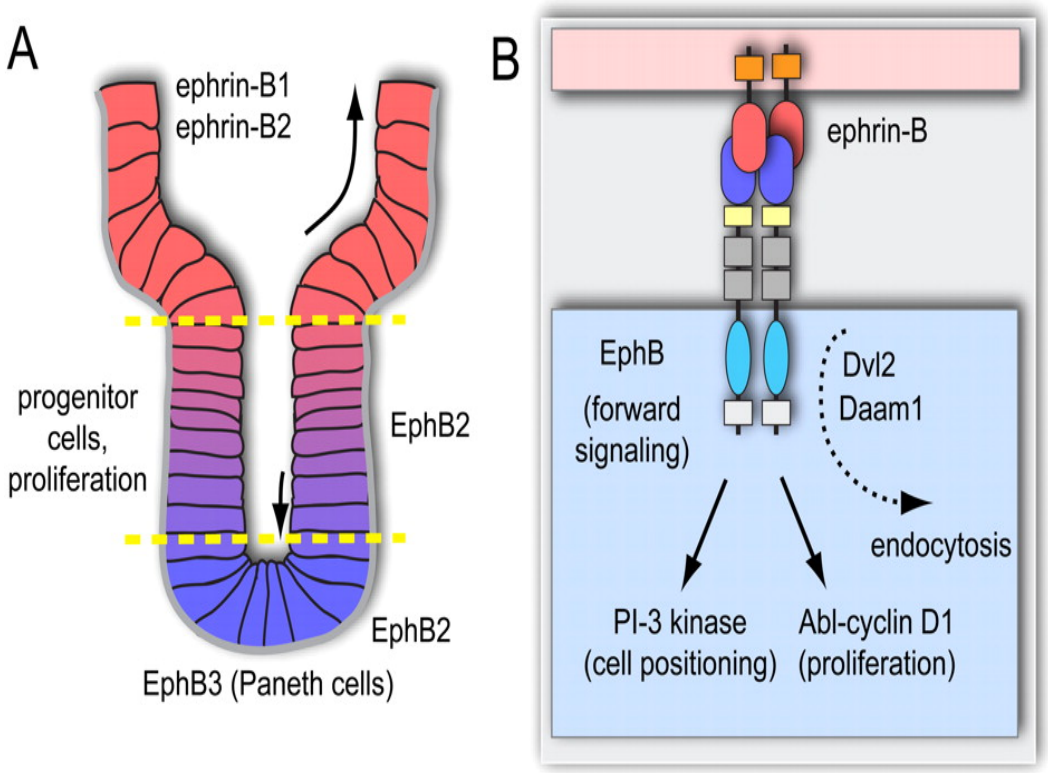

Figure 7: Eph-ephrin signaling in the intestine.
(A) EphB2-positive stem and progenitor cells are located near the bottom (lower dashed line) of the intestinal crypts, while ephrin-B1/ephrin-B2-positive differentiated epithelial cells are concentrated at the crypt–villus boundary (upper dashed line). In the small intestine, EphB3-expressing Paneth cells are found at the crypt bottom. (B) Intestinal cell sorting and positioning involves PI3K activity downstream from Eph/ephrin signaling. Cell proliferation is promoted by Abl kinase and cyclin D1. Figure and legend adapted with permission from Pitulescu and Adams 2010. (Pitulescu and Adams, 2010)

throughout the crypt-villus axis. Similarly, Paneth cells are mislocalized upon loss of ephrinB1, which also has a role in suppressing colorectal cancers by compartmentalizing hyperproliferative EphB2 positive cells (Cortina et al., 2007).

Tgf-β signaling

Another important pathway involved in development and maintenance of the intestinal epithelium is the TGF-β signaling pathway. The TGF-beta superfamily signals through serine/threonine kinase receptors (TBRI and TBRII) to specific intracellular mediators known as Smad proteins (Roberts, 1999; Shi and Massague, 2003). Smad proteins are classified into three functional groups: (i) receptor activated Smads (R-Smads): Smad1, Smad2, Smad3, Smad5 and Smad8; (ii) co-mediator Smads (co-Smads): Smad4; and (iii) inhibitory Smads (I-Smads): Smad6 and Smad7. R-smads and co-smads are primarily cytoplasmic until activated which occurs via phosphorylation by a TGF-beta receptor. Adaptor proteins, such as SARA and ELF, facilitate the phosphorylation of R-Smads by the receptors to modulate their activity (Itoh et al., 2002; Runyan et al., 2009; Tang et al., 2003). Once phosphorylated R-Smads join with Co-Smads to translocate into the nucleus where they enhance transcription of target genes. Among the genes activated by the TGF-beta signaling pathway are the I-Smads, which are part of the negative feedback mechanism to control TGF-beta signaling (Nakao et al., 1997; Nakao et al., 2002)(Figure 8).

A number of studies demonstrate a prominent role for the TGF-beta pathway in gastrointestinal cell progenitor formation, differentiation of intestinal cells and it has been found that disruption in proper TGF-beta signaling can result in cancer.

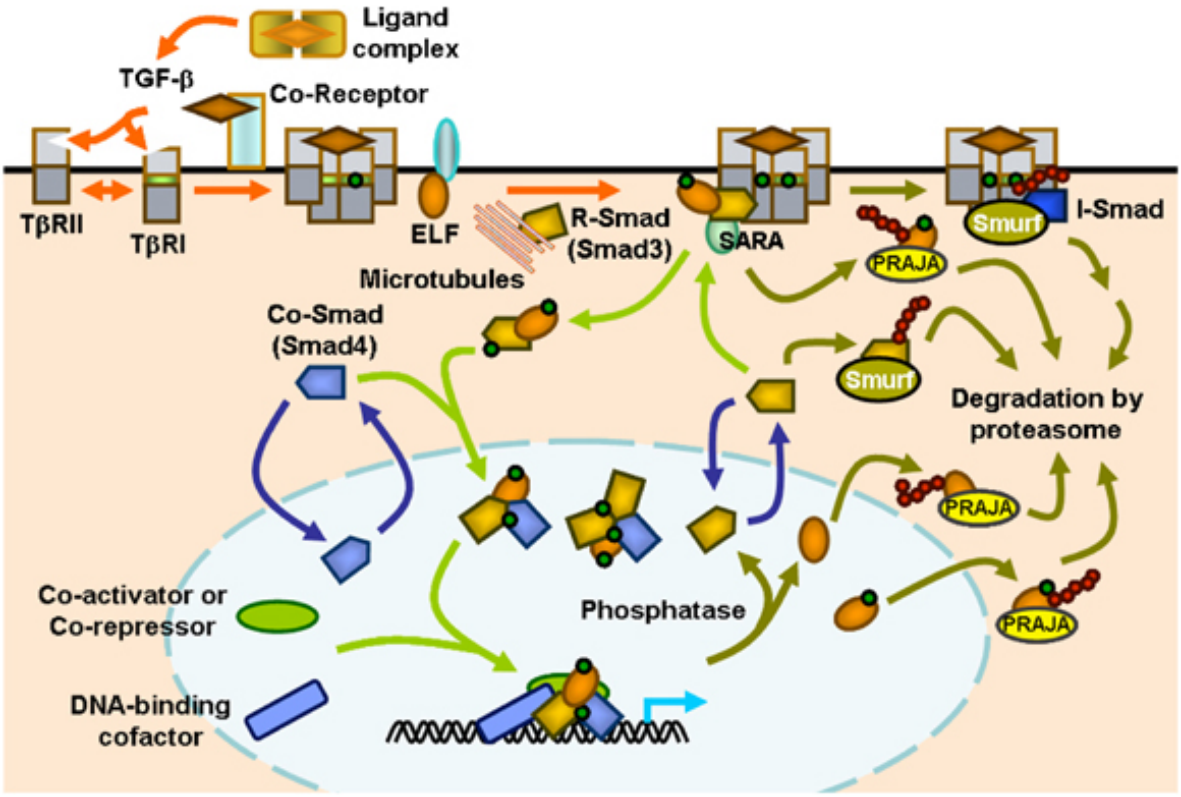

Figure 8: TGF-β signaling
TGF- binds to serine threonine kinase receptor complexes that phosphorylate R-Smads as well as adaptor proteins such as SARA and ELF. R-Smads and Smad4 form a heteromeric complex and translocate to the nucleus, activating target genes. Arrows indicate signal flow and are colour coded: red for ligand and receptor activation, olive green for Smad and receptor inactivation, light green for Smad activation and formation of a transcriptional complex, and blue for Smad nucleocytoplasmic shuttling. Phosphate groups and ubiquitin are represented by green and red circles, respectively. Figure and legend adapted with permission from Mishra et al., 2005. (Mishra et al., 2005)

For example, abnormalities in gut epithelial morphology have been observed in *elf*$^{-/-}$ mutants and include severely flattened epithelium with loss of villi (Tang et al., 2003; Tang et al., 2005). TGF- beta II receptors have been localized to both undifferentiated cells of the crypt and differentiated cells of the intestinal villi (Barnard et al., 1989; Koyama and Podolsky, 1989), while Smad4 and ELF expression has been observed in the crypts and the tips of the villi (Tang et al., 2005). Due to positional expression it was hypothesized that TGF-beta signaling in the crypts may be important for stem cell progression to progenitor cells. To this end, it has been shown that TGF-beta signalling, in the absence of Wnt signalling, promotes a rapid cell cycle arrest and differentiation in the villus compartment (Tang et al., 2005). Also, the TGF-beta target gene *p21*$^{cip1/waf1}$ can induce differentiation in colorectal cancer cells when stimulated by TGF-beta (Rorke et al., 2000).

The role of TGF-beta signaling has also been investigated in the context of colorectal cancer. For example, Smad3 mouse mutants develop spontaneous metaplastic lesions in the distal colon (Zhu et al., 1998), while disruption of Smad2 or Smad4, together with loss of APC, results in increased tumor growth and invasion (Alberici et al., 2006; Hamamoto et al., 2002; Takaku et al., 1998). Thus, Smads 2, 3 and 4 have been deemed as tumor suppressors in colorectal cancer.

Cdx proteins and the intestine

Mammalian Cdx genes

Homeodomain transcription factors play crucial roles in many developmental processes. A number of homeodomain proteins also play critical roles in the adult,

and alterations in their function are linked to a number of disorders, including metabolic diseases and cancers (McGinnis and Krumlauf, 1992). The homeobox gene *caudal* was originally identified in *Drosophila*. *Caudal (cad)* exhibits a posterior high gradient of expression in the gut, gonads and genital disc and is important for anterior-posterior (A-P) patterning in *Drosophila* (Macdonald and Struhl, 1986; Mlodzik et al., 1985; Mlodzik and Gehring, 1987). Homologues of *cad* have been subsequently identified in zebrafish, mice and humans. Of the three murine homologues *Cdx1*, *Cdx2* and *Cdx4,* only *Cdx1* and *Cdx2* are expressed in the intestinal epithelium (James and Kazenwadel, 1991).

In the embryo proper, *Cdx1* and *Cdx2* display a biphasic pattern of expression characterized by early transcription in the tail bud in all three germ layers, with later expression confined to the intestinal epithelium which persists throughout the life of the animal (Hierholzer and Kemler, 2009). In the adult the expression of both *Cdx1* and *Cdx2* increases along the A-P axis in the small intestine, while in the colon, the level of *Cdx1* increases posteriorly, with *Cdx2* expression being maximally higher in the proximal region (James et al., 1994). Cdx1 and Cdx2 are also expressed along the crypt-villus axis, with *Cdx1* expression decreasing towards the tip of the villi; Cdx2 does not vary along this axis although it is differentially phosphorylated (Figure 9).

Structure of Cdx

Cdx1 and *Cdx2* genes extend over 40 and 7 kb, respectively, and are composed of three exons with the DNA binding homeodomain encoded primarily in the second exon. The Cdx1 and Cdx2 proteins are very conserved within the homeodomain, but

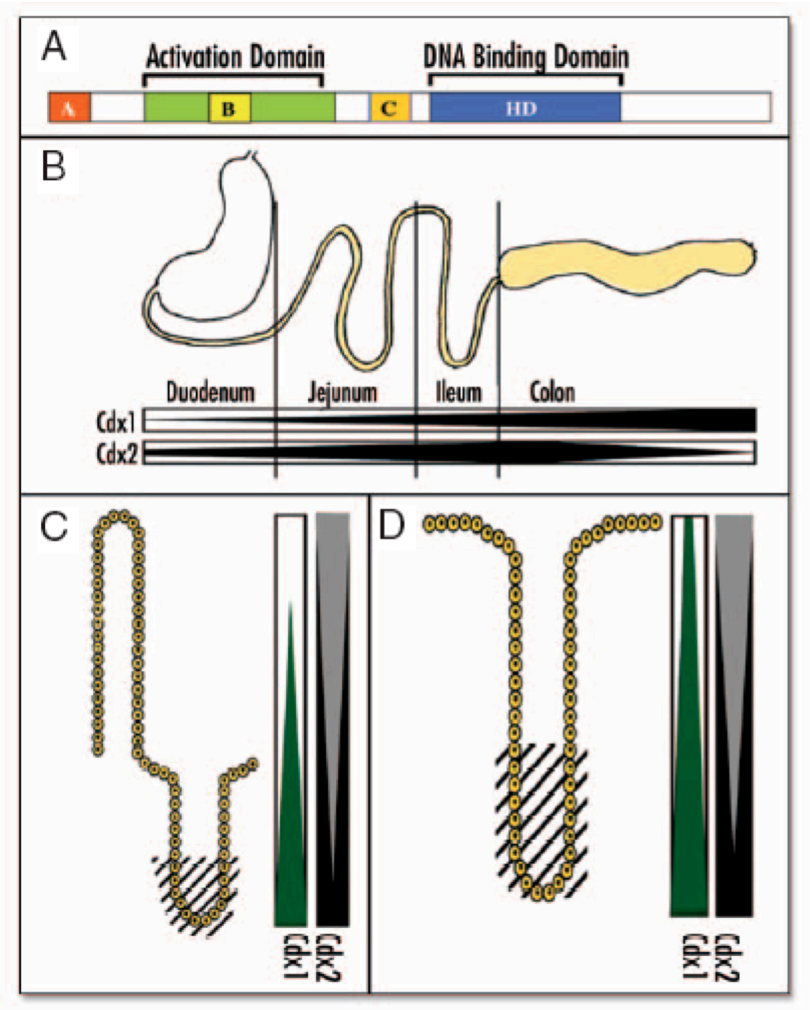

Figure 9: Conserved domains between Cdx proteins and *Cdx* expression along the anterior-posterior and crypt-villus axes.
Box A shows the conserved domains A, B and C between Cdx1 and Cdx2 along with the homeodomain (HD). Box B shows the expression levels. Boxes C and D show expression along the crypt-villus axis in the small intestine and colon, respectively. Note that Cdx2 expression is unchanged and the gray triangle represents the differential phosphorylation. The hatched areas represent the proliferating zones in the crypts. Figure and legend adapted with permission from Guo et al., 2004. (Guo et al., 2004)

are otherwise of limited similarity (Figure 9). In *Drosophila* and mice, a canonical Cdx response element (CDRE) has been identified with the sequence of TTTATG which is recognised by all Cdx members (Dearolf et al., 1989). Variations of the canonical CDRE have also been documented to impact the protein-DNA interaction (Margalit et al., 1993). Cdx proteins are transcriptional activators of their target genes, however there is some evidence to suggest that Cdx's may repress some targets (Chun et al., 2007; Faber et al., 2013; Wang et al., 2010).

Cdx mutant mice

Previous studies of *Cdx* knockout mice have yielded informative observations as to the function of *Cdx1* and *Cdx2*. *Cdx1* null mice exhibit homeotic transformations of the axial skeleton (Subramanian et al., 1995) but do not exhibit an overt intestinal phenotype; $Cdx1^{+/-}$ mice develop normally. *Cdx2* null mice are embryonic lethal and die at embryonic day 3.5 due to implantation failure, preventing analysis of the null phenotype at later stages. However, *Cdx2* heterozygotes display vertebral defects similar to those seen in *Cdx1* null mice (Beck et al., 1999). In contrast to Cdx1 null mutants, *Cdx2* heterozygous mice have an intestinal phenotype characterized by lesions in the pericaecal region and proximal colon (Beck et al., 1999; Chawengsaksophak et al., 1997). These lesions exhibit regions of epithelium reminiscent of the entire gastrointestinal tract, including stratified keratinized esophageal, glandular stomach, and small and large intestine. Other studies have mis-expressed Cdx1 or Cdx2 in the stomach or the esophagus, which resulted in a conversion of the epithelium to small intestinal epithelium (Kong et al., 2011; Mutoh et al., 2002; Mutoh et al., 2004; Mutoh et al., 2009; Silberg

et al., 2002). It is also interesting to note that, in Barretts Esophagus, the distal esophageal epithelium undergoes a fate switch from a stratified squamous mucosa to an intestinal phenotype which occurs concomitant with ectopic Cdx2 expression (Colleypriest et al., 2009). Taken together, these findings suggest a critical role for Cdx2 in A-P intestinal patterning.

The embryonic lethality of the *Cdx2* null mice necessitated derivation of a conditional knock out model, which was generated by our laboratory using the Cre-lox system (Figure 10). This system makes use of Cre recombinase of the P1 bacteriophage which catalyzes recombination between two 34-bp loxP sites. Two loxP sites in *cis* results in Cre-mediated recombination removing the intervening DNA sequence. In the mouse, there are neither endogenous LoxP sequences, nor any Cre-like recombination activity, thus the excision event is completely dependent on the expression of an exogenous Cre transgene.

The conditional *Cdx2* knockout model makes use of the Cre/*loxP* system in which exon two of *Cdx2* is flanked by *loxP* sites. Loss of this domain was predicted to result in a functionally null protein as exon two encodes the Cdx2 DNA binding domain. The Cre recombinase used for these studies is fused to a modified estrogen receptor ligand binding domain that responds exclusively to tamoxifen (ER^T), and is believed to be sequestered in the cytoplasm by Hsp90 in the absence of ligand. Tamoxifen administration results in the release of the Hsp90 protein and subsequent translocation of the Cre-ER^T fusion protein to the nucleus, where it excises the *Cdx2* sequences within the *loxP* sites. To excise Cdx2 specifically in the

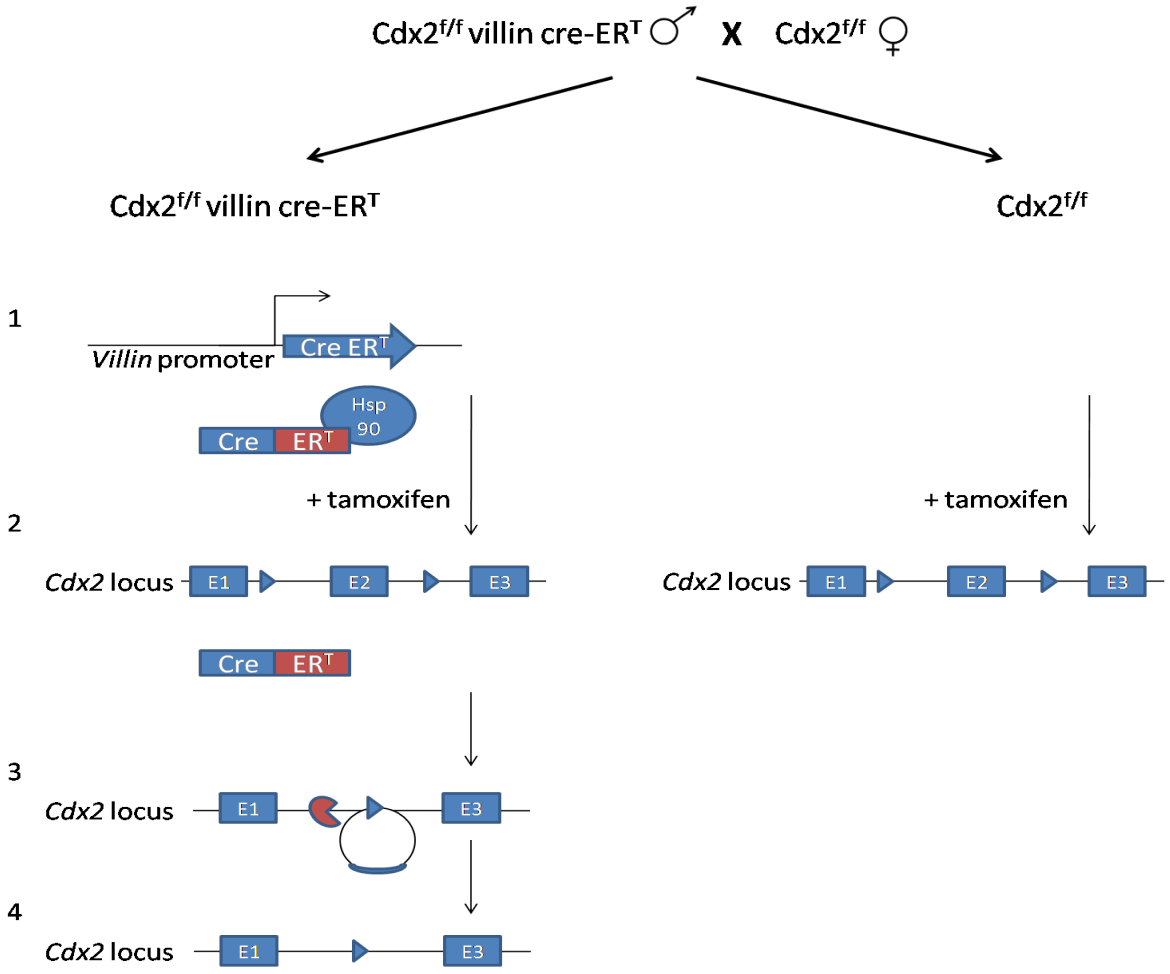

Figure 10: Cre-lox based knockout strategy.
A Cdx2$^{f/f}$ villin cre-^{ERT} male is bred with a Cdx2$^{f/f}$ female to produce offspring with or without villin cre in a Mendelian predicted 1:1 ratio. [1] Cre is transcribed under the spatial control of the villin promoter. The Cre protein is fused to a modified estrogen receptor ligand binding domain (ER) that will only respond to tamoxifen. In the absence of tamoxifen, association with Hsp90 results in cytoplasmic sequestration of the chimeric protein. [2] The Cdx2 locus is flanked by two loxP (location of cross-over) sites, which are not found in mammalian genomes. Adult mice are given a dose of tamoxifen to release Hsp90 from the Cre-ERT fusion protein, resulting in the translocation of the Cre fusion protein to the nucleus. [3-4] The Cre protein recognizes the loxP sites, and a recombination event occurs, resulting in the excision of critical Cdx2 sequences. Similarly, Cdx1$^{-/-}$Cdx2f$^{/f}$ females can be bred with Cdx1$^{-/-}$Cdx2$^{f/f}$ villin cre-^{ERT} males to produce double mutants.

developing and adult intestines the Cre transgene is driven by the intestinal-specific *villin* promoter.

Several groups have used conditional Cdx2 knockout approaches to help elucidate the role of Cdx2 in the developing intestine. Loss of *Cdx2* from the early definitive endoderm results in an anterior transformation of the small intestinal epithelium to an esophageal fate caused by ectopic activation of the foregut/esophageal differentiation program (Gao et al., 2009). Similarly, our group disrupted Cdx2 expression in the endoderm at later stages, alone or in conjunction with knockout of Cdx1, which led to transformation of the intestinal tissue to a partial gastric identity (Grainger et al., 2010). Loss of Cdx2 in the endoderm also leads to defects in epithelial cell polarity (Gao and Kaestner, 2010). These studies suggest that Cdx2 is necessary for posteriorization of the endoderm to an intestinal identity. The importance of Cdx transcription factors in the adult intestinal epithelium, however, has not yet been investigated.

Functional overlap and specificity of Cdx members

Due to overlapping expression domains of Cdx1 and Cdx2 in the adult intestine, it is possible that there may be some functional overlap between these two family members. In this regard, several groups have used tissue culture models to investigate functional specificity with conflicting results. For example Cdx2 can more readily transactivate the expression of the intestinal *apical sodium-dependent bile acid transporter (ASBT)* (Ma et al., 2012). In Caco-2 cells, Cdx1 activates the *intestinal alkaline phosphatase* (*IAP*) gene while Cdx2 did not (Alkhoury et al., 2005), thus demonstrating differential activation of target genes by the Cdx family

members. However, in other contexts, Cdx1 and Cdx2 appear to act comparably on target genes. For example, intestinal genes *solute carrier family 5, member 8 (SLC5A8)* and *DNA replication-related element binding factor (DREF)* both have been identified as Cdx target genes that are comparably activated by both Cdx1 and Cdx2 (Kakizaki et al., 2010; Park et al., 2011).

Functional redundancy between Cdx family members has also been assessed in animal models. Compound mutant studies revealed that in the developing small intestine the additional loss of Cdx1 in a Cdx2 mutant background did not play a critical role in development, at least after E13.5 (Grainger et al., 2010). This is in stark contrast to the axial defects of Cdx1 or Cdx2 conditional mice which are compounded by loss of both Cdx members (Savory et al., 2009; Savory et al., 2011). A "gene swap" mouse model, wherein the endogenous *Cdx1* transcript was replaced by *Cdx2* cDNA has also been used to assess functional similarity between Cdx family members and revealed that Cdx2 can fully compensate for loss of Cdx1 in axial patterning (Savory et al., 2009). Conversely, Cdx2 could not fully substitute for the Cdx1 in the developing intestine (Grainger et al., 2013). Taken together, these observations suggest that, in the intestine, there is the potential for both functional overlap of the Cdx transcription factors as well as context-dependent transcriptional specificity.

Intestinal cancer and Cdx

Colorectal Cancer

As a result of its high turnover rate, the intestinal epithelium is frequently subject to malignant transformation leading to colorectal cancers (CRC), which are

one of the top three cancer-related causes of death worldwide (Siegel et al., 2012). Under normal conditions, the Wnt pathway plays a major role in the self-renewal process of the intestinal epithelium and disruptions to this pathway are associated with the development of CRC, with activating mutation in the Wnt pathway underlying the majority of CRC cases (Fodde and Brabletz, 2007). Inactivating mutations to the *Adenomatous polyposis coli (APC)* gene are an early causative event in most cases of sporadic intestinal cancer and also underlie the familial adenomatous polyposis (FAP) syndrome in humans (Nathke, 2004; Powell et al., 1992). Other contributing mutations in the Wnt pathway that can give rise to CRC are loss of the tumor suppressor Axin2 (Liu et al., 2000) or activation of the proto-oncogene β-catenin (Morin et al., 1997; Rubinfeld et al., 1997). Common to all of these mutations is accumulation of β-catenin in the nucleus where it constitutively increases transcription of Wnt target genes leading to hyperproliferation and the appearance of adenomatous polyps which are predisposed to malignant transformation to carcinoma (Korinek et al., 1997; Morin et al., 1997).

Fearon and Vogelstein have proposed that CRC follows an ordered sequence of mutations termed the adenoma-carcinoma sequence (Fearon and Vogelstein, 1990). They propose that the initiating mutation occurs in the Wnt pathway, leading to the formation of long-lived benign adenomas. The transition from benign adenomas to metastatic carcinomas proceeds as a result of subsequent mutations to other intestinal proto oncogenes and tumor suppressor genes such as Kras, Smad4 and p53 (Fearon and Vogelstein, 1990)(Figure 11).

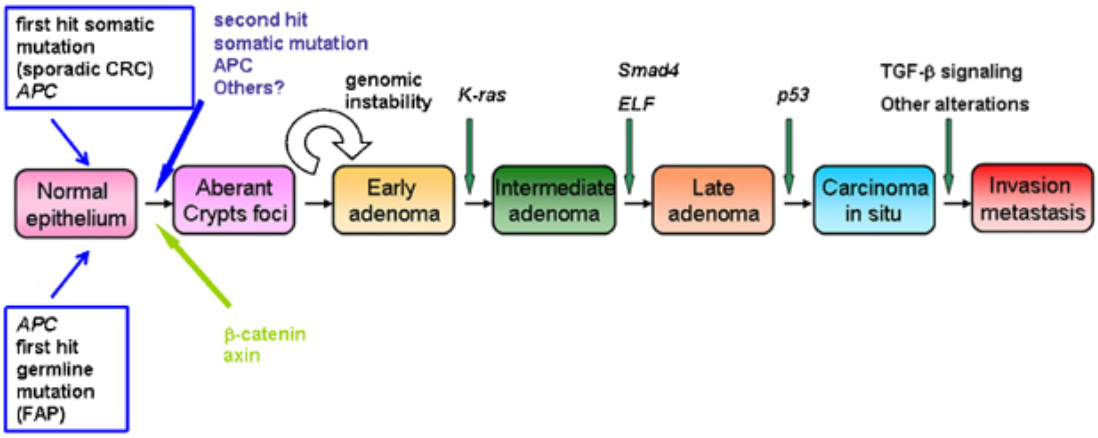

Figure 11: Fearon and Vogelstein's CRC adenoma-carcinoma sequence.
Correlation between CRC progression and the accumulation of genetic alterations according to Fearon & Vogelstein (1990). Figure and legend adapted with permission from Mishra et al., 2005. (Mishra et al., 2005)

Mouse Models of CRC

Mouse models activating the Wnt pathway have been a useful tool in the study of CRC. A mutagenesis study was the first to develop a mouse strain with hereditary intestinal adenoma formation (Moser et al., 1990). This strain was termed the multiple intestinal neoplasia (Min) mice which harbors a heterozygous inactivating mutation of the *Apc* gene. Due to spontaneous mutations to the remaining wild-type *Apc* allele in the intestinal epithelial cells, the Min mice develop multiple adenomas in the intestine within a few months (Su et al., 1992). Subsequently, several other *Apc* mutant models have been developed all of which lead to rapid accumulation of β-catenin in the nucleus with varying degrees of intestinal tumor formation (Gaspar and Fodde, 2004). Another approach that results in polyp formation in the mouse is an activating mutation of β-catenin. Conditional deletion of the third exon of β-catenin results in a protein that can no longer be phosphorylated and targeted for degradation, leading to constitutive transcription of Wnt target genes (Harada et al., 1999).

The above models have thus been used as the murine counterpart to the hereditary FAP in humans and extensively used for the study of CRCs. While they are very good models for adenoma formation there are some notable shortcomings: (i) compared to human adenoma formation which occurs almost exclusively in the colon, in mice the polyps preferentially form in the small intestine and pericecal areas and; (ii) murine polyps due to mutations in the Wnt pathway rarely progress to invasive carcinomas (Halberg et al., 2000; Moser et al., 1990). In comparison to the Fearon/Vogelstein hypothesis, additional mutations in conjunction with Wnt

disruption enhance the progression of murine adenomas to carcinomas as well as their distribution to the colon. Such combinations include activating mutations of KRas or lesions in the TGF-β signaling pathway in a Min mouse background (Hamamoto et al., 2002; Sansom et al., 2006; Takaku et al., 1998). These studies have yielded further knowledge of CRC progression in murine models that better recapitulate some aspects typical of human CRC.

Cdx in CRC

Cdx2 has also been implicated in the development of CRCs. For example, *CDX2* loss is observed in ~30% of colorectal cancers (Brabletz et al., 2004; Subtil et al., 2007) and this is correlated with high tumor grade, late stage disease and microsatellite instability (MSI) (Baba et al., 2009; Hinoi et al., 2001; Lugli et al., 2008). CDX2 loss is also associated with a significant increase in mortality in patients with a family history of colorectal cancer (Baba et al., 2009). Conversely, overexpression of CDX2 in human CRC cells induces a less malignant phenotype, inhibiting proliferation, invasion and migration, and increases expression of markers of enterocyte differentiation (Brabletz et al., 2004; Hinoi et al., 2003; Lorentz et al., 1997; Mallo et al., 1998). These observations have led to the suggestion that CDX2 functions as a tumor suppressor. In marked contrast, CDX2 has also been shown to be overexpressed in a subset of human colorectal cancers (Salari et al., 2012; Witek et al., 2005). The basis for these divergent observations is presently unknown, but implies that misregulation of Cdx2 in either context has an impact on the tumorigenic potential of colorectal cancer.

Based on the above observations, the impact of Cdx2 loss has been investigated in mouse models of colorectal cancers. Mice that are compound heterozygotes for *Cdx2* and *Min* develop more adenomatous polyps in the colon than their heterozygous littermates, however, these polyps are not distally located (Aoki et al., 2003). Furthermore, the incidence of polyps induced by azoxymethane in the colon is increased in Cdx2 heterozygous mice (Bonhomme et al., 2003). Interestingly, in colonic polyps of the $Apc^{+/\Delta 716}$ mutant mice, Cdx2 expression was also found to be reduced (Oshima et al., 1995). These results suggest that reduced levels of Cdx2 accelerate colonic tumorigenesis, however, whether an increase in tumor formation is due to Cdx2-dependent development effects or can be invoked by somatic deletion later in adulthood is yet to be determined.

Although Cdx1 is also present in the intestinal tract of adults, its role in CRC is less clear. Cdx1 expression is decreased or lost in the majority of Wnt or azoxymethane -induced adenocarcinomas (Domon-Dell et al., 2003; Pilozzi et al., 2004; Silberg et al., 1997). Similar to Cdx2, Cdx1 expression is downregulated or lost in a significant number of human colorectal cancer cell lines (Mallo et al., 1998; Vider et al., 2000). Loss of Cdx1 alone, however, has no impact on the frequency of intestinal tumors induced by azoxymethane or APC loss (Bonhomme et al., 2008). The lack of phenotype in Cdx1 mutant intestine may be due to functional compensation by Cdx2, which can be addressed using Cdx1 and Cdx2 compound mutants.

RATIONAL

The intestine, being one of the most rapidly renewable epithelia in the human body, needs strict control over its stem cell and differentiation programs to function normally over the lifetime of any individual. The mechanisms by which it regulates this function have not been fully elucidated. Since Cdx members are expressed exclusively in the intestines of adults, we sought to determine their role in the adult intestinal tract. It has been previously suggested that during development, Cdx members are involved in anterior patterning as well as cell differentiation. This may extend to roles in homeostasis of the adult intestinal tract. Moreover, although Cdx members have been implicated in the development of colorectal cancers, the means by which Cdx functions to impact the development of these cancers is poorly understood.

HYPOTHESIS

I hypothesize that *Cdx1* and *Cdx2* play critical, and potentially overlapping, roles in maintenance of the intestinal epithelium in the adult as well as in intestinal cancers.

OBJECTIVES

1) Characterize the effects of acute and chronic loss of Cdx2, or Cdx1 and Cdx2 on the adult intestine.

2) Identify the mechanisms by which Cdx1 and Cdx2 regulate the differentiation of the adult intestine.

3) Assess a role for Cdx1 and Cdx2 in an intestinal cancer model

CHAPTER 2: Cdx Function is Required for Maintenance of Intestinal Identity in the Adult

Cdx Function is Required for Maintenance of Intestinal Identity in the Adult

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Author contributions:

Alexa Hryniuk designed and carried out experiments, wrote the manuscript and made figures.

Stephanie Grainger assisted with generating experimental material.

Joanne G.A. Savory generated *Cdx2f/f* mice.

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

Abstract

The homeodomain transcription factors Cdx1 and Cdx2 are expressed in the intestinal epithelium from early development, with expression persisting throughout the life of the animal. While our understanding of the function of Cdx members in intestinal development has advanced significantly, their roles in the adult intestine is relatively poorly understood. In the present study, we found that ablation of *Cdx2* in the adult small intestine severely impacted villus morphology, proliferation and intestinal gene expression patterns, resulting in the demise of the animal. Long-term loss of *Cdx2* in a chimeric model resulted in loss of all differentiated intestinal cell types and partial conversion of the mucosa to a gastric-like epithelium. Concomitant loss of Cdx1 did not exacerbate any of these phenotypes. Loss of *Cdx2* in the colon was associated with a shift to a caecum-like epithelial morphology and a gain of cecum-associated genes which was more pronounced with subsequent loss of *Cdx1*. These findings suggest that Cdx2 is essential for differentiation of the small intestinal epithelium, and that both Cdx1 and Cdx2 contribute to homeostasis of the colon.

Introduction

Following specification, definitive endoderm undergoes extensive anterior-posterior (AP) patterning which is orchestrated by signaling molecules and transcription factors that are typically expressed in a tightly regulated spatial-temporal manner. This patterning yields the various specialized anatomical structures of the mature digestive tract, each with its own unique epithelial characteristics. For example, the esophagus becomes lined with a stratified keratinized epithelium, while the stomach exhibits a glandular epithelium with the exception of the stratified keratinized epithelium of the cardiac region. The small intestine is characterized by a simple columnar epithelial layer, which contains invaginations (the intestinal crypts) and villi that project into the intestinal lumen. The crypts are more pronounced in the large intestine, where a flat epithelial surface is found instead of the protruding villi (Wells and Melton, 1999; Zorn and Wells, 2009).

The intestinal epithelium is composed of the absorptive enterocytes, as well as the secretory goblet, enteroendocrine and Paneth cells. These lineages are derived from intestinal stem cells which are located at or near the base of the intestinal crypts (Li and Clevers, 2010; Wells and Melton, 1999). These progenitor cells initially yield a highly proliferative transit amplifying (TA) population that subsequently differentiates and migrates up the villi, where they are eventually shed, turning over every 5–6 days. The exception to this are the Paneth cells which migrate downwards to the base of the crypt where they reside with a lifespan of approximately 30 days (Lipkin, 1985).

Homeodomain transcription factors play crucial roles in diverse developmental processes as well in the adult, with alterations in their function being linked to a number of disorders including metabolic diseases and cancer (Jones, 2003; Samuel and Naora, 2005; Wigle and Eisenstat, 2008). The three murine *Cdx* genes (*Cdx1*, *Cdx2* and *Cdx4*) encode homeodomain transcription factors related to *Drosophila cad*. In the adult, Cdx1 and Cdx2 (but not Cdx4) are expressed to varying levels along the AP axis of the gut, with Cdx1 exhibiting a caudal-high gradient maximal in the distal colon while Cdx2 peaks in the proximal colon (James et al., 1994; Silberg et al., 1997).

Initial studies using germline knockout alleles yielded limited information as to the function of Cdx1 and Cdx2 in the adult intestine. *Cdx1* null mice exhibit homeotic transformations of the axial skeleton (Subramanian et al., 1995), but do not display any overt intestinal phenotype. *Cdx2* null mutants are peri-implantation lethal, however, *Cdx2* heterozygous mice survive and exhibit an intestinal phenotype characterized by metaplastic lesions in the pericecal region and proximal colon. These lesions appear devoid of Cdx2 expression and exhibit properties of more anterior gastrointestinal tissue, including esophagus and stomach, suggesting that *Cdx2* is required to posteriorize endoderm to an intestinal fate (Beck et al., 1999; Beck et al., 2003; Chawengsaksophak et al., 1997). This hypothesis is supported by more recent studies using conditional knockout approaches which demonstrate that loss of *Cdx2* from the early definitive endoderm results in an anterior transformation of the small intestinal epithelium to an esophageal fate (Gao et al., 2009), while disruption at later stages leads to transformation to a partial gastric

identity (Grainger et al., 2010) as well as defects in epithelial cell polarity (Gao and Kaestner, 2010). Conversely, ectopic expression of *Cdx2* in the postnatal stomach causes a transformation of the gastric epithelium to intestinal mucosa (Mutoh et al., 2011; Silberg et al., 2002; Tsukamoto et al., 2004). In addition, in Barrett's Esophagus, the distal esophageal epithelium undergoes a fate switch from a stratified squamous mucosa to an intestinal phenotype concomitant with gain of *Cdx2* expression (Colleypriest et al., 2009; Debruyne et al., 2006). Together, these findings suggest that Cdx2 plays an important role in patterning of endoderm and early intestinal epithelium to a posterior intestinal nature and that the adult gastrointestinal epithelium exhibits considerable plasticity with respect to Cdx2 gain-of-function.

Outside of the DNA-binding homeodomain, Cdx proteins exhibit little sequence similarity. However, the phenotype of compound Cdx mutants, as well as gene substitution models, indicate that Cdx members are functionally similar in regards to their role in developmental programs such as vertebral patterning (Savory et al., 2009; van den Akker et al., 2002; van Nes et al., 2006), hematopoiesis (Davidson and Zon, 2006; Wang et al., 2008) and axial elongation and neural tube closure (Savory et al., 2011). These observations suggest that Cdx1 and Cdx2 may also functionally overlap in the intestinal tract, offering a potential basis for the lack of a gut phenotype in *Cdx1* null mutants. Consistent with this, loss of Cdx1 in a Cdx2 null intestine has been reported to have a marked impact on epithelial proliferation (Verzi et al., 2010).

In the present study we used a tamoxifen-inducible Cre under the control of the intestinal epithelium-specific *villin* promoter (Grainger et al., 2010) to delete a floxed *Cdx2* allele (Savory et al., 2009) in the adult intestine alone or in a *Cdx1* null background (Subramanian et al., 1995). *Cdx2* or *Cdx1*-*Cdx2* deletion caused a rapid decline in health concomitant with loss of Cdx2 protein necessitating endpointing 6–7 days post-treatment. This was accompanied by a marked disruption of villus morphology and a decrease in the TA and stem cell compartments. This phenotype was comparable between the single and double mutants, suggesting that Cdx1 does not play a significant role in the adult small intestine in the absence of Cdx2. In the large intestine, loss of *Cdx2* elicited a transformation of the proximal colon epithelium to a cecum-like nature. Unlike the small intestine, this transformation increased in severity with subsequent loss of *Cdx1*, providing evidence for a novel role for Cdx function in the adult colon and a critical requirement for Cdx1 in this process. Finally, chimeric disruption of *Cdx2* was effected in order to observe the consequences of long-term loss of Cdx in the adult intestine. Using this approach, we provide novel evidence that Cdx2 is required for differentiation of all intestinal cell types.

Materials and methods

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

Cdx1$^{-/-}$, *Cdx2*$^{f/f}$ and *Villin*-Cre ER^{T} mice have been previously described (Grainger et al., 2010; Savory et al., 2009; Subramanian et al., 1995). To effect *Cdx2* deletion, *Cdx2*$^{f/f}$-*Villin*-Cre ER^{T} progeny were treated with 0.25 mg–1.0 mg of tamoxifen by oral gavage at approximately 2 months of age. *Cdx1-Cdx2* double mutants were similarly generated from *Cdx1*$^{-/-}$-*Cdx2*$^{f/f}$-*Villin*-Cre ER^{T} animals. Age-matched animals lacking the *Cre* transgene were used as controls in all instances. Animals were maintained according to the guidelines established by the Canadian Council on Animal Care and Animal Care & Veterinary Services of the University of Ottawa.

Histology and immunohistochemistry

Intestinal tracts were dissected free of accessory organs in phosphate-buffered saline (PBS), flushed with ice cold PBS, dissected longitudinally and Swiss rolls were prepared as previously described (Moolenbeek and Ruitenberg, 1981). Paraffin-embedded material was sectioned at 5 μmfor histological and immunohistochemical analysis. Hematoxylin and eosin, Alkaline Phosphatase, Periodic acid-Schiff, Alcian Blue, Churukian Silver, Phloxine-tartrazine and immunostaining were carried out using standard methods. Primary antibodies used were rabbit polyclonal anti-Cdx1 and anti-Cdx2 (1/1000 dilution, (Savory et al., 2009); anti-Ki67 (1/1000 dilution, Abcam); anti-lysozyme (1:500 dilution, Dako); and anti-$H^{+}K^{+}$ATPase (β subunit; 1/1000 dilution, Abcam). Secondary antibodies used were HRP-conjugated goat antimouse or goat anti-rabbit, as appropriate (1/1000 dilution, Santa Cruz

Biotechnologies). Slides were mounted using Permount (Fisher) and images captured using a Zeiss Mirax Midi Scanner (Zeiss).

Semi-quantitative reverse-transcriptase PCR (RT-PCR)

RNA was extracted from stomach, cecum, small intestine or large intestine with Trizol reagent (Invitrogen) and used to generate cDNA by standard procedures. cDNA was subsequently amplified by semi-quantitative RT-PCR using oligonucleotides directed against *Delta-like ligand 1 (Dll1)*, *Intestinal Alkaline Phosphatase (IAP)*, *Gastrokine 1 (Gkn1)*, *Gastrokine 2 (Gkn2)*, *Ghrelin (Ghrl)*, *Intestinal Fatty Acid Binding Protein (IFABP)*, *Sucrase Isomaltase (SI)*, *Hoxb13, Defensin alpha 5 (Defa5)*, *Sodium channel non-voltage gated 1 beta (Scnn1B)*, or *β-actin*. PCR products were resolved by electrophoresis on a 2% agarose gel, stained using ethidium bromide and products from the linear range of amplification quantified by ImageJ (NIH) using β-actin values to normalize for input. Primers used for amplification are listed in the supplemental materials.

In situ hybridization

Intestinal sections were cut at 10 μm and slides were processed as described above. Probes for *Olfactomedin 4* (*Olfm4*) were synthesized using the DIG RNA labeling system (Roche) according to manufacturer's recommendations. In situ hybridization was carried out as previously described (Gregorieff and Clevers, 2010) and slides mounted using Faramount Aqueous Mounting Medium (Dako).

Results

Cdx2 is essential in the adult intestine

In order to investigate the role of Cdx2 in the adult intestinal tract, we circumvented the embryonic lethality inherent to *Cdx2* loss using a previously described floxed allele (*Cdx2$^{f/f}$;* (Savory et al., 2009) and effected tamoxifen-induced deletion in the intestinal epithelium with a *villin*-cre ER^{T} transgene (Grainger et al., 2010). In addition, we bred the *Cdx2* floxed allele into the *Cdx1* null background (Subramanian et al., 1995) and elicited Cre recombination as above to generate a *Cdx1*- *Cdx2* double null intestine.

Immunohistochemistry 5–48 h following a 1 mg oral gavage of tamoxifen revealed almost complete absence of Cdx2 24 h post treatment, with residual protein present only in Paneth cells (Figure 12). These results indicated highly efficient excision of the floxed allele followed by rapid loss of Cdx2 throughout the adult small intestinal tract. This disruption resulted in diarrhea and rapid wasting, necessitating humane endpointing by 6–7 days posttreatment. Upon dissection, animals exhibited severely distended intestines, especially evident in the cecum (Figure 13). Histological analysis revealed that the villi of the small intestine became progressively shorter, with a severe reduction in villus length by day 6 compared to controls (Figure 14A). The rapid and near-complete loss of Cdx2 perdured throughout the course of the analysis (Figure 14C), consistent with ablation of *Cdx2* in intestinal stem cells.

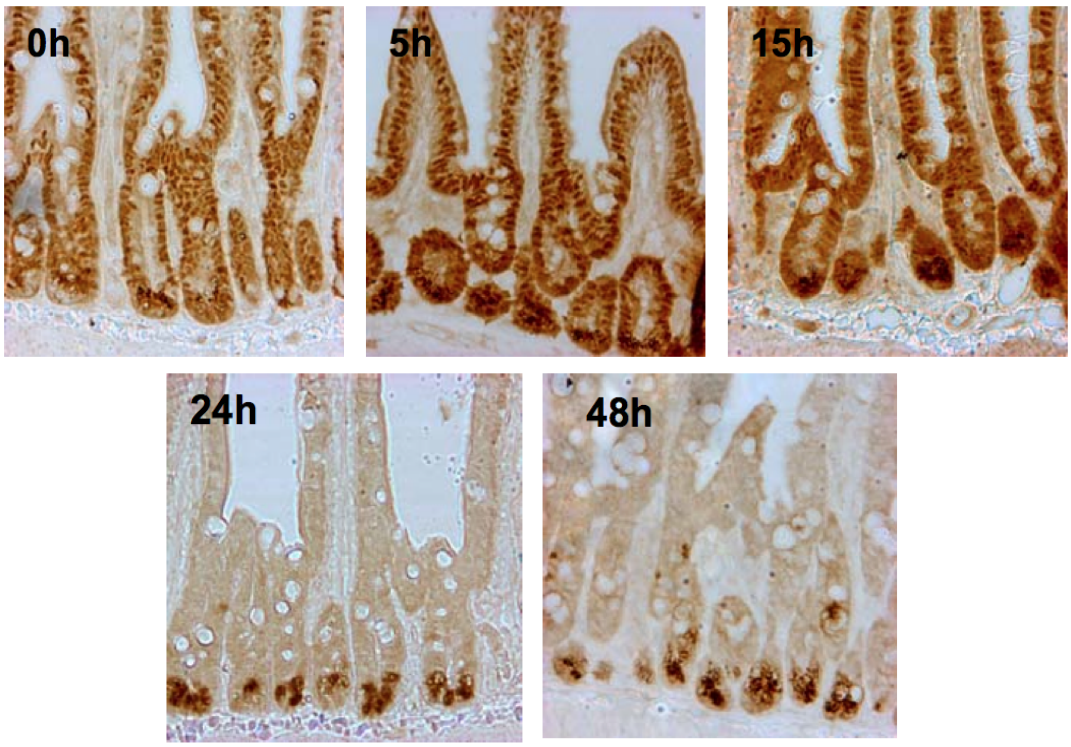


Figure 12: Ablation of Cdx2 from the intestinal epithelium.
Immunohistochemistry for Cdx2 in the small intestine of *Cdx2*$^{-/-}$ mice 0-48 hours post deletion.

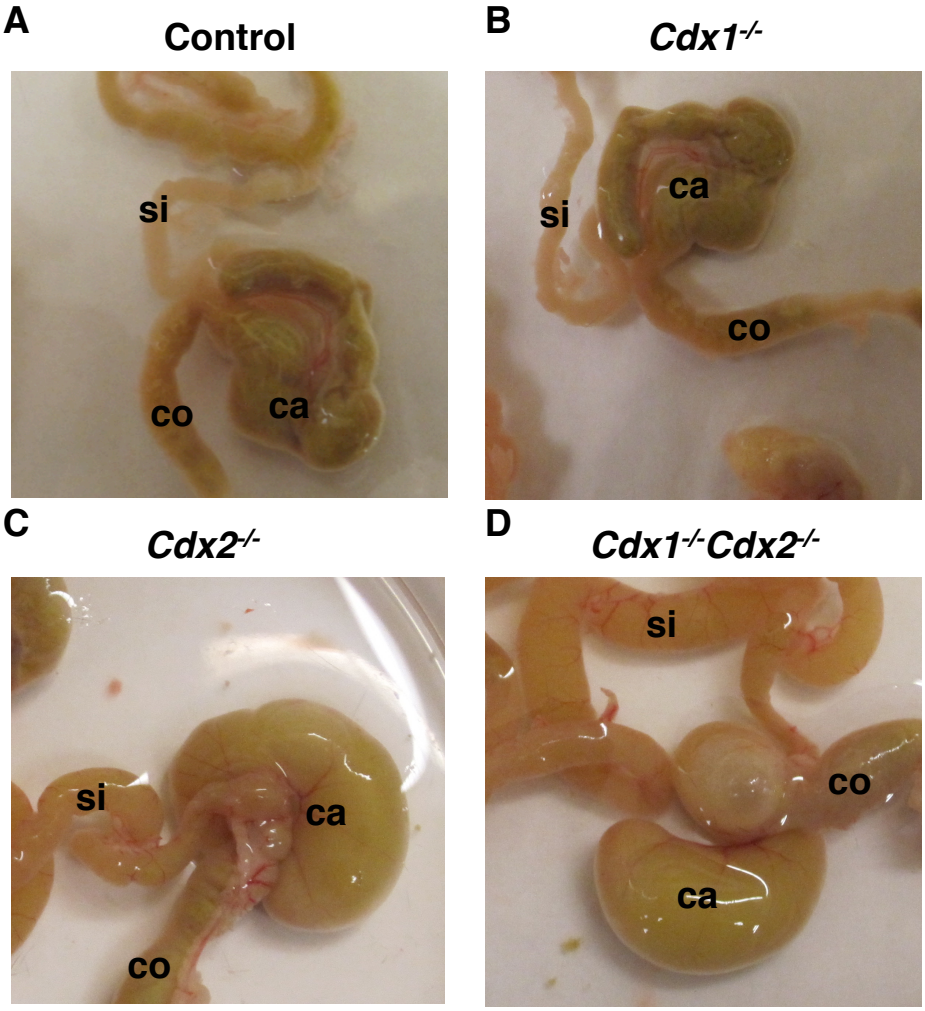


Figure 13: Morphology of *Cdx2*$^{-/-}$ and *Cdx1*$^{-/-}$*Cdx2*$^{-/-}$ intestinal tracts. (A) Control, (B) *Cdx2*$^{-/-}$, (C) *Cdx1*$^{-/-}$, and (D) *Cdx1*$^{-/-}$*Cdx2*$^{-/-}$ intestines. Note the distension of the intestines, most notable in the caecum, in the *Cdx2*$^{-/-}$ and *Cdx1*$^{-/-}$*Cdx2*$^{-/-}$ specimens. Abbreviations: si, small intestine; ca, caecum; co, colon.

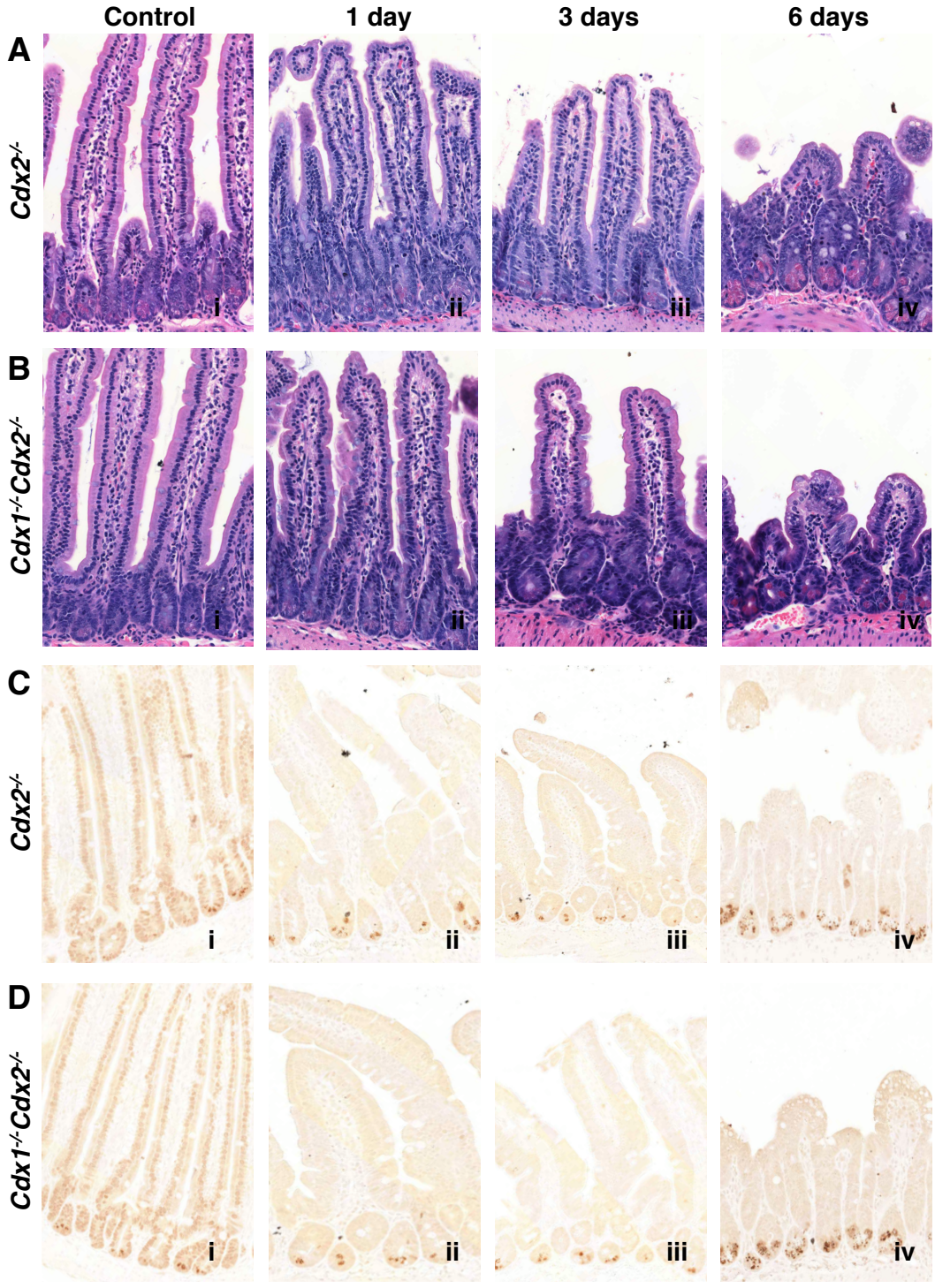


Figure 14: Cdx2 is essential for morphogenesis of the small intestine. Hematoxylin and eosin staining of the small intestine of *Cdx2*$^{-/-}$ (A) or *Cdx1*$^{-/-}$*Cdx2*$^{-/-}$ (B) animals 0-6 days post *Cdx2* deletion. Immunohistochemistry for Cdx2 in the small intestine of *Cdx2* $^{-/-}$ (C) or *Cdx1*$^{-/-}$*Cdx2*$^{-/-}$ (D) at 0-6 days post deletion. Note the loss of Cdx2 protein within 24 hours, and the altered villus morphology by 3 days post-deletion.

Mice lacking both *Cdx1* and *Cdx2* in the intestine exhibited a trajectory of wasting identical to that of Cdx2 single mutants, and loss of *Cdx1* concomitant with *Cdx2* did not accelerate the rate or the extent to which intestinal morphology was affected (Figure 14B). Immunohistochemistry revealed that the kinetics of Cdx2 disruption was comparable to that seen in the single mutants (Figure 14D). These findings suggest that loss of Cdx1 does not result in additional phenotypic impact in the absence of Cdx2 in the small intestine.

Cdx2 impacts on proliferation and intestinal stem cells

Intestinal stem cells are housed at or near the base of the intestinal crypts and give rise initially to TA cells which rapidly divide and subsequently differentiate into the four cell types of the intestinal epithelium (Pinto and Clevers, 2005). The finding that loss of *Cdx2* resulted in a rapid and profound reduction in villi outgrowth and morphology prompted us to evaluate each of these populations.

Immunohistochemistry for Ki67 revealed a decrease in the proliferative population of the small intestine which was comparable in both *Cdx2* and *Cdx1*-*Cdx2* deficient intestine. Loss of proliferation became apparent as early as day 2 post-treatment and was markedly reduced in the small intestine at 6 days (Figure 15A and B). This reduction occurred concomitant with loss of villus outgrowth, consistent with a reduction in the TA cell population impacting on intestinal morphology.

To determine if Cdx loss impacted TA progenitors, in situ hybridization for *Olfm4*, an intestinal stem cell marker (van der Flier et al., 2009), was conducted. *Olfm4* expression decreased upon *Cdx2* ablation in a manner reflective of the

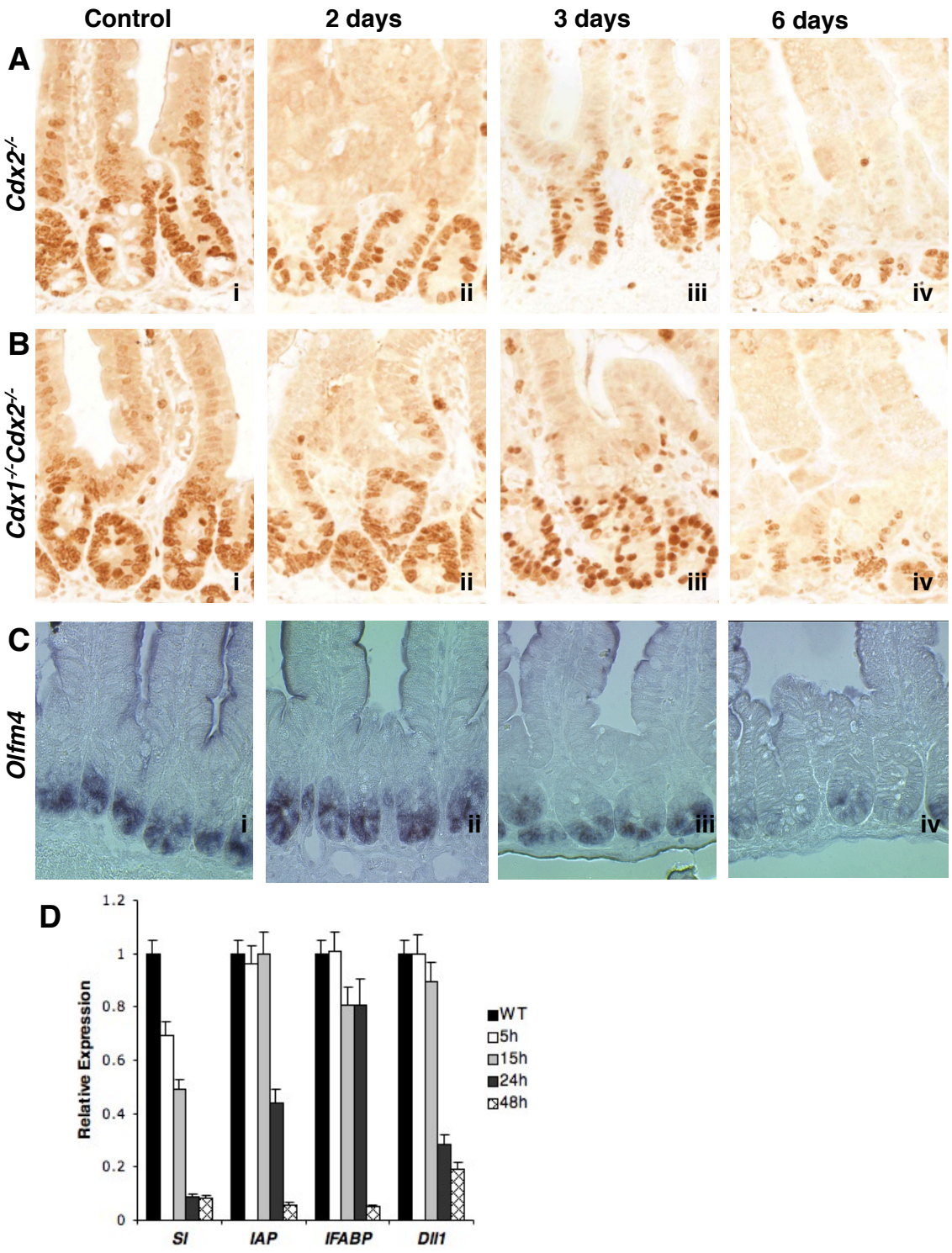


Figure 15: Cdx2 is necessary for intestinal homeostasis.
Immunohistochemistry for Ki67 in the small intestine of *Cdx2*$^{-/-}$ (A) and *Cdx1*$^{-/-}$*Cdx2*$^{-/-}$ (B) at 0-6 days post *Cdx2* deletion. (C) *In situ* hybridization for *Olfm4* in the small intestine of *Cdx1*$^{-/-}$*Cdx2*$^{-/-}$ at 0-6 days post Cdx2 deletion. (D) Semi-quantitative RT-PCR for *SI, IAP, IFABP,* and *Dll1* from small intestine of WT and *Cdx2* mutants 0-48 hours post-treatment. Error bars represent s.d. from the mean of 3 independent samples.

diminution of TA cell proliferation (Figure 15C). These data illustrated that Cdx2 is required to maintain the intestinal stem cell population and their TA progeny, although the protracted period following Cdx2 ablation required to impact these populations suggests that it is not a primary event.

IAP, *IFABP* and *SI* are markers of intestinal enterocytes (Agellon et al., 2002; Xie et al., 1997), while the latter is also a Cdx2 target gene (Boudreau et al., 2002). *Dll1*, a Notch ligand, is also expressed in the intestine where it impacts on intestinal cell fates (Crosnier et al., 2005) and is also a Cdx2 target gene(Grainger et al., 2011). Expression analysis revealed that the loss of Cdx2 was associated with a marked reduction in the levels of all of these genes at 48 h post recombination; the more rapid loss of *Dll1* and *SI* are consistent with their position as direct Cdx2 targets (Figure 15D). The kinetics of these effects indicate a critical role for Cdx2 in governing intestinal differentiation in the adult (Grainger et al., 2010; Grainger et al., 2011; Mutoh et al., 2005; Verzi et al., 2010; Verzi et al., 2011) that may be independent of the impact of Cdx2 loss on TA and stem cell populations, which manifests at later stages.

A model for chronic loss of Cdx function in the intestine

To circumvent the lethality evoked by ablation of *Cdx2* in the intestine to perform longer-term analyses, we employed dose-seeking studies and found that administration of a single 0.25 mg dose of tamoxifen had no overt effect on health or longevity. Analysis of animals 6 weeks post-treatment, however, revealed extensive areas in the intestinal tract which were devoid of Cdx2 immunoreactivity (Figure 16B). These regions were consistently associated with a loss of the normal crypt-

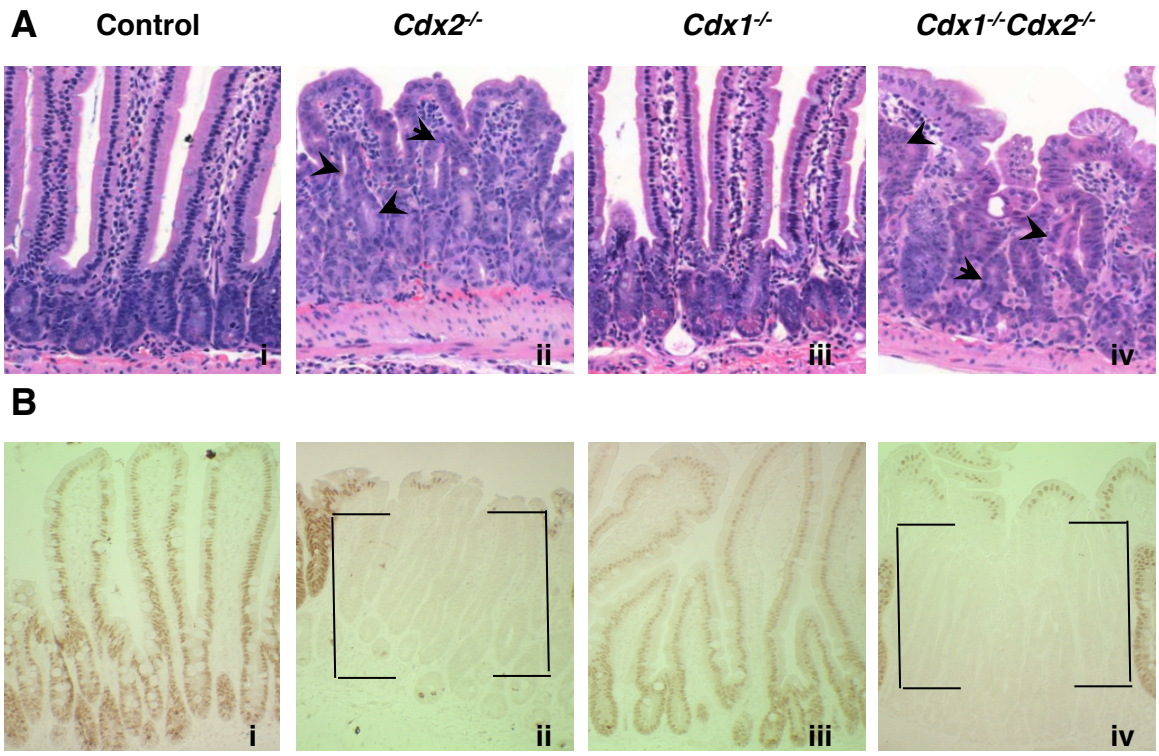


Figure 16: Chronic loss of Cdx2.
(A) Hematoxylin and eosin staining of control (i), *Cdx2*$^{-/-}$ (ii), *Cdx1*$^{-/-}$(iii), and *Cdx1*$^{-/-}$*Cdx2*$^{-/-}$ (iv) small intestine 6 weeks after inactivation of *Cdx2*. Note metaplasias in the mutants (arrows). (B) Immunohistochemistry for Cdx2 in small intestines 6 weeks post disruption. Note the lack of Cdx2 associated with the epithelial dysplasia (brackets in ii and iv).

villi axis and aberrant morphology (Figure 16A). Further analysis by differential staining indicated that these metaplasia were devoid of mature enterocytes, as evidenced by loss of Alkaline Phosphatase activity (Figure 17A), and goblet cells (as seen with loss of Alcian Blue and Periodic Acid-Schiff staining; Figures 17B and C). Similarly, Churukian silver and Phloxine-tartrazine staining suggested a loss of enteroendocrine and Paneth cells, respectively (Figures 17D and E). Immunoreactivity for lysozyme was also absent from these metaplastic regions (Figure 18A), again consistent with a loss of Paneth cells in the mutant epithelium (Chung et al., 1988).

In contrast to the partial reduction in intestinal stem cells seen in acute Cdx2 ablation, above, ectopic, but robust, expression of *Olfm4* was seen in Cdx2 metaplastic tissues (Figure 18B). Thus, chronic loss of Cdx2 does not appear to have a deleterious effect on the number of *Olfm-4* positive cells, but rather appears to impact on localization of this population. Cdx1 appears to be incapable of supporting this function in the absence of Cdx2, as similar outcomes were seen in both Cdx2 single and Cdx1-Cdx2 double mutant intestines (Figure 18B).

Cdx2 suppresses gastric identity

Previous studies suggested fundamental roles for *Cdx2* in intestinal patterning during development (Beck et al., 1999; Chawengsaksophak et al., 1997; Gao et al., 2009; Grainger et al., 2010; Stringer et al., 2008). However, loss of Cdx function has not been reported to elicit any overt intestinal transformation in the adult (Verzi et al., 2010; Verzi et al., 2011). In the present study, we found that long-term loss of *Cdx2* led to loss of all intestinal differentiation programs concomitant

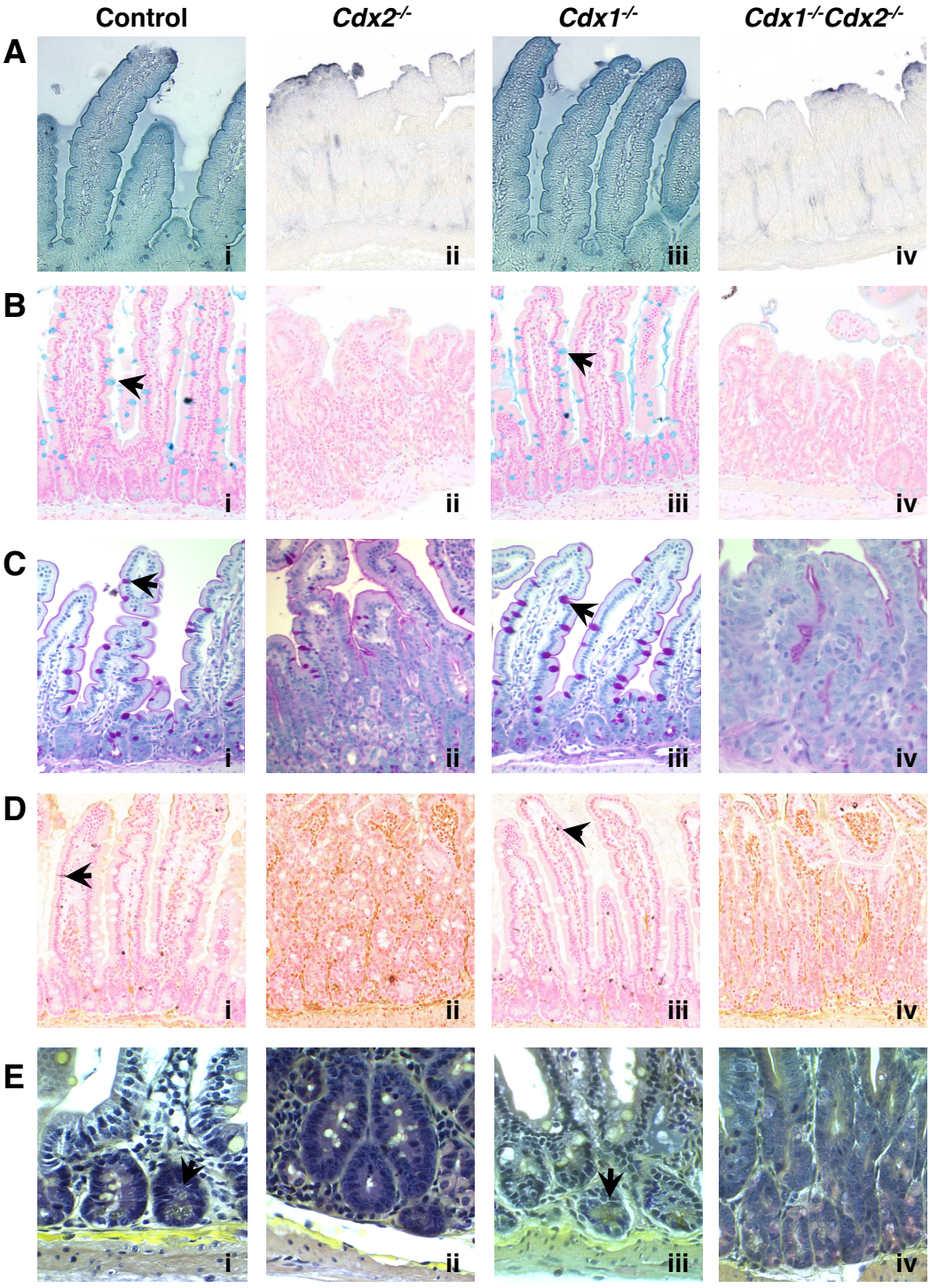


Figure 17: Cdx2 is required for differentiation of all intestinal epithelial cell types. Small intestine of control (i), *Cdx2*$^{-/-}$ (ii), *Cdx1*$^{-/-}$ (iii), and *Cdx1*$^{-/-}$*Cdx2*$^{-/-}$ (iv) offspring 6 weeks post-deletion of *Cdx2*. (A) Alkaline phosphatase staining for enterocytes. (B) Alcian blue and (C) PAS staining for goblet cells (black arrow in i and iii). (D) Churukian silver staining for enteroendocrine cells (black arrows in i and iii). (E) Phloxine-tartrazine staining to detect Paneth cells (black arrows in i and iii).

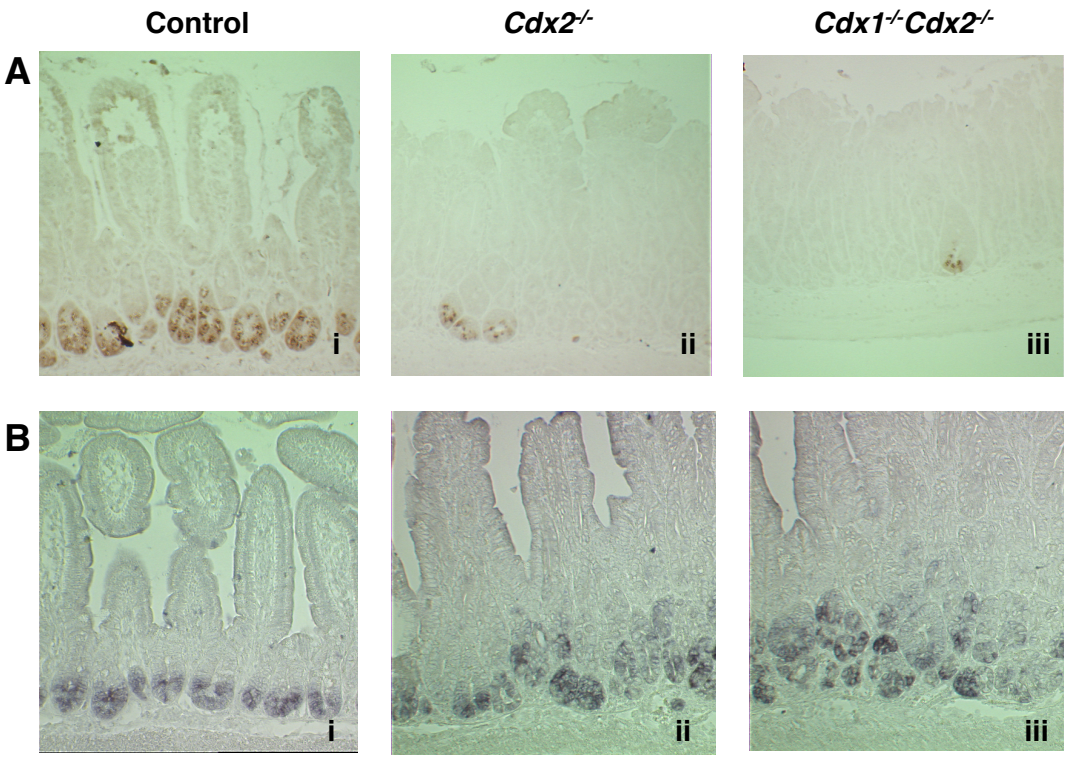


Figure 18: Long-term Cdx2 loss affects stem cells and their niche.
(A) Immunohistochemistry for lysozyme in control (i), *Cdx2*$^{-/-}$ (ii) and *Cdx1*$^{-/-}$*Cdx2*$^{-/-}$ (iii) small intestines. (B) *In situ* hybridization for *Olfm4* in control (i), *Cdx2*$^{-/-}$ (ii) and *Cdx1*$^{-/-}$ *Cdx2*$^{-/-}$ (iii) small intestines.

with the appearance of an atypical cell type which, upon closer inspection, resembled gastric parietal cells (Figure 19A). Consistent with this, H^+K^+ATPase, a marker of parietal cells (Yang et al., 2001), was expressed by these aberrant cells (Figure 19B). In addition, relative levels of the stomach markers *Ghrl* (Kojima et al., 1999), *Gkn1* (Oien et al., 2004) and *Gkn2* (Baus-Loncar et al., 2007) were all increased in the Cdx mutant intestine, while the intestinal marker *IFABP* exhibited a concomitant decrease (Figure 19D). Expression of the esophageal markers *Krt5* and *Krt14* were undetectable in Cdx2 and Cdx1-Cdx2 mutant intestine (Figure 20).

As with the intestine, coordinated proliferation and differentiation of stomach cells is essential for the renewal of the short-lived gastric epithelium. However, in contrast to the basally-located TA cell population characteristic of the intestine, the proliferative stem cell progeny of the stomach are located at the isthmus of the gastric pits (Mills and Shivdasani, 2011). In this regard, Ki67 positive cells in the Cdx2 metaplastic regions appear to be ectopically located in a manner reminiscent of this more superficial proliferative zone (Figure 19C). Taken together with the finding of ectopic expression of stomach markers, these results are consistentwith long-termablation of Cdx2 leading to partial conversion of the intestinal epithelium to a stomach-like nature.

Cdx and patterning of the colon

The colon is composed of the cecum proximally, and the colon proper and is distinguished from the small intestine by a lack of villi and deeper crypts (Sancho et al., 2004). As with the small intestine, immunohistochemistry revealed rapid and near-complete loss of Cdx2 in the colon of both *Cdx2*$^{-/-}$ and *Cdx1*$^{-/-}$*Cdx2*$^{-/-}$ mutants

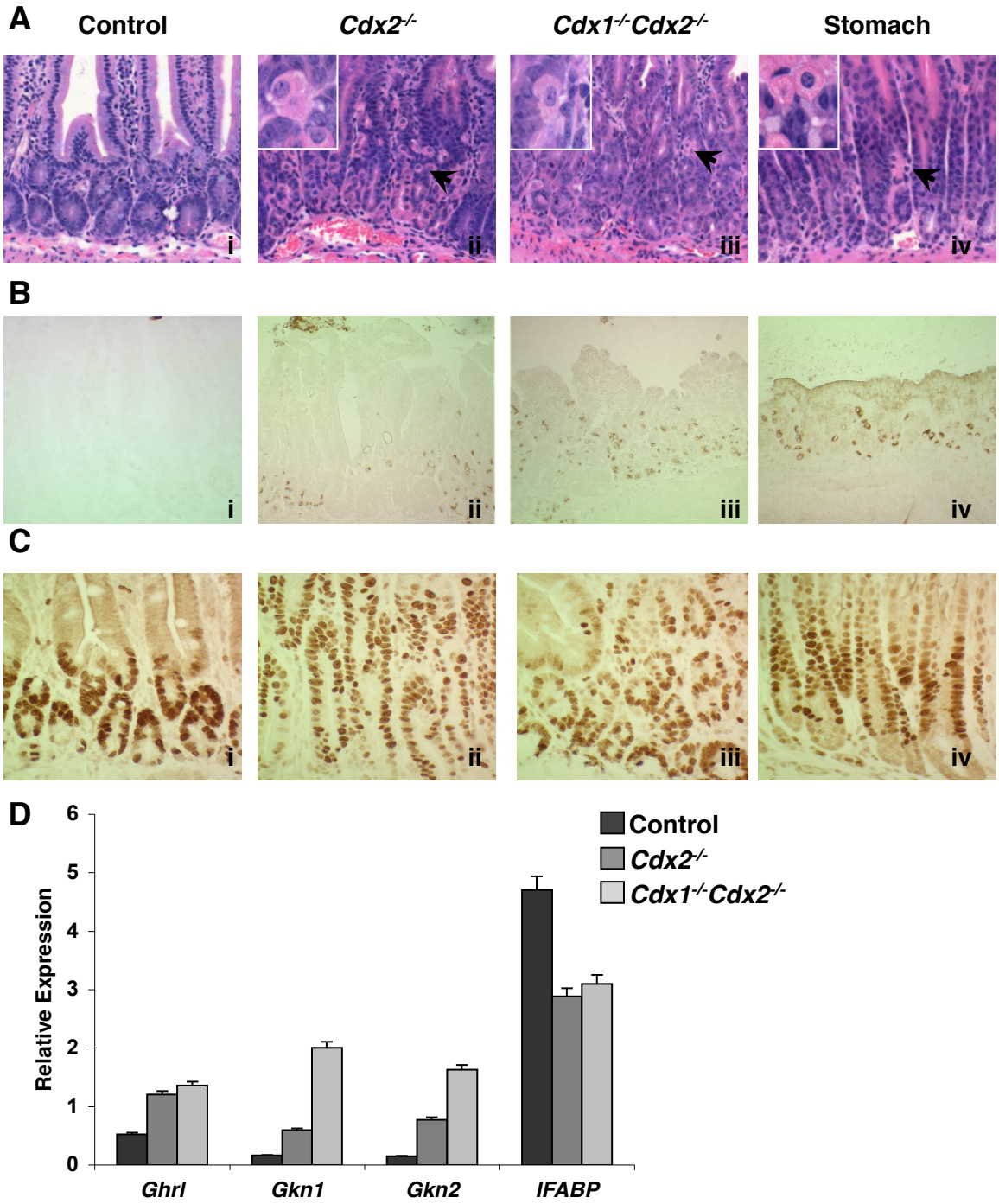


Figure 19: Long-term loss of Cdx2 causes a shift to a stomach-like phenotype. (A) Hematoxylin and eosin staining of small intestines from control (i), *Cdx2*$^{-/-}$ (ii) and *Cdx1*$^{-/-}$*Cdx2*$^{-/-}$ (iii) offspring as well as wild-type stomach (iv). Note the appearance of parietal-like cells in mutants (arrows and insets in i and iii). (B) Immunohistochemistry for H^+K^+ATPase and (C) Ki67 in control (i), *Cdx2*$^{-/-}$ (ii), *Cdx1*$^{-/-}$*Cdx2*$^{-/-}$ (iii) intestines and wild-type stomach (iv). (D) Semi-quantitative RT-PCR for *Ghrelin* (*Ghrl*), *Gastrokine 1* and *2* (*Gkn 1* and *Gkn2*), and *IFABP*. Error bars represent s.d. from the mean of 3 independent samples.

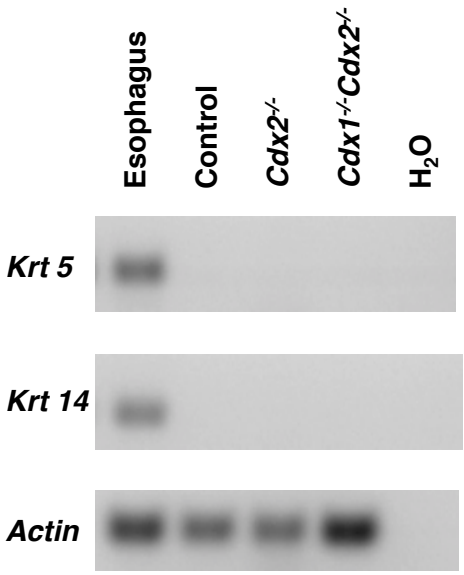


Figure 20: Esophageal characteristics do not appear in the small intestine after chronic loss of Cdx2.
Semi-quantitative RT-PCR for *Krt5* and *Krt 14* from esophagus, control small intestine, *Cdx2*$^{-/-}$ small intestine and *Cdx1*$^{-/-}$*Cdx2*$^{-/-}$ small intestine.

following a single 1 mg dose of tamoxifen (Figures 21A and B). Cdx1 protein was still present in *Cdx2* single mutants (Figure 21C), indicating that Cdx2 is not required for expression of *Cdx1* in the adult large intestine.

Histological analysis revealed a change in the morphology of the proximal colon of Cdx2 mutants into a cecum-like nature (Figure 22A), which may be ascribed to differences in mucosal folding (Treuting and Dintzis, 2011). While more distal regions of the Cdx2 mutant colon were unaffected, in marked contrast, *Cdx1*$^{-/-}$*Cdx2*$^{-/-}$ offspring exhibited a cecum-like morphology throughout the extent of the large intestine (Figure 22B). To further assess this putative transformation, expression of markers characteristic of the colon (*Hoxb13*) and cecum (*Defa5* and *Scnn1b*) (Birkenkamp-Demtroder et al., 2005) were evaluated by RT-PCR. This analysis revealed that a reduction in *Hoxb13* levels, with a concomitant increase in *Defa5* and *Scnn1b*, in the proximal colon of Cdx2 mutants. In contrast, and consistent with histological analysis, *Defa5* expression markedly increased in both the proximal and distal *Cdx1*$^{-/-}$*Cdx2*$^{-/-}$ colon (Figure 22C). These observations suggested a novel function for both Cdx1 and Cdx2 in maintaining normal morphology of the colon.

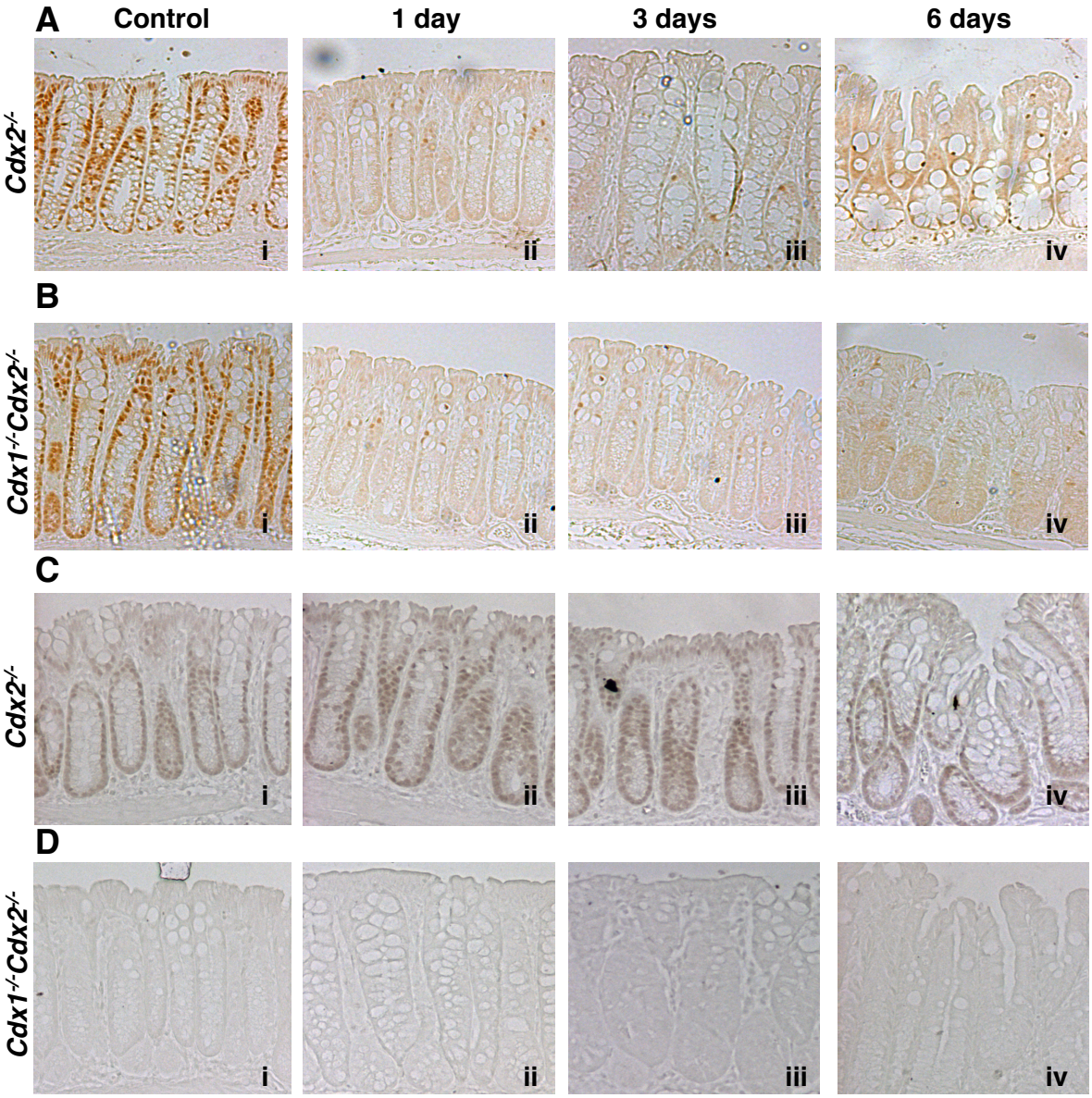
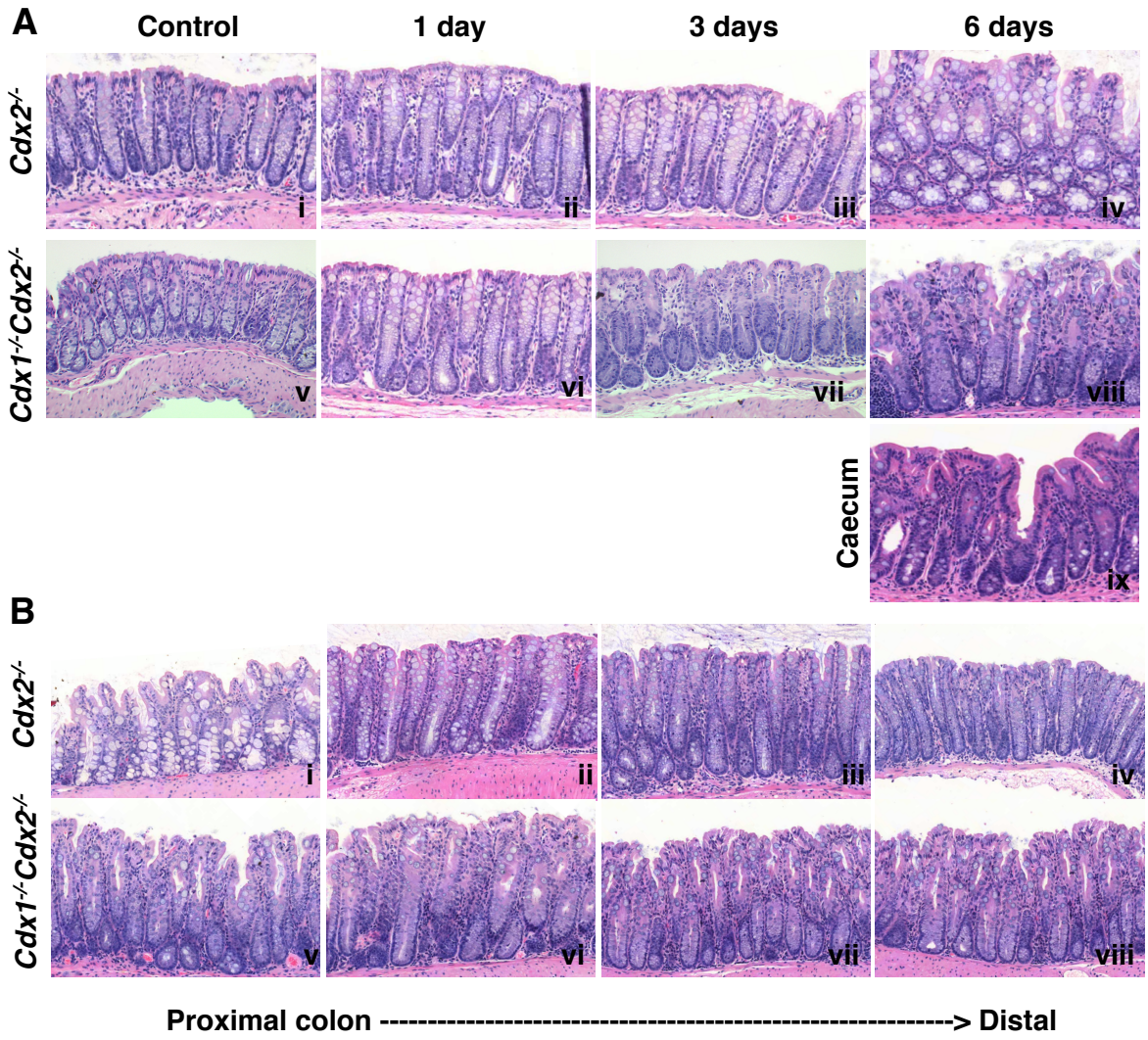


Figure 21: Conditional inactivation of Cdx2 in the colon.
Immunohistochemistry for Cdx2 (A and B) and Cdx1 (C and D) from the colon of *Cdx2*$^{-/-}$ and *Cdx1*$^{-/-}$*Cdx2*$^{-/-}$ offspring 0-6 days post-Cdx2 disruption.



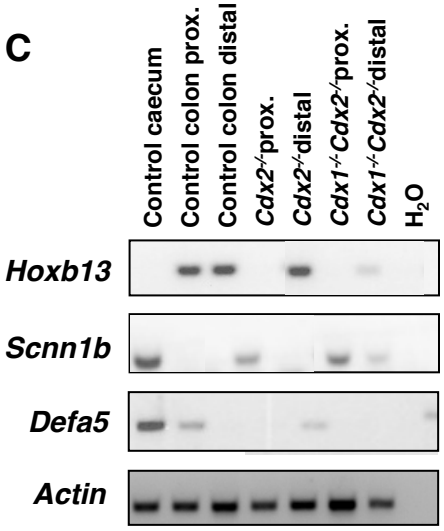


Figure 22: Cdx functions in the colon.
(A) Hematoxylin and eosin staining of the proximal colon from *Cdx2*$^{-/-}$ (i-iv) or *Cdx1*$^{-/-}$*Cdx2*$^{-/-}$ (v-viii) offspring 0-6 days post Cdx2 deletion. Wild-type caecum (ix) is shown for comparison. Note that the colon of Cdx mutants appears to take a caecum-like morphology of the caecum. (B) Proximal to distal images of the colon from *Cdx2*$^{-/-}$ and *Cdx1*$^{-/-}$*Cdx2*$^{-/-}$ offspring 6 days post Cdx2 inactivation. Note the caecum-like morphology persists throughout the extent of the colon of the double mutant. (C) Semi-quantitative RT-PCR for *Hoxb13, Scnn1b,* and *Defa5* from control colon, *Cdx2*$^{-/-}$, *Cdx1*$^{-/-}$*Cdx2*$^{-/-}$ proximal and distal colon.

Discussion

To understand better the roles of the Cdx transcription factors in the adult GI tract, we utilized a conditional mutagenesis strategy to delete *Cdx2* in the intestinal epithelium alone or in a *Cdx1* null background. This approach resulted in the efficient and rapid loss of Cdx2 throughout the intestinal tract, leading to rapid death within 7 days, likely due to intestinal dysfunction. Villus morphology and proliferation, as well as the expression of a number of intestinal markers, were all impacted following ablation of Cdx2. These indices were not further affected by subsequent loss of *Cdx1*, suggesting that Cdx1 cannot fulfill Cdx2-dependent functions in the small intestine. In the distal GI tract, *Cdx2* ablation resulted in an apparent transformation of the epithelium of the proximal colon to a cecal-like identity, and this outcome was exacerbated with the subsequent loss of *Cdx1*. In contrast to acute loss-of-function studies, long-term loss of Cdx2 resulted in metaplasia throughout the small intestine in which all normal differentiated intestinal cell types were lost. These lesions assumed a partial gastric nature and malpositioned stem cells. These findings are consistent with a degree of plasticity in the small intestine, and underscore new roles for Cdx1 and Cdx2 in the GI tract.

Cdx2 is essential for intestinal homeostasis

We found that acute loss of *Cdx2* compromised the intestinal mucosa, leading to death within 1 week. This was preceded by a reduction in both the precursor and proliferative compartments, as well as a more acute reduction in expression of a number of markers of intestinal differentiation. Concomitant loss of *Cdx1* did not

exacerbate these outcomes, suggesting that *Cdx1* cannot support any Cdx2- related roles in the adult small intestine in the absence of Cdx2. This may be due to functional specificity and/or differences in expression levels, with Cdx2 predominating in the small intestine (James et al., 1994; Silberg et al., 1997). Consistent with this is the finding that Cdx1 does complement Cdx2 loss in programs related to vertebral patterning, axial extension and neural tube closure (Davidson and Zon, 2006; Savory et al., 2009; van den Akker et al., 2002). Additional experimentation will be required to address more rigorously issues related to functional specificity between Cdx members in the intestine.

In contrast to the present study, prior work illustrated that ablation of *Cdx1* and *Cdx2* in the adult small intestine induced a more severe phenotype compared to deletion of *Cdx2* alone (Verzi et al., 2010; Verzi et al., 2011). In this regard, it is notable that considerable Cdx2 protein persisted for some time post-recombination in these former studies. This was reflected with respect to the relative longevity of the Cdx2 null animals, which lived for approximately 3 weeks, and with the finding of no apparent effect on the morphology of the villi. Deletion of Cdx1 in this background led to an impact in health, with mice surviving ~8 days, and with a modest impact on villi morphology. In the current study, we observed essentially complete loss of *Cdx2* within 24 h following a single dose of tamoxifen throughout the intestinal tract, and with a strong and rapid impact on morphology and gene expression patterns. These differences are likely due to the use of a more efficacious *villin*-Cre transgenic line in the present study (D. Metzger, personal communication). Under these conditions, subsequent loss of Cdx1 has no apparent impact, perhaps

due to relatively low protein level of Cdx1 compared to Cdx2 in the small intestine, as discussed above.

Cdx2 is essential for differentiation of all cell types of the small intestine

Acute ablation of *Cdx2* led to a decrease in enterocyte markers, including alkaline phosphatase reactivity, and a reduction in expression of the pro-secretory lineage marker *Dll1*, a known Cdx target gene (Grainger et al., 2011). Paneth, enteroendocrine and goblet cells were, however, not strongly impacted over the 6 day course of this study (data not shown), consistent with prior findings (Verzi et al., 2010). It is notable, however, that the TA and *Olfm4*-positive progenitor populations were reduced, particularly at later time points, suggesting that Cdx2 disruption had more widespread effects on these precursor populations. Analysis of any longer-term impact was, however, negated by the lethality evoked by Cdx2 loss.

To circumvent the lethality evoked by intestinal Cdx2 loss, we developed a sub-optimal dosing strategy to evoke partial ablation compatible with long-term survival. Examination of animals treated with a low dose of tamoxifen 6 weeks post-gavage revealed a number of dysplastic regions in the small intestine that were devoid of Cdx2. Differential staining and immunohistochemistry revealed that these metaplasias were associated with a loss of all differentiated intestinal cell types. This is in marked contrast to prior observations following short-term Cdx disruption, which suggested an impact primarily on enterocyte differentiation and TA cell proliferation (Verzi et al., 2010; Verzi et al., 2011). Rather, these data suggest a broader role for Cdx2 in governing differentiation of all intestinal cell types in the adult.

Dysplastic *Cdx2*-null areas exhibited an atypical, parietal-like cell type, characterized by expression of H^+K^+ATPase, suggesting a partial transformation of the small intestine to a gastric identity. It is interesting to note that the levels of *Gkn1* and *Gkn2* increased with concomitant loss of Cdx1 in the Cdx2 mutant background, although there was no apparent morphological impact. This finding suggests that Cdx1 may elicit some function in the absence of Cdx2, but that it is without overt consequence, and may be confined to only a subset of Cdx-dependent pathways.

The increase in gastric character following loss of *Cdx2* suggests that the adult small intestine exhibits considerable plasticity. Plasticity of the gastrointestinal tract has been seen in development where changes can occur over a protracted period of time, and impacting certain organs (Li and Edlund, 2001; Shen et al., 2003), as seen in *Cdx2* mutant embryos (Grainger et al., 2010). Similar plasticity is also evident in the adult gastrointestinal tract under conditions of chronic tissue damage, such as the switch from a keratinized stratified squamous epithelium to an intestinal mucosa seen in Barrett's esophagus (Bonino and Sharma, 2004). Interestingly, this transformation coincides with, and may be driven by, aberrant expression of *Cdx2* (Kong et al., 2011; Souza et al., 2008). In a similar manner, ectopic postnatal expression of *Cdx2* in the stomach results in replacement of gastric mucosal cells by an intestinal epithelium (Mutoh et al., 2002; Mutoh et al., 2005). Taken together, these data suggest that Cdx2 is necessary and sufficient to direct gut epithelial cells to an intestinal nature in the adult. The converse transformation observed here suggests similar, albeit more limited, plasticity in the

adult small intestine. In this regard, the hamartomas previously described in Cdx2 heterozygous offspring comprised a broad spectrum of tissues suggestive of intercalary regenerates from the esophagus through the large intestine (Beck et al., 1999). The much more limited transformation seen in Cdx2-null adult intestine suggests that these hamartomas are developmental in nature, likely reflecting the need for Cdx2 in the specification of definitive endoderm, as previously described (Gao et al., 2009).

Cdx2 and intestinal precursor populations

Stem cells are present in the epithelium of the stomach as well as in the intestine (Mills and Shivdasani, 2011; Pinto and Clevers, 2005). Prior to terminal differentiation, each of these stem cell populations gives rise to rapidly dividing TA cells. Following long-term loss of *Cdx2*, both presumptive stem cells and TA cells were displaced from the intestinal crypt, reminiscent of the normal localization of gastric TA cells.

Paneth cells contribute to a stem cell niche in the base of the intestinal crypt (Sato et al., 2011), and it is tempting to speculate that the loss of Paneth cells, elicited by ablation of *Cdx2*, may impact on the intestinal stem cell niche leading to the observed repositioning of the stem and TA cell populations. In this regard, conditional ablation of Paneth cells led to the disappearance of *Olfm4*-positive cells (Sato et al., 2011), while loss of Paneth cells in the current study did not lead to ablation of this population. This suggests that the microenvironment of the Cdx2 null metaplasia suffices to provide an adequate niche, the basis of which is presently unknown. Interestingly, ectopic expression of *Cdx2* in the stomach results in

displacement of gastric TA cells from the isthmus towards the base of the glands, reflecting the localization of the proliferative compartment in the intestine (Mutoh et al., 2002). Irrespective of the basis for the localization of these precursor niches, our present findings illustrate that Cdx2 is not essential for long term maintenance of *Olfm-4* positive progenitor cells, but is absolutely required for both their proper positioning and subsequent differentiation.

The reemergence of *Olfm4* positive and proliferative cells following long-term *Cdx2* deletion contrasts with the decrease in these populations seen following acute *Cdx2* loss. This may be attributed to a transient reduction in the *Olfm4*-positive precursor population, followed by their regeneration either through an unknown adoptive process or via a previously described reserve stem cell population (Li and Clevers, 2010; Scoville et al., 2008; Tian et al., 2011). Further work will be required to more fully understand this process.

Novel function for Cdx1and Cdx2 in the colon

Immunohistochemistry revealed effective deletion of Cdx2 in the colon. However, expression analysis suggested that it was not transformed into more anterior derivatives (Figure 23). This implies that the mechanisms underlying the plasticity seen in the small intestine do not operate in the colon, or require greater time. In this regard, the suboptimal tamoxifen dosing regimen necessary to circumvent lethality did not result in sufficient long-term excision of Cdx2 in the colon to allow us to address this latter possibility.

Prior work has shown a critical requirement for Cdx2 in specification of the colon from definitive endoderm (Gao et al., 2009), however a role for Cdx in the

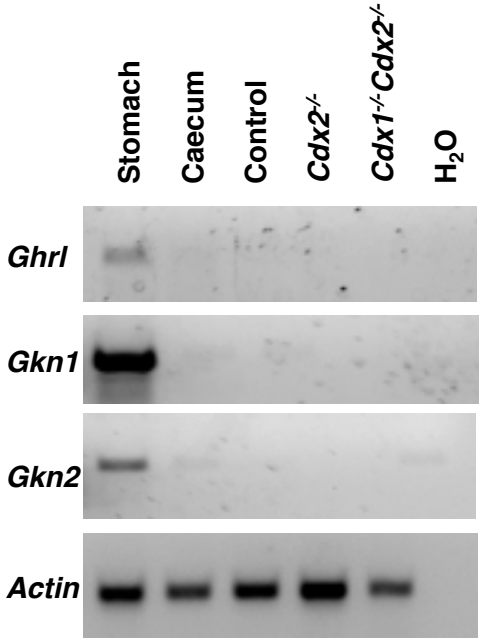


Figure 23: Gastric characteristics do not appear in the colon after chronic loss of Cdx2.
Semi-quantitative RT-PCR for *Ghrl*, *Gkn 1* and *Gkn2* from stomach, caecum, control colon, *Cdx2*$^{-/-}$ colon and *Cdx1*$^{-/-}$*Cdx2*$^{-/-}$ colon.

adult distal intestine has not been described. We found that ablation of *Cdx2* resulted in transformation of the typical colon epithelium with a more scalloped morphology similar to that of the cecum. Moreover, while this phenotype was restricted to the proximal colon in *Cdx2* mutants, it extended throughout the large intestine in *Cdx1-Cdx2* double mutants, and was accompanied by altered expression of cecum- or colon-associated genes in a manner reflective of this altered morphology. The relative contribution of Cdx1 and Cdx2 to this putative anterior transformation is in close agreement with their relative distribution along the longitudinal gut axis, with a posterior high gradient for Cdx1 and a peak of expression for Cdx2 in the proximal colon which dissipates distally (James et al., 1994; Silberg et al., 1997). These data are consistent with functional overlap between Cdx1 and Cdx2 in the colon, and suggest a novel role for Cdx in the homeostasis of this tissue.

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CHAPTER 3: Cdx1 and Cdx2 Function as Tumor Suppressors

Cdx1 and Cdx2 Function as Tumor Suppressors

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Author contributions:

Alexa Hryniuk designed and carried out experiments, wrote the manuscript and made figures.

Stephanie Grainger assisted with generating experimental material and with figure generation.

Joanne G.A. Savory generated *Cdx2f/f* mice.

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

Capsule

Background: Cdx function is essential for intestinal homeostasis and may impact tumorigenesis.

Results: Cdx loss increases tumor incidence and alters tumor phenotype in an APC mutant background.

Conclusion: Cdx transcription factors impact intestinal tumorigenesis.

Significance: Cdx members modulate both the frequency and phenotype of intestinal tumors through previously unrecognized mechanisms.

Abstract

In humans, colorectal cancer is often initiated through APC loss-of-function which leads to crypt hyperplasia and polyposis driven by unrestricted canonical Wnt signaling. Such polyps typically arise in the colorectal region and are at risk of transforming to invasive adenocarcinomas. Although colorectal cancer is the third most common cause of cancer related death worldwide, our understanding of the processes impacting initiation, transformation and invasion are incompletely understood. Murine $APC^{Min/+}$ mutants are often used to model colorectal cancers, however, they develop non-metastatic tumors confined largely to the small intestine and are thus not entirely representative of the human disease. $APC^{Min/+}$ alleles can collaborate with mutations impacting other pathways to recapitulate some aspects of human colorectal cancer. To this end, we assessed $APC^{Min/+}$-induced polyposis following somatic loss of the homeodomain transcription factor Cdx2, alone or with a Cdx1 null allele, in the adult gastrointestinal tract. $APC^{Min/+}$-Cdx2 mutants recapitulated several aspects of human colorectal cancer, including an invasive phenotype. Notably, the concomitant loss of Cdx1 led to a significant increase in the incidence of tumors in the distal colon, relative to $APC^{Min/+}$-Cdx2 offspring, demonstrating a previously unrecognized role for this transcription factor in colorectal tumorigenesis. These findings underscore previously unrecognized roles for Cdx members in intestinal tumorigenesis.

Introduction

Colorectal cancer (CRC) leads to more than 600,000 deaths annually, making it the third most deadly cancer worldwide. Despite this, our understanding of the mechanisms driving intestinal tumorigenesis is incomplete. Inactivating mutations in the *Adenomatous polyposis coli (APC)* gene are an early causative event in most cases of sporadic intestinal cancer and also underlie the familial adenomatous polyposis (FAP) syndrome in humans (Nathke, 2004). Loss of APC function in the human intestine results in protracted activation of the canonical Wnt signaling pathway, resulting in hyperproliferation and outgrowth of intestinal polyps which are predisposed to subsequent malignant conversion (Halberg et al., 2000; Moser et al., 1990; Polakis, 2007). APC mutant mice, such as the multiple intestinal neoplasia ($APC^{Min/+}$) mouse (Moser et al., 1990), harbor a comparable inactivating mutation and develop polyps similar to human FAP patients (Clevers, 2006). However, in contrast to humans, $APC^{Min/+}$ mice exhibit lesions primarily affecting the small intestine and pericecal area and which rarely progress to adenocarcinomas (Halberg et al., 2000; Moser et al., 1990).

Considerable work has revealed the nature of additional pathways that impact on tumorigenesis in murine APC models. Such pathways include activating mutations of KRas and lesions in the TGF-β and Eph-Ephrin signaling pathways (Cortina et al., 2007; Hamamoto et al., 2002; Sansom et al., 2006; Takaku et al., 1998). These studies have yielded murine models that recapitulate some aspects typical of human CRC. The Cdx family of transcription factors are also potential contributors to the CRC phenotype. Cdx1 and Cdx2 are necessary for the proper

development of the intestinal tract and homeostasis of the intestinal epithelium throughout life (Chawengsaksophak et al., 1997; Gao et al., 2009; Grainger et al., 2010; Hryniuk et al., 2012; Verzi et al., 2010; Verzi et al., 2011). Our understanding of Cdx function in intestinal tumorigenesis has, until recently, been restricted by the peri-implantation lethality of Cdx2 null mutants (Chawengsaksophak et al., 1997). Moreover, although Cdx1 null mice do not display any overt intestinal phenotype (Subramanian et al., 1995), Cdx members likely play overlapping roles in the intestine (Verzi et al., 2011) and colon (Hryniuk et al., 2012), and the impact of concomitant loss of Cdx1 and Cdx2 on intestinal tumorigenesis has not been reported to date.

There is considerable evidence suggesting that CDX2 suppresses CRC. For example, loss of CDX2 expression is seen in ~30% of human CRC and is associated with higher tumor grade (Baba et al., 2009; Hinoi et al., 2001; Lugli et al., 2008). Furthermore, the frequency of polyps in $APC^{Min+/-}$ offspring, or those induced by azoxymethane (Bonhomme et al., 2003), is increased in Cdx2 heterozygotes. While these latter observations support a tumor-suppressive function for Cdx2, it is unclear if this increase in polyposis is reflective of neoplastic-related Cdx2 functions or to Cdx2-dependent developmental events. Moreover, the potential functional overlap between Cdx1 and Cdx2 has confounded a clearer understanding of a role for Cdx in CRC.

In the present study, Cdx2 was conditionally deleted from the adult intestine in a mosaic manner to circumvent the lethality associated with complete loss of the protein. This mosaic deletion, alone or in conjunction with a Cdx1 germ line

mutation (Subramanian et al., 1995), was assessed for impact on *APC*$^{Min/+}$-associated polyposis. Loss of Cdx2 or Cdx1-Cdx2 in an *APC*$^{Min/+}$ background resulted in a marked acceleration of lethality associated with an increase in tumor incidence impacting the small intestine. Ablation of Cdx2, also resulted in a significant increase in tumor formation in the colon which was exacerbated with concomitant loss of Cdx1, particularly in the distal colon. Tumors were associated with coincident with alterations in TGF-β signaling and invasion. Finally, Cdx2 ablation resulted in the formation of villous tumors which were associated with loss of ephrinB1 expression. These findings reveal previously unrecognized roles for Cdx1 in CRC and underscore new roles for Cdx in modifying the CRC phenotype.

Experimental Procedures

Generation of Cdx-Min mutants

Cdx1$^{-/-}$, *Cdx2*$^{f/f}$, APC$^{Min/+}$ and *Villin*-CreER^{T} mice have been previously described (Hryniuk et al., 2012; Savory et al., 2009; Su et al., 1992; Subramanian et al., 1995). These animals were intercrossed and inbred for a minimum of 5 generations. To effect *Cdx2* deletion, non-transgenic control and Cre positive animals were treated with either a single 0.25mg dose of tamoxifen by oral gavage at 2 months (for mosaic deletion) or 2 mg of tamoxifen for complete deletion. Animals were maintained according to the guidelines established by the Canadian Council on Animal Care and the Animal Care & Veterinary Services of the University of Ottawa.

Histology and immunohistochemistry

Intestines were prepared as previously described (Hryniuk et al., 2012). Paraffin-embedded material was sectioned at 5 μm and frozen sections at 8 μm. Immunostaining was carried out using standard methods. Primary antibodies used were α-Cdx1 and α-Cdx2 [1;1000 dilution (Savory et al., 2009)]; α-Ki67 (1:200 dilution, Abcam); α-lysozyme (1:500 dilution, Dako); α-beta-catenin (1:50 dilution, Millipore); α-ephrin B1 (1:200 dilution, R&D systems); α-EphB2 (1:200 dilution, R&D systems); α-ZO-1 (1:200, Invitrogen); and α-pSmad2 (Ser465/467) (1/1000 dilution, Millipore). Linker antibody for EphB2 and ephrin B1 reactivity was rabbit α-goat IgG (H+L) (1:1000 dilution, Jackson ImmunoResearch Laboratories). Secondary antibodies used were HRP-conjugated goat α-mouse, goat α-rabbit, donkey α-rabbit (1/1000 dilution, Santa Cruz Biotechnologies) or goat α-rabbit

Alexa Fluor 594 (1/1000 dilution, Invitrogen). Slides were mounted using Permount (Fisher) and images captured using a Mirax Midi Scanner (Zeiss).

Quantitative polymerase chain reaction

Total RNA was extracted using Trizol reagent (Invitrogen) according to the manufacturer's instructions and used to generate cDNA by standard procedures. cDNA was subsequently amplified using gene-specific oligonucleotides with SsoFast EvaGreen Supermix (BioRad) according to the manufacturer's recommendations. PCR was performed using the MX3005P (Agilent Technologies) and results 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. Data is reflective of a minimum of 3 different biological samples and expressed as the mean +/- standard deviation. Primer sequences are available upon request.

Chromatin immunoprecipitation (ChIP)

ChIP for Cdx genomic occupancy was performed as previously described (Savory et al., 2009) using chromatin generated from C2bbe1 cells. PCR was directed across regions encompassing potential CDREs, using *Dll1* and an internal exon as positive and negative control, respectively. Oligonucleotide sequences used for ChIP are available upon request.

Promoter Analysis

P19 cells were seeded onto 6 well plates and triplicate samples transfected with the appropriate combination of expression plasmids 24 hours later. Luciferase activity was measured 48 h post-transfection using the Luciferase Assay System (Promega)

with a luminometer (Synergy H1 Hybrid Multi-Mode Microplate Reader BioTek) and normalized for transfection efficiency using β-galactosidase.

Results

Cdx1 and Cdx2 suppress $APC^{Min/+}$-induced tumorigenesis

To investigate the impact of Cdx loss on intestinal tumorigenesis, an *$APC^{Min/+}$* allele (Su et al., 1992) (denoted as Min hereafter) was bred into either the *Villin-$CreER^{T}$-$Cdx2^{f/f}$* or the *Villin-$CreER^{T}$-$Cdx1^{-/-}$$Cdx2^{f/f}$* background. As widespread loss of Cdx2 in the adult intestine is lethal (Hryniuk et al., 2012; Verzi et al., 2011), a suboptimal dose (0.25 mg) of tamoxifen was used to elicit mosaic deletion of Cdx2, permitting survival as previously described (Hryniuk et al., 2012). For simplicity, animals generated in this manner are hereafter referred to as Cdx2-Min (and derivatives thereof).

A longitudinal study revealed an early, comparable, onset of lethality of Cdx2-Min and Cdx1-Cdx2-Min mutants relative to Min offspring (Moser et al., 1990) (Figure 24A). Macroscopic examination of the intestinal tracts 12-14 weeks after tamoxifen administration revealed an approximately three-fold increase in tumor burden in the small intestine of Cdx-Min mutants compared to Min controls, with no significant difference between Cdx2-Min and Cdx1-Cdx2-Min cohorts (Figure 24B and C). While Min animals exhibited a modest bias for polyposis in the proximal small intestine, tumor incidence was uniform along the small intestine of Cdx-Min offspring (data not shown). Of note Cdx1-Min mutant mice were identical to Min offspring with respect to tumor distribution, phenotype and longevity (data not shown) suggesting that Cdx1 loss alone does not impact Min-induced tumorigenesis. Cdx2-Min mice exhibited a significant increase in tumor burden in the colon compared to Min offspring; this was increased significantly by concomitant loss of

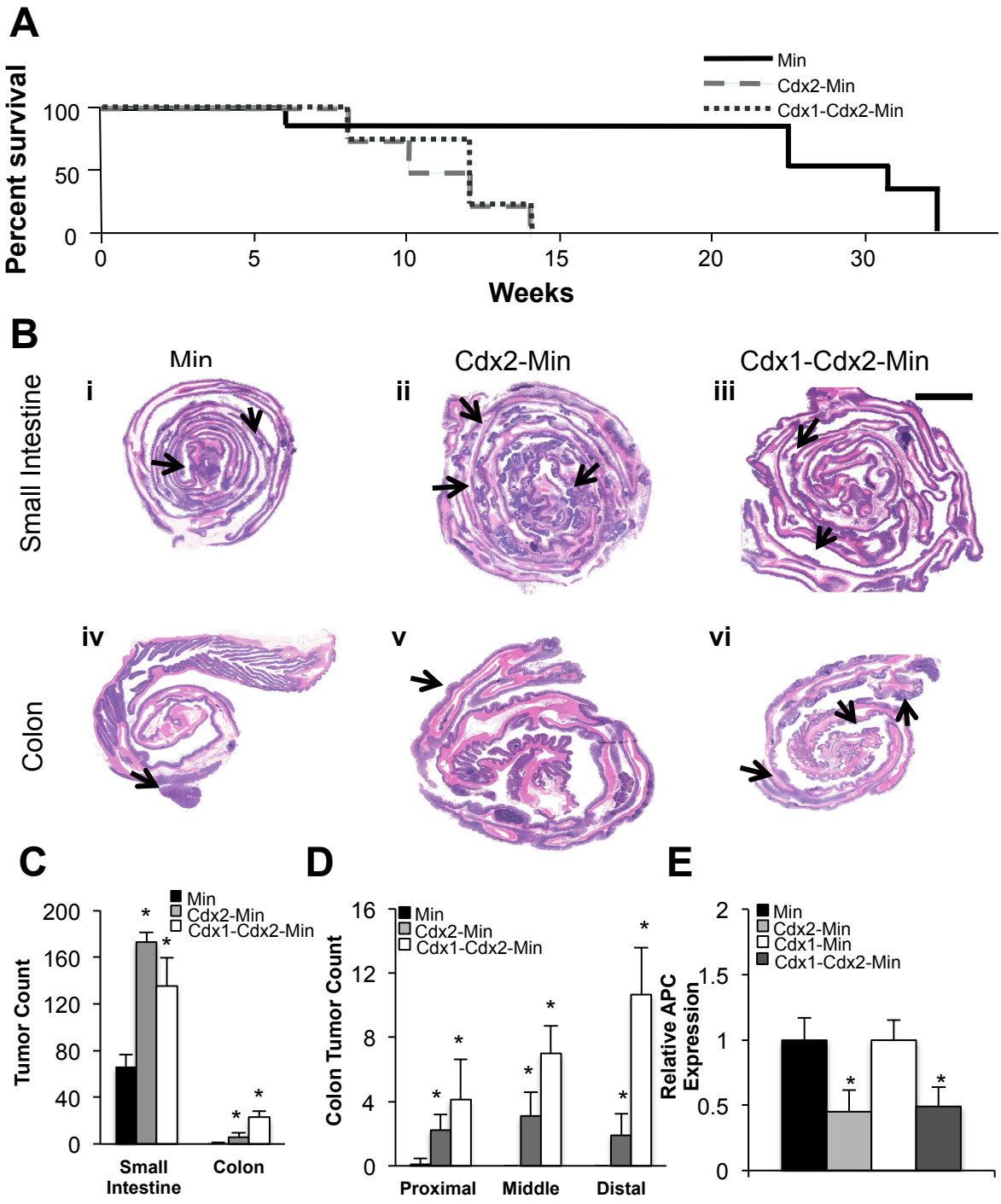

Figure 24: Cdx impacts tumor incidence and survival in Min mice.
(**A**) Kaplan-Meier curve for survival of Min, Cdx2-Min and Cdx1-Cdx2-Min mice after tamoxifen administration (n=10). P values were determined by log-rank test compared to controls. (**B**) Hematoxylin and eosin staining of small intestine and colon from Min, Cdx2-Min and Cdx1-Cdx2-Min mice. Arrows denote representative macroscopic tumors. **(C)** Incidence of macroscopic tumors in the intestinal tract and **(D)** proximal, middle and distal colon of Min, Cdx2-Min and Cdx1-Cdx2-Min mice. (**E**) qPCR analysis of *APC* expression in the colon of Min, Cdx2-Min, Cdx1-Min and Cdx1-Cdx2-Min mice. Data is expressed as the mean of 5 independent mice +/- standard deviation. Scale bar in B represents 5 mm. * $P < 0.05$ relative to Min controls by Student's *t*-test.

Cdx1 (Figure 24B and C). Polyps in Min offspring occurred exclusively in the proximal colon (Figure 24D). In contrast, Cdx2-Min mutants exhibited a uniform distribution of polyps throughout the colon, while Cdx1-Cdx2-Min mutants exhibited a biased localization towards the distal large intestine (Figure 24D).

Prior work has shown an increase in tumor incidence in the distal colon of Cdx2-Min compound heterozygotes suggested to be due to Cdx-dependent regulation of APC (Aoki et al., 2003). However, *APC* expression in the colon did not differ between Cdx1-Cdx2-Min and Cdx2-Min offspring (Figure 24E). This suggests that Cdx1 increases distal polyposis independent of effects on *APC*.

Cdx impacts cell sorting

Loss of APC results in β-catenin stabilization leading to an increase in Wnt target gene expression and aberrant proliferation (Clevers, 2006). Consistent with this, high levels of β-catenin and hyperproliferation (as evidenced by increased Ki67 expression) were seen in all tumors, irrespective of genotype (Figure 25A and B). β-catenin positive control Min tumors appeared to be encased in a sheet of normal epithelium, consistent with the ingressed morphology typical of the tubular adenomas seen in this model (Figure 25C; (Batlle et al., 2005). In marked contrast, β-catenin positive hyper-proliferative cells were distributed on the luminal surface in Cdx-Min offspring (Figure 25C), a morphology typical of villous tumors (Cortina et al., 2007).

Defects in cues directing cell sorting have been implicated in the altered distribution of neoplastic cells such as seen in villous tumors (Batlle et al., 2002; Gregorieff and Clevers, 2005; Pasquale, 2005). Consistent with this, expression of

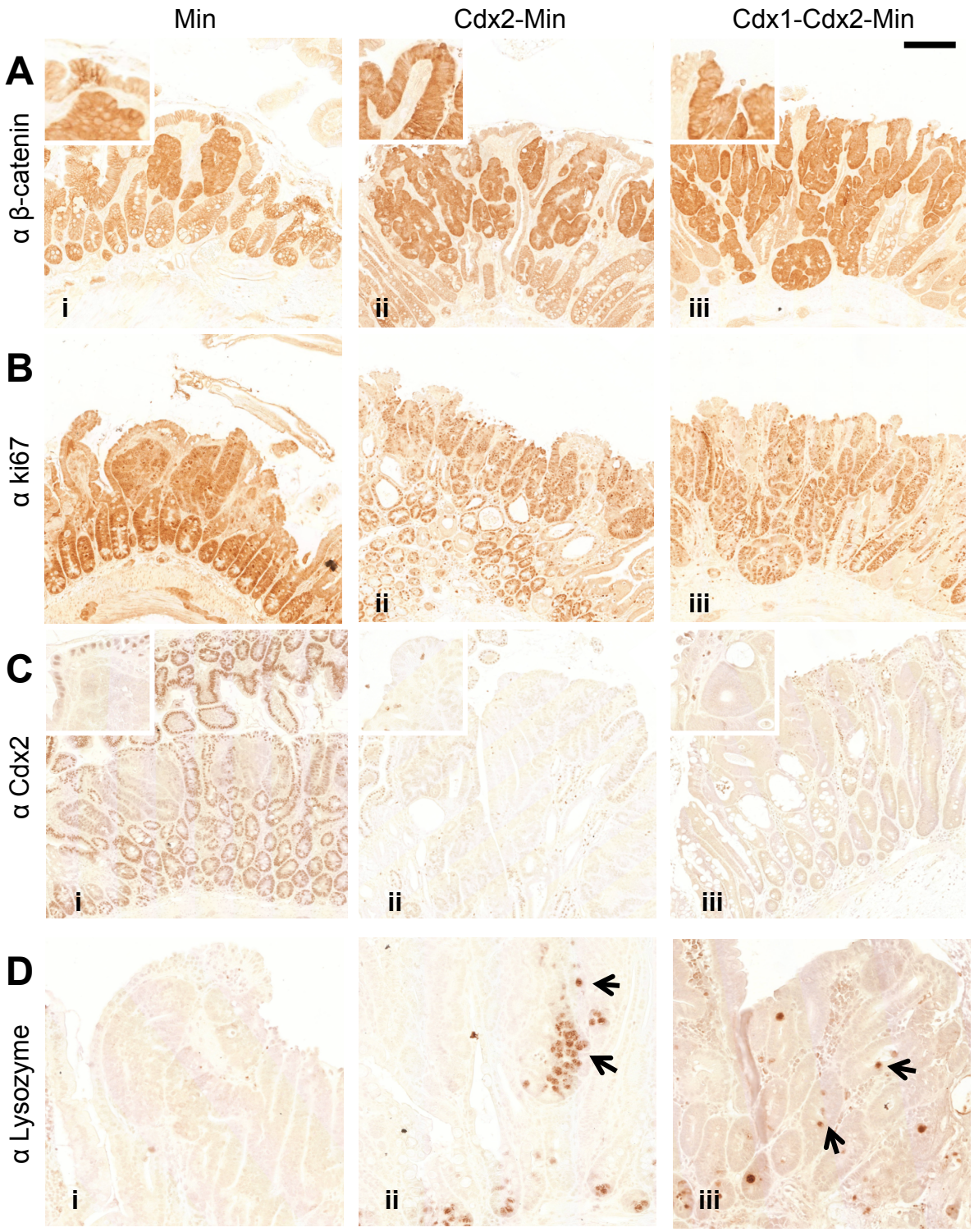

Figure 25: Cdx ablation impacts tumor cell sorting.
Immunohistochemistry for (**A**) β-catenin (**B**) Ki67 (**C**) Cdx2 and (**D**) lysozyme in the small intestine of Min, Cdx2-Min and Cdx1-Cdx2-Min mice. Note the Cdx2 positive, Ki67 negative, cells enveloping the β-catenin expressing cells in Min controls, while Cdx mutant tumors exhibit β-catenin expression on the luminal surface. Black arrows in **D** indicate ectopic lysozyme expression. Scale bar represents 200 μm.

lysozyme was observed dispersed throughout the Cdx2-Min and Cdx1-Cdx2-Min tumors (Figure 25D), indicative of a mislocalization of Paneth cells from their normal crypt niche and consistent with lesions in cell sorting processes. This phenomenon was not seen in Min tumors (Figure 25D) nor has it been observed in Cdx2 or Cdx1-Cdx2 null intestine (Hryniuk et al., 2012).

In the intestine, Eph-ephrin signaling establishes a boundary between the EphB2 and EphB3 positive cells in the crypt and ephrin B1 expressing cells of the villous epithelium involved in cell compartmentalization. Mislocalization of Paneth cells has been observed as a consequence of loss of Eph-ephrin signaling in the intestine (Batlle et al., 2002; Cortina et al., 2007), as in Cdx-Min mutant tumors. Moreover, EphB2-ephrin B1 interactions have been suggested to suppress colorectal cancers by compartmentalizing hyperproliferative intestinal cells, while lesions in this pathway can result in the migration of transformed cells over the villous, again as observed in the Cdx-Min mutants. Consistent with this, ephrin B1 protein and mRNA was decreased in the Cdx2-Min and Cdx1-Cdx2-Min tumors, but was maintained in the villus epithelium enveloping Min control polyps (Figure 26A and C). EphB2, which is usually expressed in the intestinal crypt, was seen throughout tumors irrespective of genotype, consistent with a crypt origin of these cells (Figure 26B; (Clevers and Batlle, 2006)). These observations suggest that ephrin-B1 is dependent on Cdx, and that loss of Eph-ephrin signaling contributes to the villous nature of Cdx-Min tumors.

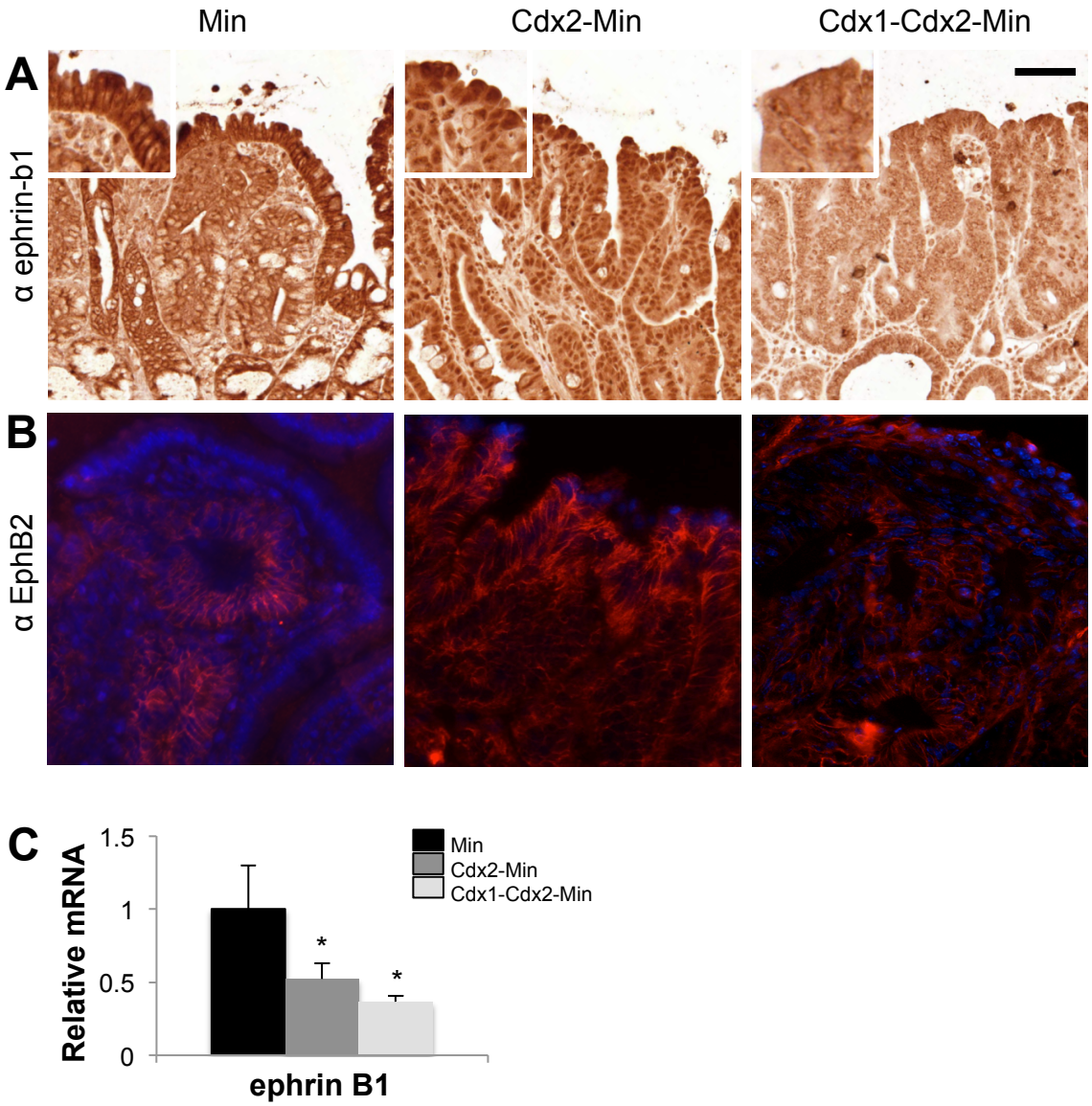

Figure 26: Cdx2 impacts ephrin B1 expression.
Immunohistochemistry for ephrin B1 (**A**) and (**B**) EphB2 (red) counterstained with Dapi (blue) in the tumors of the small intestine in Min, Cdx2-Min and Cdx1-Cdx2-Min mice. (**C**) qPCR analysis for *ephrin B1* expression in tumors from Min, Cdx2-Min and Cdx1-Cdx2-Min offspring. Results are expressed and the mean of 3 independent tumors from separate mice +/- standard deviation. * $P < 0.05$ relative to Min controls by Student's *t*-test. Scale bar represents 100μm.

Cdx impacts tumor progression and TGF-β signaling

CDX2-deficiency has been correlated with high CRC tumor grade and poor prognosis (Bakaris et al., 2008; Hinoi et al., 2001; Kaimaktchiev et al., 2004), however, it is unclear if loss of CDX2 is causal to these events or if expression is lost secondarily. Using differential staining with Periodic Acid-Schiff (PAS, goblet cells), and Churukian silver (enteroendocrine cells) we found that Cdx2-Min tumors were devoid of mature goblet and enteroendocrine cells, while Min control tumors maintained both (Data not shown). These results suggested that loss of Cdx2 contributes to the poor differentiation of Min-induced tumors (Baba et al., 2009; Hinoi et al., 2001).

Tumor progression requires the acquisition of the ability to invade neighboring tissue leading to metastases, which are responsible for 90% of cancer deaths (Sporn, 1996). We found areas of tumor cell invasion into the submucosa in both Cdx2-Min and Cdx1-Cdx2-Min offspring, but not Min tumors (Figure 27A). One of the steps associated with solid tumor extravasation and metastasis is epithelial-mesenchymal transition (EMT) (Thiery, 2003). Consistent with this, Cdx-Min tumors lost the expression of the epithelial marker *E-cadherin* and gained expression of *vimentin*, *Twist1, Zeb1* and *Zeb2* (Figure 27B) as is typical of EMT (Sipos and Galamb, 2012). Loss of epithelial character was also evidenced by mis-expression of the tight junction protein ZO-1 in Cdx null tumors (Figure 27D, (Stevenson et al., 1986)).

The TGF-β signaling pathway is a potent inducer of EMT (Akhurst and Derynck, 2001; Thiery, 2003), and TGF-β ligands have been shown to enhance invasion and metastasis of diverse cancers (Derynck et al., 2001). Consistent with

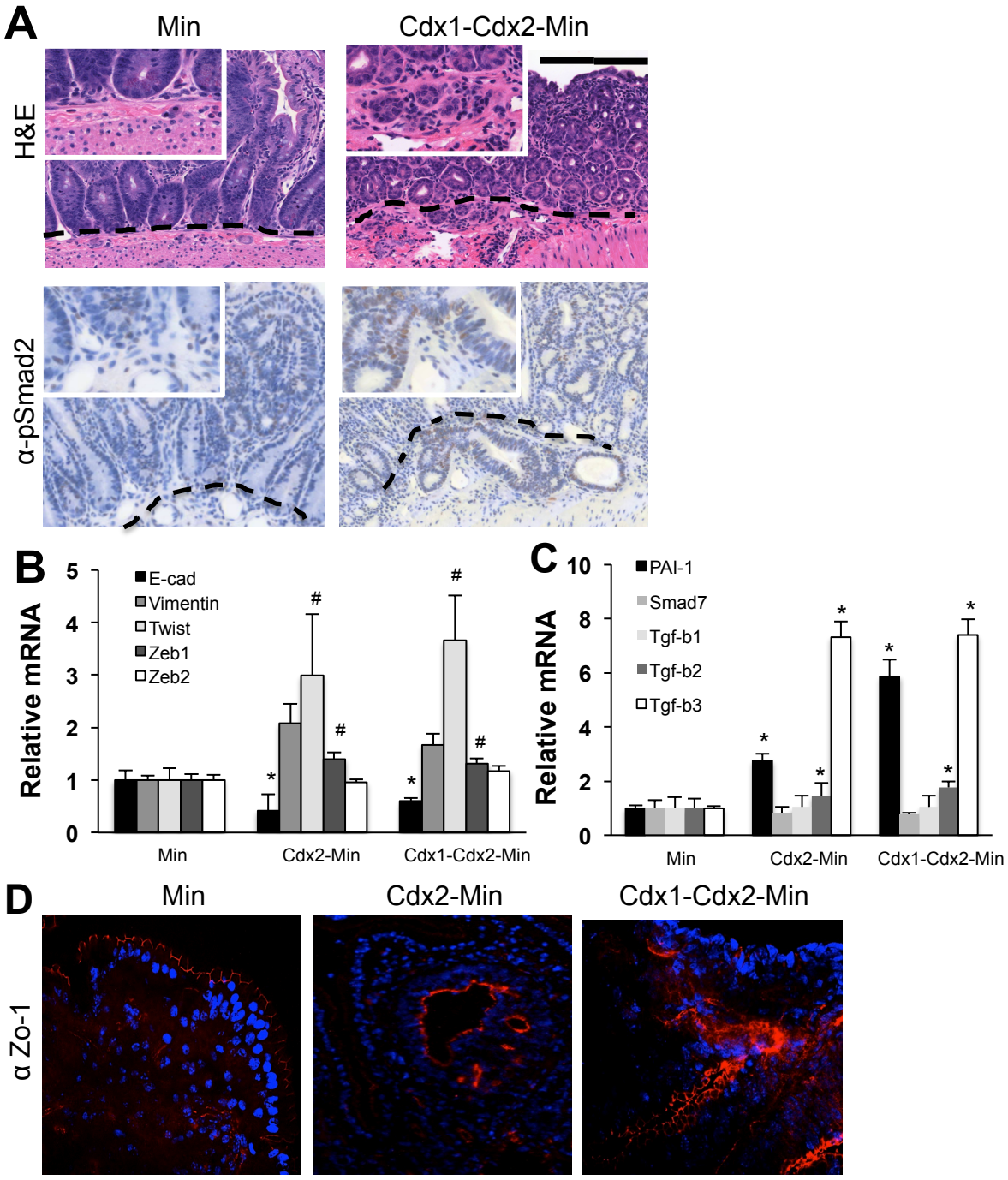

Figure 27: Loss of Cdx impacts tumor invasion.
(**A**) Hematoxylin and eosin, and pSmad2 staining of small intestine from control and Cdx1-Cdx2-Min mice. Dotted line indicates the basement membrane; note the dispersion of tumor cells into the mucosa in the Cdx1-Cdx2-Min specimen. (**B**) qPCR for *E-cadherin* (*E-cad*), *Vimentin, Twist1, Zeb1* and *Zeb2* from control and Cdx null intestinal tumors. (**C**) qPCR for *PAI-1*, *Smad7*, *Tgf-β1*, *Tgf-B2* and *Tgf-β3* from control and Cdx null tumors. (**D**) Immunohistochemistry for Zo-1 (red) counterstained with Dapi (blue) in small intestine of Min, Cdx2-Min and Cdx1-Cdx2-Min mice. Scale bar represents 200 μm. Values represent the mean of 3 independent mouse tumors +/- standard deviation. * $P < 0.005$ by Student's *t*-test; # $P < 0.05$ by students *t*-test compared to Min controls.

this, in the small intestine, we found an increase in expression of *TFG-β2*, *TFG-β3* and the TGF-β target gene *PAI-1* in Cdx-Min polyps 12-14 weeks post-deletion (Figure 27C). pSmad2 levels were also increased in the leading edge of invasive Cdx-Min tumors (Figure 27A). These findings suggest that aberrant activation of the TGF-β signaling pathway may contribute to tumor progression in Cdx-Min mutants.

Polyps arising in the colon of Cdx-Min offspring exhibited similar characteristics to those of the small intestine, including a villous morphology, increased β-catenin expression and hyperproliferation (Figure 28A-D). The TGF-β pathway has significant influence on colorectal tumorigenesis (Sodir et al., 2006; Yoshinaga et al., 2008) and can contribute to metastatic intestinal adenocarcinomas, both with (Munoz et al., 2006; Sekiya et al., 2004) and without (Zhu et al., 1998) Wnt activation. Given the marked increase in tumor incidence throughout the colon in Cdx-Min mutants, relative to Min offspring, we assessed expression of TGF-β effectors either 48 hours after complete Cdx2 deletion or in polyps following mosaic excision. TGF-β ligand expression was unaffected following acute Cdx2 deletion in both Cdx2-Min and Cdx1-Cdx2-Min backgrounds. However, expression of *SARA* (smad anchor for receptor activation, also known as *Zfyve9*) was lost 48 hours post deletion in the colon of Cdx1-Cdx2-Min offspring (Figure 29A). The finding that deletion of both Cdx1 and Cdx2 was necessary to impact *SARA* expression is consistent with functional overlap.

SARA facilitates the phosphorylation of Smad2 and Smad3, and loss of *SARA* has been shown to impact Smad2/3 protein expression (Itoh et al., 2002; Runyan et al., 2009). Consistent with this, pSmad2 was marginally increased in Cdx2 mutant

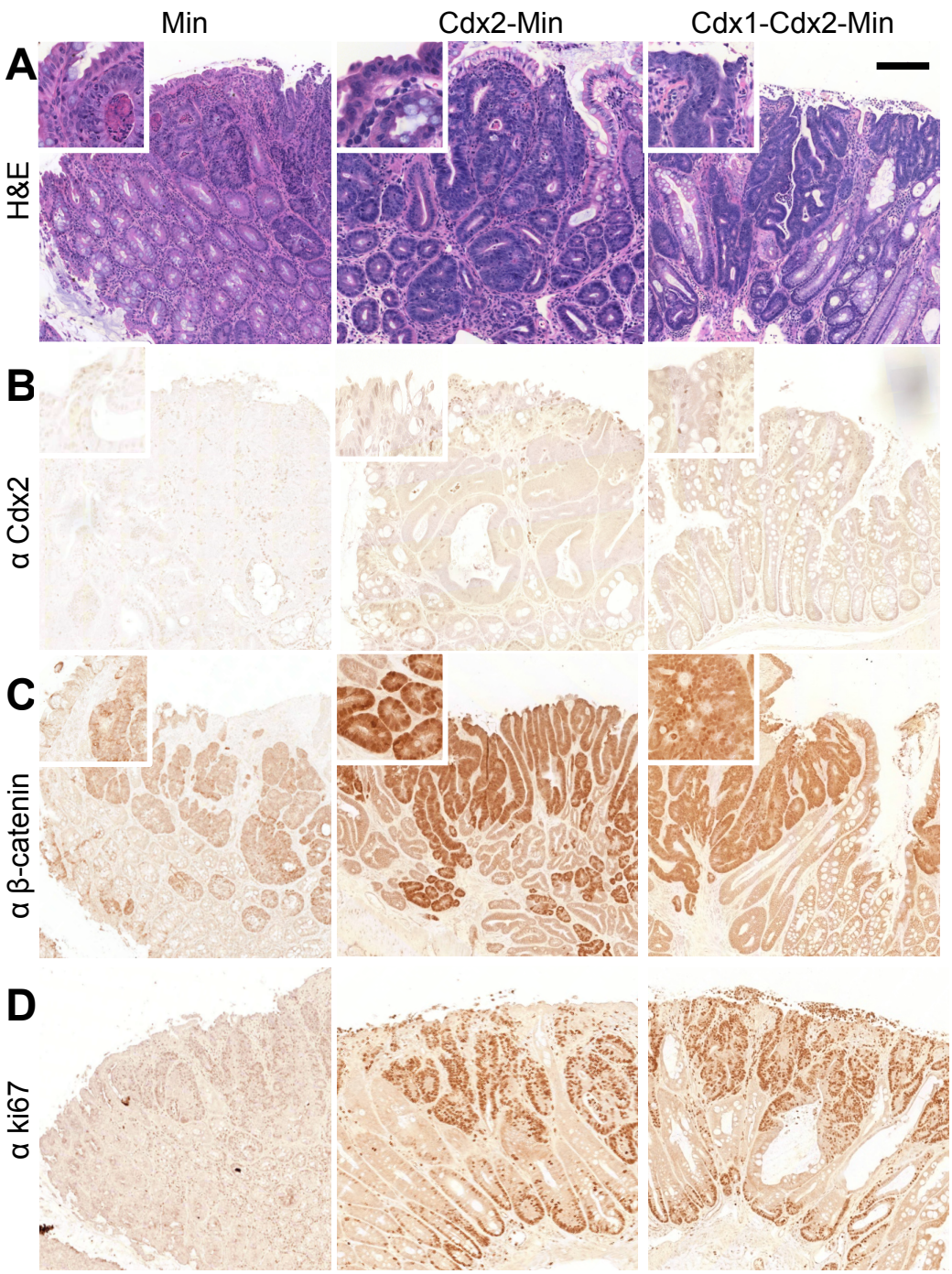

Figure 28: Tumor formation in colons of Cdx mutant mice.
Analysis of colon tumors by (A) Hematoxylin and eosin staining. Immunohistochemistry for Cdx2 (B), β-catenin (C) and Ki67 (D) from Min, Cdx2-Min and Cdx1-Cdx2-Min mice at 12-14 weeks post-tamoxifen treatment. Scale bar represents 200 μm.

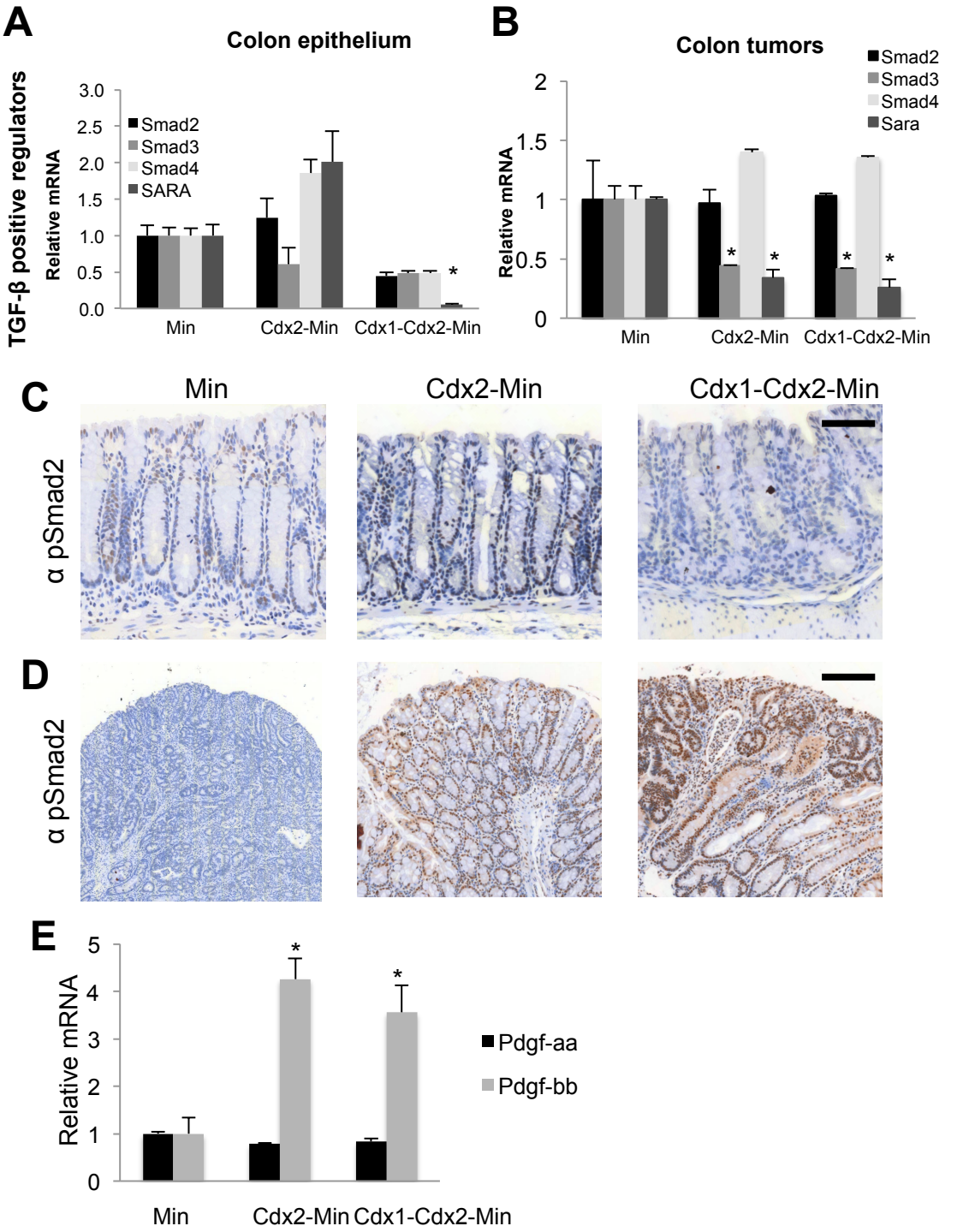

Figure 29: Cdx ablation impacts TGF-β signaling.
(**A**) qPCR analysis for expression of TGF-β intermediaries in the colon epithelium 48 hours after total Cdx2 deletion. (**B**) qPCR for TGF-β intermediaries in colon tumors from Min and Cdx-Min offspring. Immunohistochemistry for pSmad2 in colons 48 hours following Cdx2 deletion (**C**) and in tumors (**D**) from control and Cdx-mutants. (**E**) qPCR for the expression of *Pdgf-AA* and *Pdgf-BB* in colon tumors. Error bars represent standard deviation from the mean of 3 independent samples. * $P < 0.05$ by Student's *t*-test. Scale bars represents 100 (**C**) and 200 (**D**) μm.

large intestine concordant with a modest increase both *Smad2* and *SARA* transcripts in this background. While the basis for this is presently unclear, it may be indicative of a compensatory response to Cdx2 loss. In contrast, both Smad2 phosphorylation and *SARA* expression is attenuated in Cdx1-Cdx2 double mutants (Figure 29C).

In contrast to acute events, pSmad2 levels were markedly increased in polyps in both Cdx2-Min and Cdx1-Cdx2-Min colons (Figure 29D), despite loss of *SARA* expression in both of these backgrounds (Figure 29B). Phosphorylation of Smad2 can occur *via* TGF-β independent pathways (Brown et al., 1999; Funaba et al., 2002; Porsch et al., 2014). Consistent with this, induction of *Pdgf-BB* was seen in Cdx-Min colon tumors in a manner that closely correlated with both pSmad2 levels and tumor incidence (Figure 29E). pSmad3 was not appreciably altered under any condition examined (data not shown).

SARA is a Cdx target gene

The rapid loss of *SARA* expression following acute Cdx2 deletion is suggestive of direct regulation. Consistent with this, Transcriptional Element Search System analysis identified potential Cdx response elements (CDREs) in the proximal *SARA* promoter (Figure 30A) which are phylogenetically conserved (Figure 30B). ChIP analysis revealed that CDX2 was enriched in proximity to both of the putative CDREs in C2BBe1 (Figure 30C), and Cdx2 induced transcription from this promoter in cell-based reporter assays (Figure 30D). Taken together, these data suggest that Cdx may impact SARA-dependent TGF-β signaling relevant to tumorigenesis. Subsequent events promote tumorigenesis by TGF-β independent pathways (Figure

30E). Notably, this relationship appears to be limited to the colon, as *SARA* expression was not impacted in the small intestine.

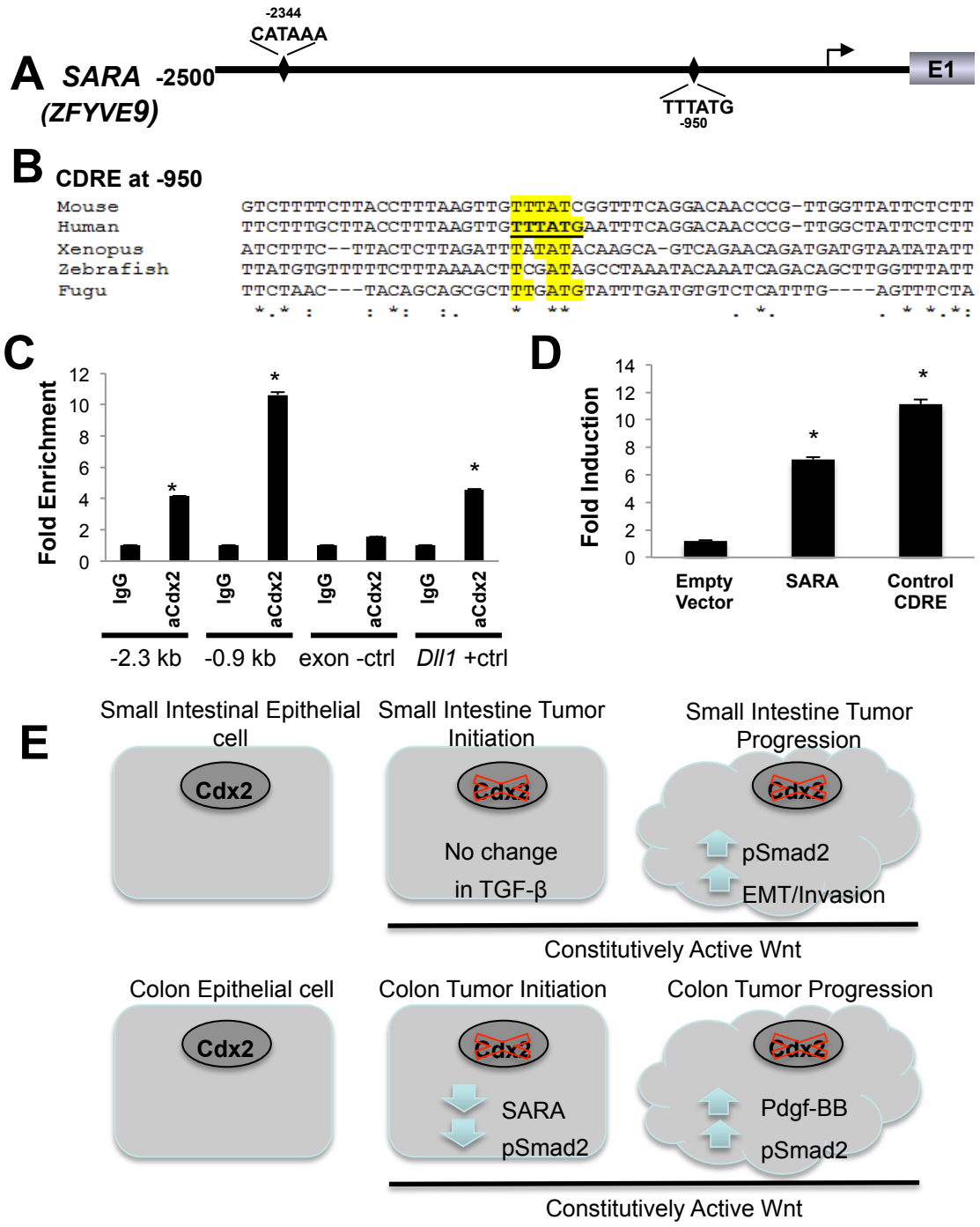

Figure 30: *SARA* is a Cdx2 target gene.
(**A**) Schematic representation of putative CDREs found in the *SARA* promoter. (**B**) Conservation of the proximal CDRE in the *SARA* promoter. (**C**) Chromatin immunoprecipitation (ChIP) analysis of the *SARA* locus from C2BBe1 cells showing Cdx2 occupancy of the regions encompassing the CDRE at -0.9kb and -2.3kb, but not the downstream exon. *Dll1* was used as a positive control. (**D**) Cdx2 induces expression from the *SARA* promoter in cell-based assays. Empty vector and a control CDRE expression vector were used as negative and positive controls respectively. (**E**) Model for tumor progression in the colon upon loss of Cdx2. * $P < 0.05$ relative to control by Student's *t*-test.

Discussion

Cdx1 and Cdx2 act as tumor suppressors

Aberrant Wnt signaling, evoked by inactivating mutation of APC, is a frequent early event in human colorectal tumorigenesis, and comparable murine APC alleles have been used to model this relationship (Grady and Markowitz, 2002; Gregorieff and Clevers, 2005; Nathke, 2004). Such models, however, develop benign polyps confined to the small intestine and proximal cecum, which differs markedly from human Wnt-induced polyps which manifest primarily in the distal colon and progress to invasive carcinoma. Prior studies have suggested Cdx2 has both tumor promoter (Domon-Dell et al., 2003; Salari et al., 2012; Subtil et al., 2007; Witek et al., 2005) and tumor suppressive (Baba et al., 2009; Beck et al., 2003; Bonhomme et al., 2003; Guo et al., 2004; Pilozzi et al., 2004; Wong et al., 2004) potential; however, a comprehensive analysis somatic inactivation of Cdx2 in colorectal tumorigenesis, and the impact of Cdx1 in this process, has not been rigorously evaluated. In the present study, we used somatic inactivation of Cdx2 alone, or with loss of Cdx1 to better understand the roles of these transcription factors in a murine Min model. Using this approach, we found novel roles for Cdx1 in suppressing colorectal tumor formation, and for Cdx function in tumor cell sorting, invasion and TGF-β signaling. The Cdx-Min model appears to represent a novel murine system that more closely reflects human CRC, and provides a tractable model to further explore the impact of Cdx in intestinal tumorigenesis.

Cdx1 expression is decreased or lost in many colorectal cancer cell lines as well as in patients (Pilozzi et al., 2004; Suh et al., 2002; Wong et al., 2004), and can

reduce proliferation when expressed in human cancer cells in culture (Guo et al., 2004). However, loss of Cdx1 alone has no impact on intestinal tumorigenesis in murine systems (Bonhomme et al., 2008). Our present observations strongly suggest that this is due to functional overlap, as concomitant loss of Cdx1 and Cdx2 had a significant impact on tumor formation in the large intestine with a biased incidence towards the distal colon, as seen in human CRC.

In contrast to the colon, somatic deletion of Cdx2 in the adult increased the incidence and growth of Min-induced tumors in the small intestine, but this was not exacerbated by concomitant deletion of Cdx1. This is consistent with prior work suggesting that Cdx1 does not contribute to Cdx2-dependent tumor suppression in the small intestine. We also found tumors to be evenly distributed throughout the large intestine of Cdx2-Min offspring, while prior work using germ-line Cdx2 heterozygotes found Min-induced tumors to be distally located, and not increased in incidence in the small intestine (Aoki et al., 2003). The bases for these discrepancies are presently unclear, but may relate to the somatic versus germ line loss of Cdx2. Differences may also be explained by the mechanisms driving loss of Cdx2; in our model system, we excised both copies of Cdx2 throughout the intestine, while polyps arise in *Cdx2*$^{+/-}$ animals only after silencing of Cdx2 expression from the residual wild type allele (Aoki et al., 2003; Bonhomme et al., 2003).

Cdx loss impacts tumor phenotype

Tumors in Cdx1-Cdx2-Min offspring were minimally differentiated, invasive and with a villous morphology. The latter two observations coincided with an EMT

signature and loss of ephrin B1, respectively, underscoring previously unsuspected roles for Cdx in CRC progression.

Cell adhesion and repulsion are important contributors to normal intestinal morphogenesis and lesions in these processes can impact CRC progression. The Wnt target genes *EphB2* and *EphB3*, expressed in the intestinal crypt, together with their ephrin B1 ligand, expressed on the villus epithelium, elicit cell repulsive mechanisms resulting in ingression of hyperproliferative $APC^{+/min}$ crypt cells, yielding the morphology typical of adenomatous polyps (Batlle et al., 2005). Loss of this compartmentalization, such as evoked by loss of ephrin B1, can lead to villous adenomas (Cortina et al., 2007). This relationship is consistent with the loss of ephrin B1 and the villous phenotype of polyps observed in Cdx-Min offspring.

Cdx and tumor progression

Unlike human CRC, the polyps arising in APC mouse models rarely progress. EMT is associated with malignant progression, and is involved in the acquisition of an invasive nature in CRC (Rubio et al., 2008). We found that Cdx-Min tumors appear to invade the basement membrane, with concomitant EMT molecular signatures. Consistent with this, E-cadherin has been previously reported to be downregulated upon loss of Cdx2 (Funakoshi et al., 2010; Lorentz et al., 1997). It is also notable that activation of the PI3K/Akt signaling cascade has also been associated with EMT (Gulhati et al., 2011). mTOR, a downstream effector of PI3K/Akt, is impacted in Cdx2-Min tumors offspring (Aoki et al., 2003), suggesting a basis for the EMT seen in the present study.

A number of pathways have been shown to interact with aberrant Wnt signaling to induce metastatic CRC, including the TGF-β signaling pathway (Grady and Markowitz, 2002; Ku et al., 2007; Takayama et al., 2006). Indeed, tumor growth and invasion in Min mice is accelerated by loss of Smad2 or Smad4 (Hamamoto et al., 2002; Takaku et al., 1998). Loss of Smad signaling in the immune compartment is thought to underlie, at least in part, the tumor phenotype seen in these mice (Kim et al., 2006). However, deletion of the transforming growth factor β receptor type II in the intestinal epithelium also leads to malignant transformation in a Min background (Munoz et al., 2006), demonstrating that disruption of TGF-β signaling in the epithelium proper can impact intestinal tumorigenesis.

Currently, a critical role for SARA in recruitment of Smad2/3 to the to the TGF-β receptor is controversial (Bakkebo et al., 2012). However, mutations in SARA have been detected in approximately 33% of colon cancers (Forbes et al., 2011; Sjoblom et al., 2006). *SARA* expression was significantly downregulated in Cdx-Min backgrounds in a manner that correlated with tumor incidence in the distal colon. Moreover, *SARA* levels declined rapidly in Cdx1-Cdx2 mutants consistent with expression profiles of direct target genes (Grainger et al., 2011). In agreement with this, the *SARA* promoter was found to harbor a number of conserved CDRE, and was occupied by Cdx2 *in vivo.* Moreover, these promoter sequences responded to Cdx2 in cell-based models.

The above findings suggest that *SARA* is a direct Cdx target gene, and that Cdx loss may impact on TGF-β signaling through *SARA,* contributing to the shift in tumor incidence to the distal colon seen in Cdx1-Cdx2-Min tumors. In agreement with this,

pSmad2 was attenuated rapidly in the colon of Cdx1-Cdx2-Min mice following Cdx2 deletion. pSmad2 was not impacted in Cdx2-Min mutants, consistent with functional overlap between Cdx members (Hryniuk et al., 2012), and underscores critical, previously unrecognized, roles for Cdx1 in this process.

pSmad2 levels were increased in colon tumors. This induction correlated with expression of Pdgf-BB, which is known to increase Smad2 phosphorylation (Porsch et al., 2014). This biphasic change in pSmad2 expression in the Cdx1-Cdx2 tumors has been observed in other models of colorectal cancer, and is consistent with a role for TGF-β signaling as a tumor- suppressor and -promoter in a stage-dependent manner (Chung, 2000; Grady and Markowitz, 2002; Pardali and Moustakas, 2007).

pSmad and *SARA* levels were unperturbed in the small intestine, irrespective of Cdx status, suggesting that Cdx exerts different influence on Min-induced polyposis along the GI tract. This is similar to observations of TGF-β signaling in intestinal tumorigenesis elicited by loss of estrogen receptors α and β (Cho et al., 2007; Hasson et al., 2014). The Cdx-Min model described in this study will likely be of relevance to a better understanding of the significant percentage of human CRC cases in which CDX2 has been lost.

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CHAPTER 4: CDX2 impacts intestinal *ephrinB1* expression through the Notch pathway

CDX2 impacts intestinal *ephrinB1* expression through the Notch pathway

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Author contributions:

Alexa Hryniuk designed and carried out experiments, wrote the manuscript and made figures.

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

Abstract

Colorectal cancer is the third most common cause of cancer-related death worldwide. In humans, the majority of colorectal cancer is initiated via loss-of-function mutations in APC, a negative regulator of the canonical Wnt signaling pathway. Although comparable murine APC alleles also cause polyps, these lesions do not progress to carcinomas in the absence of additional mutations. Cdx transcription factors are important for homeostasis of the intestinal epithelium in the adult and loss of Cdx2 has been associated with more aggressive human colorectal cancer. Moreover, loss of Cdx1 and Cdx2 in a murine $APC^{Min/+}$ background leads to an increase in tumor incidence throughout the intestinal tract. Cdx2 loss in this context also leads to an alteration in tumor morphology to a villous phenotype as well as an increase in invasive capacity. However, the Cdx-dependent mechanisms that contribute to these phenotypes are poorly understood. To this end, we modeled *CDX2* loss of function in SW480 colorectal cancer cells. CDX2 inactivation resulted in an increase in the SW480 tumorigenetic capacity, including an increase in invasive character. We also found that CDX2 loss led to an attenuation in Notch signaling, at least in part through Cdx-dependent expression of *Dll1*. Finally, we found that Notch is a direct regulator of *ephrinB1* both in SW480 cells and *in vivo.* As ephrinB1 is known to be downregulated in intestinal cancer and contributes to tumor phenotype, these findings underscore a previously unrecognized relationship for Cdx2 in Notch-dependent regulation of Eph-ephrin signaling and suggests a mechanism by which Cdx2 impacts the colorectal cancer phenotype.

Introduction

The intestinal epithelium renews itself throughout the life span of the organism. In the mouse, intestinal epithelial cells are replenished every 5-7 days by intestinal stem cells resident at or near the base of the crypt (Barker et al., 2007; Sangiorgi and Capecchi, 2008). These stem cells give rise to rapidly dividing transit-amplifying cells that subsequently differentiate into the mature cells of the intestinal epithelium. These cells are grouped into absorptive (enterocytes) and secretory (goblet, Paneth and enteroendocrine) cells (Wells and Melton, 1999).

The constant renewal of the intestinal epithelium can lead to genetic changes that can drive transition to precancerous adenomas which can progress to invasive carcinomas. The predominant initial genetic lesion in human colorectal cancer is inactivating mutations of the negative Wnt regulator *adenomatous polyposis coli* (*APC*). This leads to an increase in canonical Wnt signalling which drives unrestricted hyperproliferation of crypt cells and formation of adenomatous polyps. Accumulation of subsequent mutations, such as in *KRAS,* as well as tumour-specific genomic imbalances, results in progression of initial adenomas to carcinomas (Halberg et al., 2000; Polakis, 2007; Sansom et al., 2006).

Members of the caudal type homeobox (Cdx) family of transcription factors, Cdx1 and Cdx2, are essential for development of the intestinal tract and have roles in homeostasis of the adult intestinal epithelium (Chawengsaksophak et al., 1997; Gao et al., 2009; Grainger et al., 2010; Hryniuk et al., 2012; Verzi et al., 2010; Verzi et al., 2011). There is considerable evidence suggesting that CDX2 status impacts the colorectal cancer (CRC) phenotype. For example, ~30% of human CRC exhibit loss

of CDX2 and this is associated with higher tumor grade (Baba et al., 2009; Hinoi et al., 2001; Lugli et al., 2008). Furthermore, the frequency of polyps in *APC*$^{Min+/-}$ mice, or those induced by azoxymethane (Bonhomme et al., 2003), is increased in Cdx2 heterozygotes. However, until recently, our ability to explore murine models of Cdx function has been restricted by the peri-implantation lethality of Cdx2 null mouse mutants (Chawengsaksophak et al., 1997) and potential functional overlap between Cdx1 and Cdx2, which are co-expressed throughout the intestinal tract(Verzi et al., 2011). To address these limitations, a conditional mutagenesis strategy was used to delete Cdx2 in the intestine, either alone or with a Cdx1 germ line mutation, in an *APC*$^{Min+/-}$ background. Loss of Cdx function resulted in a marked increase in polyposis throughout the intestinal tract, and increase in invasive characteristics. In addition, Cdx mutants exhibited villous tumors coincident with loss of ephrinB1 expression, suggestive of lesions in Eph-ephrin dependent cell sorting processes (Hryniuk et al., 2014). However, *ephrinB1* appeared to be an indirect Cdx target. In the present study, we further explored the impact of Cdx on intestinal tumorigenesis using CRC-derived SW480 cells. shRNA-mediated loss of CDX2 in SW480 cells was associated with a marked acceleration of growth, as well as an increase in indices of invasion and stem cell character. In addition, attenuation of CDX2 resulted in a reduction in *ephrinB1* expression likely by indirect means, consistent with prior *in vivo* observations. In this regard, we found that Cdx2 loss impacted the Notch signaling pathway, both in SW480 cells and *in vivo,* and that *ephrinB1* is a direct Notch target. These findings reveal a previously unrecognized

mechanism for Cdx in modulating CRC through Notch-dependent regulation of ephrinB1.

Methods

Generation of stable cell lines

SW480 cells were transfected with shRNA for CDX2 or a control shRNA vector using Lipofectamine and selected by culture in the presence of 15 μg/ml of puromycin. Clones of surviving cells were isolated, expanded and CDX2 expression assessed by Western blot analysis.

Western immunoblotting

Cells were disrupted using lysis buffer, sonicated and cleared by centrifugation. Protein was resolved on a 12% SDS-PAGE gel, transferred to a PVDF membrane (Millipore) which was blocked with 5% BSA in PBS or 5% non-fat milk powder in PBS:0.1% Tween 20 (PBST), then incubated overnight with appropriate primary antibody at 4°C. Primary antibodies used were rabbit polyclonal anti-Cdx2 (1/1000 dilution, (Savory et al., 2009); anti-NICD (1/500 dilution, BD Biosciences); anti-beta-actin (1/2000 dilution, Abcam); and anti-CyclophilinB (1/10000 dilution, Abcam). Secondary antibodies (HRP-conjugated anti-rabbit or anti-mouse IgG; 1/25 000 dilution, Santa Cruz Biotechology) were detected by ECL (Millipore) according to the manufacturer's instructions.

Semi-quantitative reverse transcriptase PCR (RT-PCR)

RNA was extracted from cells or mouse small intestines using Trizol reagent (Invitrogen) and used to generate cDNA by standard procedures. cDNA was subsequently amplified by PCR using oligonucleotides primers directed against *Cdx2*, *Dll1*, *ephrinB1*, *Hes1*, *Tff3*, *Math1, Lgr5, Smoc2* or *β-actin*. PCR products were

resolved by electrophoresis on a 2% agarose gel, visualized by ethidium bromide and quantified by ImageJ (NIH) using β-actin values to normalize for input from the linear range of amplification. Primers used for PCR amplification are available upon request.

Animals

Cdx1$^{-/-}$, *Cdx2*$^{f/f}$, and *Villin-CreER*^{T} mice have been previously described (Hryniuk et al., 2012; Savory et al., 2009; Subramanian et al., 1995). To effect *Cdx2* deletion, Cre positive animals were treated with a single 2 mg dose of tamoxifen; non-transgenic animals, treated in an identical manner, were used as negative controls. Inhibition of Notch signaling was effected by treatment with 3 mMol/kg of DAPT by oral gavage for 5 consecutive days at 2 months of age. Animals were maintained according to the guidelines established by the Canadian Council on Animal Care and the Animal Care & Veterinary Services of the University of Ottawa.

Histology and immunohistochemistry

Intestines were prepared as previously described (Hryniuk et al., 2012). Paraffin-embedded material was sectioned at 5 μm. Immunostaining was carried out using standard methods. Primary antibodies used were α-Cdx2 [1;1000 dilution (Savory et al., 2009)]. Secondary antibodies used were HRP-conjugated goat α-rabbit (1/1000 dilution, Santa Cruz Biotechnologies). Slides were mounted using Permount (Fisher) and images captured using a Mirax Midi Scanner (Zeiss).

In situ hybridization

Intestinal sections were cut at 10 μm and slides were processed as described above. Probes for *Hes1* were synthesized using the DIG RNA labeling system (Roche) according to manufacturer's recommendations. *In situ* hybridization was carried out as previously described (Gregorieff and Clevers, 2010) and slides mounted using Faramount Aqueous Mounting Medium (Dako).

Chromatin immunoprecipitation (ChIP)

ChIP was performed as previously described (Savory et al., 2009) using chromatin generated from colorectal cancer cells (SW480). PCR was directed across regions encompassing potential Notch binding sites using Hes1 as a positive control. Oligonucleotide sequences used for ChIP are available upon request.

Promoter analysis

P19 cells were seeded onto 6 well plates and triplicate samples transfected with the appropriate combinations of plasmids 24 hours later. Luciferase activity was measured 48 h post-transfection using the Luciferase Assay System (Promega) with a luminometer (Synergy H1 Hybrid Multi-Mode Microplate Reader BioTek) and normalized for transfection efficiency using β-galactosidase.

DAPT and valproic acid treatment

SW480 CDX2 knockdown cells were treated with either DAPT (0-100 uM) (Sigma) or Valproic acid (VPA) (5 mM)(ICN Biochemicals) and harvested 36 hours post treatment.

Transwell assay

Matrigel invasion assays were performed with SW480 cells using Corning transwell inserts (24-well plates, 6.5 mm diameter, 8 μm pore size, Fisher). Cells were serum starved (0.25% FBS media) overnight, then collected and resuspended in media with 0.25% FBS at a density of 5×10^5 cells/ml. Culture media with 20% FBS was added to the lower chamber and 100 μl of the resuspended cells were added onto the top of a 250 ug/ml Matrigel layer. Forty hours later, cells and Matrigel on the topside of the transwell were removed. Cells on the lower surface of the membrane were fixed with 4% PFA in PBS, stained with 25 ug/ml PI and viewed with Zeiss AxioObserver D1 using AxioVision Rel. 4.8.

Anchorage independent growth

SW480 cells were collected and suspended in 2x media with 20% FBS at a density of 1×10^5 cells/ml and 100 ul of resuspended cells was plated in 0.3% LMP agarose on a 0.5% LMP agarose base layer. Media (500 ul) was replenished twice weekly and colonies visualized with a dissecting microscope after 14 days in culture.

Results

CDX2 deficient SW480 cells.

CDX2 loss is associated with increased tumor grade and decreased survival, however, the mechanism underlying this observation is poorly understood. To further explore the basis for CDX2 in intestinal tumorigenicity, we assessed the consequence of CDX2 loss in SW480 cells, which are derived from human colorectal cancer. Independent CDX2 deficient SW480 clonal lines, designated Sh1 and Sh2, were derived using shRNA and confirmed by Western blot and RT-PCR analysis (Figure 31A and B). CDX1, the only other Cdx member found in the intestine, is not expressed in SW480 cells (data not shown).

Cdx2 impacts Notch signaling in SW480 cells

CDX2 is necessary for intestinal epithelial differentiation (Beck and Stringer, 2010; Hryniuk et al., 2012; Verzi et al., 2010; Verzi et al., 2011) and impacts tumor phenotype (Hryniuk et al., 2014). A survey for markers of differentiation was therefore assessed between control and CDX2 knockdown SW480 cell lines. This survey revealed an increase in expression of the secretory cell makers *Tff3* and *Math1* in the cell lines lacking CDX2 (Figure 31C). An increase in the levels of such secretory cell markers is also seen following disruption in the Notch signaling pathway(van Es et al., 2005; van Es et al., 2010). Consistent with this, *Dll1*, which encodes a Notch ligand, is downregulated in Cdx2 deficient SW480 cells (Figure 31E), in agreement with prior work illustrating that *Dll1* is a Cdx target gene (Grainger et al., 2011).

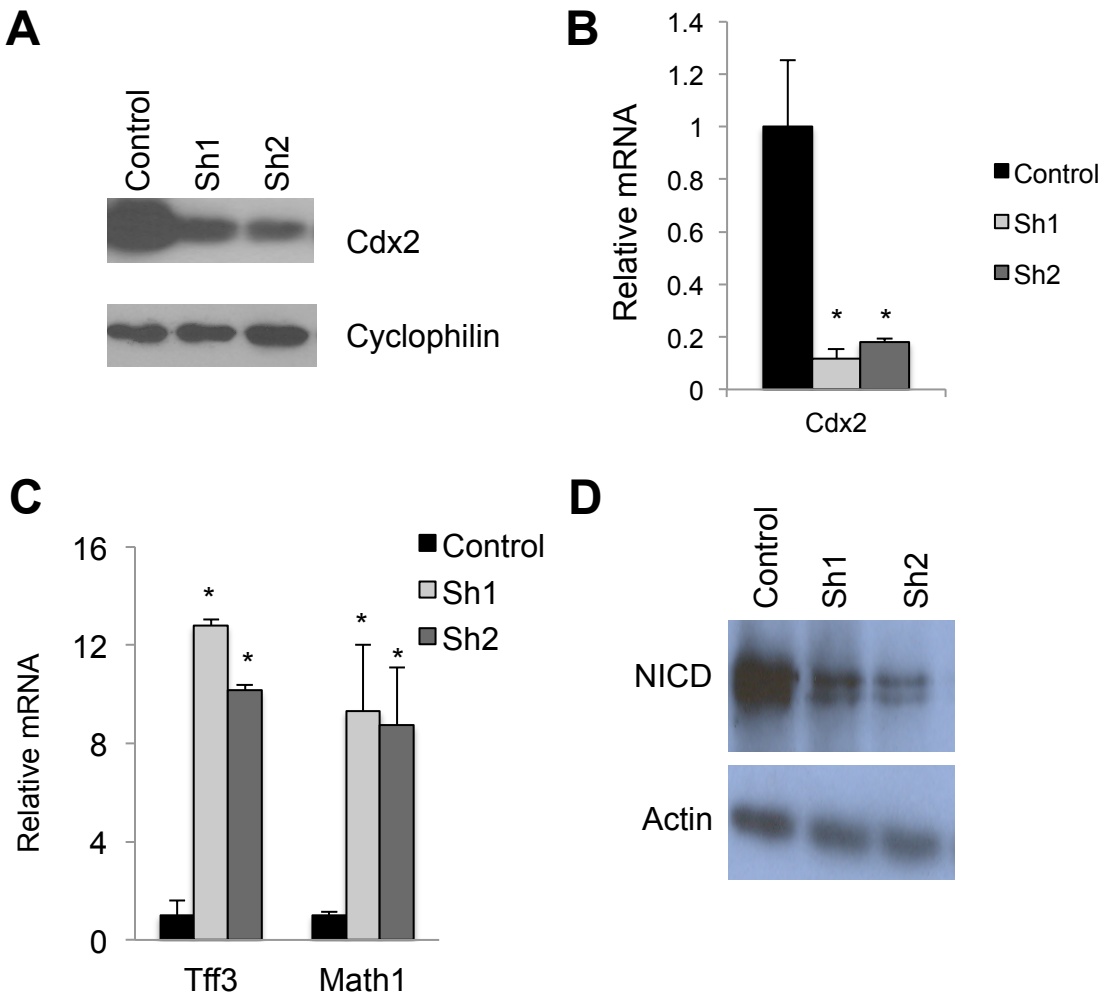

Figure 31: Loss of Cdx2 by shRNA knockdown in SW480 cells disrupts Notch signaling.
(A) Western blot analysis for Cdx2 in control and knockdown cells. CyclophilinB was used as a loading control. (B) RT-PCR analysis for expression of Cdx2 from control compared to Sh knockdowns. (C) RT-PCR form SW480 cells for secretory cell markers in control and CDX2 knockdowns. (D) Western blot for the Notch Intracellular Domain (NICD) in control and CDX2 sh knockdown SW480 cells. Actin was used for a loading control. (E) RT-PCR form SW480 cells for Dll1 and ephrin B1 in control and CDX2 knockdowns. Error bars represent the standard deviation from 3 independent samples. * $p<0.01$ by Student's T-test.

Activation of the Notch pathway results in proteolytic release of the Notch intracellular domain (NICD) from the membrane. The NICD then translocates to the nucleus where it binds with RBP-Jκ on Notch target genes to increase their transcription(D'Souza et al., 2008; Fortini, 2009). Knockdown of CDX2 in the SW480 cells resulted in a decrease in the NICD compared to controls (Figure 31D) illustrating that Cdx impacts functionally on Notch signaling in SW480 cells.

Cdx2 regulates ephrinB1 through the Notch pathway

We recently reported that Cdx2 deletion in an $APC^{min/+}$ background results in the formation of villous, rather than ingressed tumors, and that this altered morphology is associated with loss of *ephrinB1* (Hryniuk et al., 2014); a similar loss of *ephrinB1* expression was seen in CDX2 deficient SW480 cells (Figure 31E). *EphrinB1*, however, does not appear to be a direct Cdx target, as its expression is not impacted by acute Cdx2 deletion in murine small intestines, in contrast to *Dll1* (Figure 32A).

Prior work has shown that *ephrinB1* may be regulated by Notch signaling (Koo et al., 2009), suggesting that Cdx2 may impact *ephrinB1* expression through the Notch pathway. Consistent with this, expression of both *ephrinB1* and *Hes1*, a known Notch target gene (Schroder and Gossler, 2002), are attenuated in the intestine five days post Cdx2-deletion (Figure 32A and B). To further assess this, we inhibited Notch signaling *in vivo* using DAPT. Mice treated with DAPT exhibited a decrease in intestinal expression of *ephrinB1* and *Hes1*, similar to that observed five days post-deletion of Cdx2 (Figure 32C). Treatment with DAPT did not perturb Cdx2

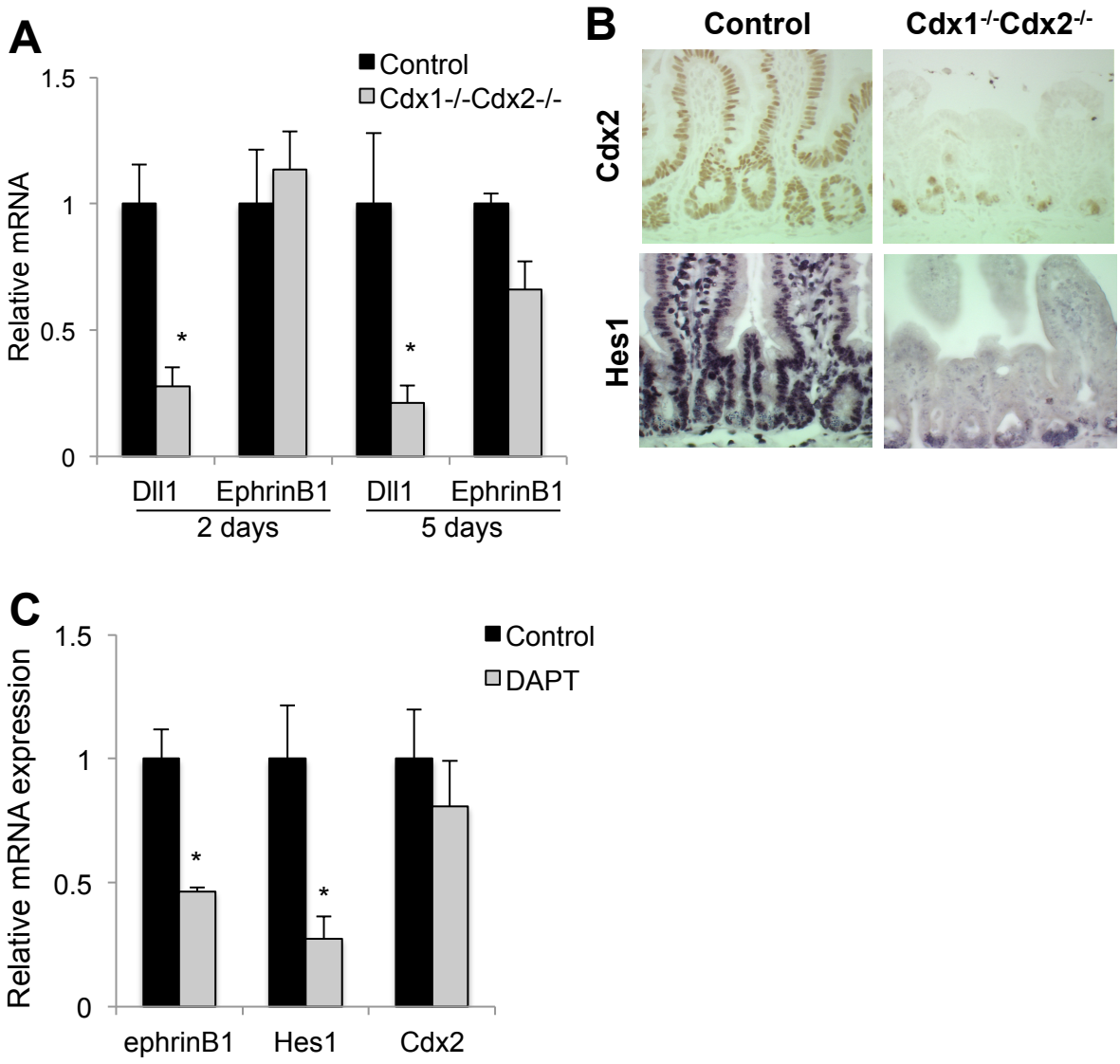

Figure 32: Ephrin B1 is downregulated by Cdx2's regulation of Notch signaling. (A) RT-PCR for Dll1 and ephrinB1 2 and 5 days post knockout of Cdx2 from the small intestine of control and $Cdx1^{-/-}Cdx2^{-/-}$ treated mice. (B) Expression of Cdx2 and Hes1 from the small intestine of control and $Cdx1^{-/-}Cdx2^{-/-}$ mice. (C) RT-PCR for ephrinB1, Hes1 and Cdx2 after 5 days of treatment with DAPT from the small intestine of control and $Cdx1^{-/-}Cdx2^{-/-}$ treated mice. Error bars represent the standard deviation from 3 independent samples. * $p<0.01$ by Student's T-test.

levels (Figure 32C) consistent with a role for Notch in regulating *ephrinB1* expression.

The above observations are consistent with a pathway wherein Cdx2 impacts Notch signaling which subsequently impacts on *ephrinB1*. Consistent with this, Transcriptional Element Search System (TESS) identified potential Notch response elements in the proximal *ephrinB1* promoter (Figure 33A). ChIP analysis revealed that the NICD was enriched on this *ephrinB1* promoter region in a manner similar to the Notch target gene *Hes1*. In contrast, Cdx2 did not appear to associate with the *ephrinB1* promoter (Figure 33B).

Cell based reporter assays revealed that the NICD was able to induce transcription from the *ephrinB1* promoter; this response was lost upon mutation of the distal Notch binding site (Figure 33C). A role for Notch in activating *ephrinB1* was further supported by the observation that DAPT treatment of SW480 cells attenuated *ephrinB1* expression in a manner comparable to that of *Hes1* (Figure 34A). Conversely, valproic acid (VPA), which has been shown to induce Notch signaling (Greenblatt et al., 2007) caused an increase in the expression of both *ephrinB1* and *Hes1* in SW480 CDX2 deficient cell lines (Figure 34B), suggesting that Cdx2 impacts Notch signaling upstream of the receptor. Consistent with this, reintroduction of the NICD into CDX2 knockdown SW480 cells also restored expression from the wild type *ephrinB1* reporter. In contrast, the reporter harboring a mutation in the Notch binding site was inactive in SW480 cell lines irrespective of Cdx2 status, and was not responsive to the NICD (Figure 34C). Taken

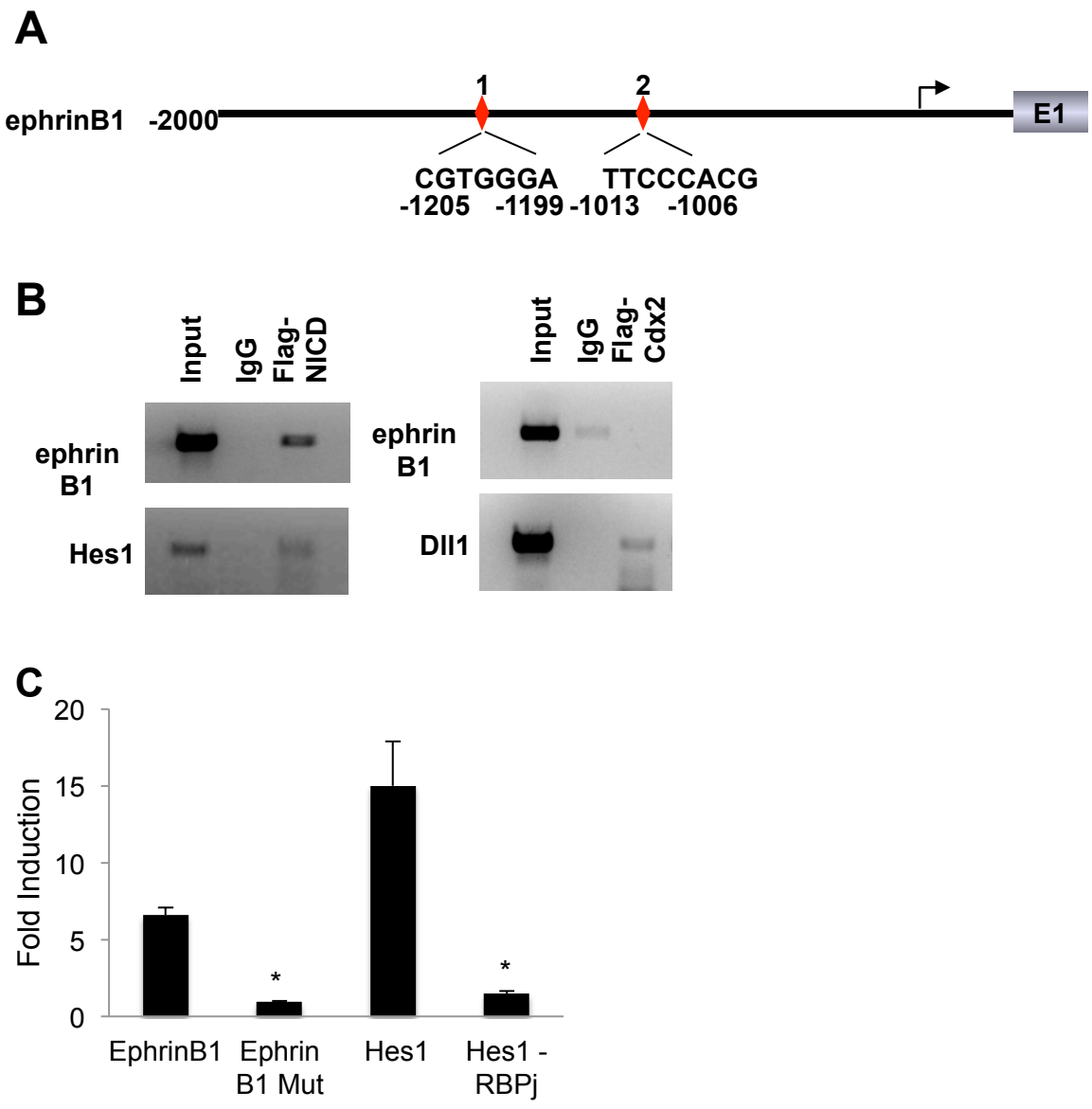

Figure 33: Ephrin B1 is a target gene of Notch signaling.
(A) Schematic representation of putative Notch binding sites found in the *ephrin B1* promoter. (B) Chromatin immunoprecipitation (ChIP) analysis of the *ephrin B1* locus from SW480 cells showing NICD occupancy of the regions encompassing the Notch binding sites. Cdx2 could not bind the *ephrin B1* promoter. *Hes1* and *Dll1* were used as positive controls for NICD and Cdx2 respectively. (C) Notch signaling induces expression from the *ephrin B1* promoter in cell-based assays but not when distal Notch binding site was mutated. *Hes1* and *Hes1 –RBPJ* binding site expression vectors were used as positive and negative controls respectively. Error bars represent the standard deviation from 3 independent samples. * $P < 0.05$ relative to control by Student's *t*-test.

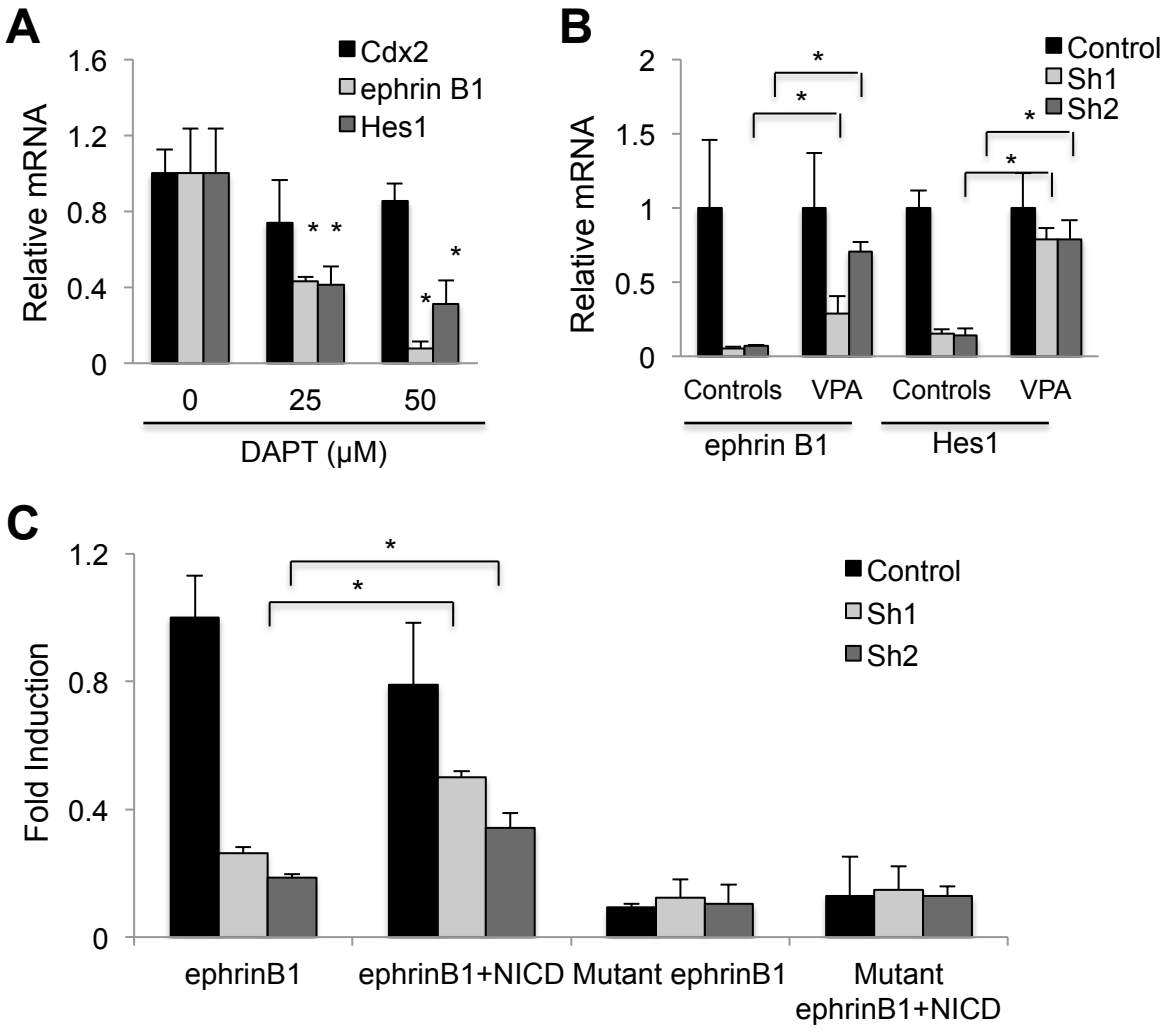

Figure 34: Notch signaling impacts the expression of ephrinB1.
RT-PCR from control and Sw480 cells treated with the Notch inhibitor DAPT (A) or with the Notch activator VPA (B). (C) Relative luciferase expression of ephrinB1 or ephrinB1 containing a mutant Notch binding site in control and Sw480 Cdx2 knockdown cells treated with or without NICD. Error bars represent the standard deviation from 3 independent samples. * $P < 0.05$ relative to control by Student's *t*-test.

together, these data suggest that Cdx2 regulates *ephrinB1* expression through the Notch pathway in the intestine and SW480 cells.

Loss of CDX2 leads to a more invasive stem-like CRC phenotype

Both CDX2 and Notch signaling have been proposed to be oncogenic in CRC. However, there is evidence that both of these factors can also impair CRC progression(Baba et al., 2009; Kim et al., 2012) and that their expression is lost in some human carcinomas (Fre et al., 2009; Hinoi et al., 2001). Therefore, the effect of CDX2 deficiently in CRC was investigated in SW480 cells. SW480 cells are composed of two populations of cells: cuboidal shaped epithelial cells (e-cells) and round shaped cells (r-cells). R-cells grow faster, produce larger colonies in soft agar and develop into larger undifferentiated tumors in nude mice (Tomita et al., 1992). CDX2 knockdown led to an abundance of r-like cells, while the control cells maintained mostly an e-cell morphology (Figure 35A). Consistent with this, CDX2 deficient lines grew at a faster rate (Figure 35B), an outcome that is also observed in CRC cells deficient in Notch signaling (Jagle et al., 2014).

Intestinal stem cells are thought to be the cells of origin in CRC (Schwitalla et al., 2013). Gene expression analyses revealed an increase in the intestinal stem cell markers *Lrg5* and *Smoc2* in lines lacking CDX2 (Figure 35C), suggesting that cells deficient in CDX2 are more stem-like.

SW480 cells were derived from a primary adenocarcinoma of the colon prior to gaining metastatic potential (Leibovitz et al., 1976). To evaluate the influence of CDX2 in this transition, transwell assays were conducted. This analysis revealed that CDX2 knockdown cells had an increased migratory potential compared to

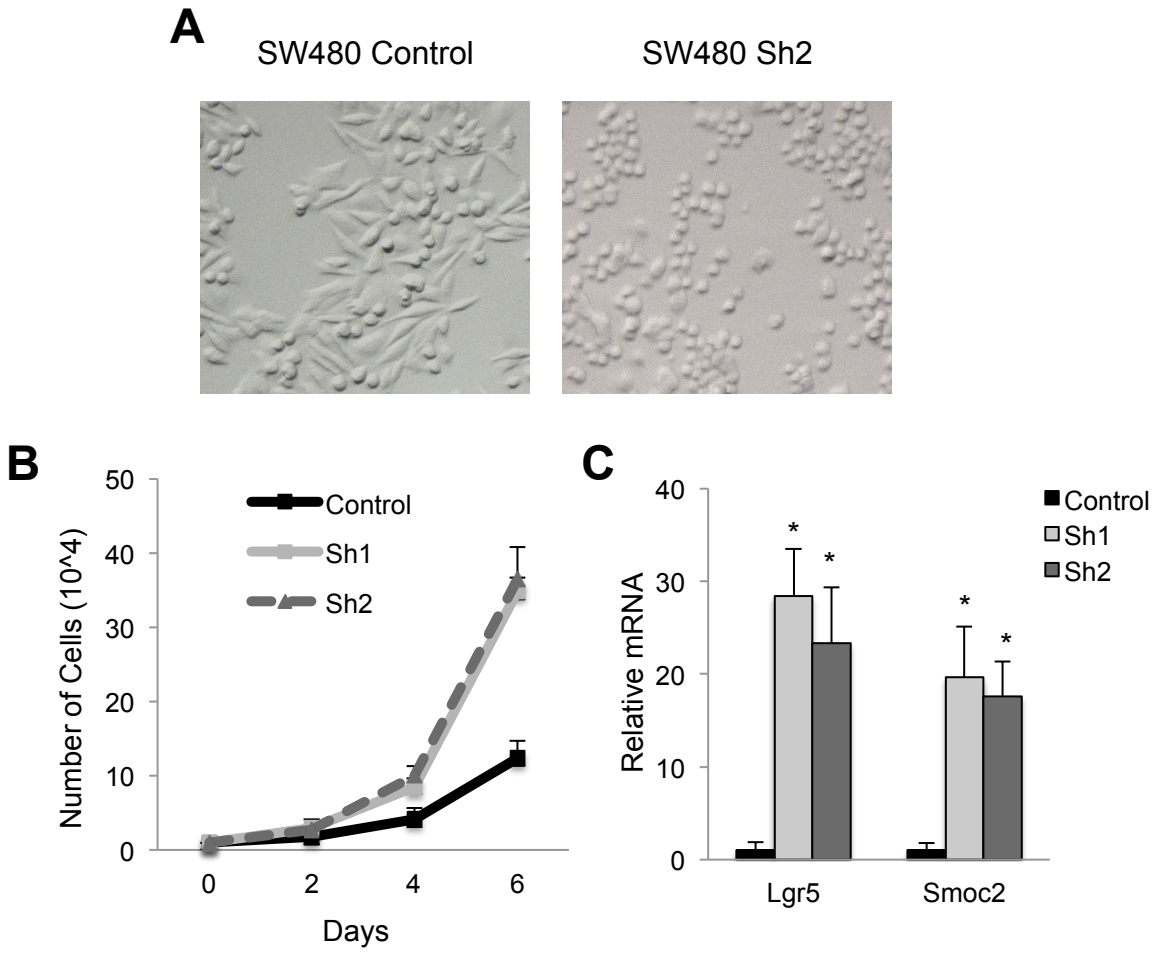

Figure 35: Cdx2 loss causes increased stemness.
(A) SW480 cells from control and Cdx2 Sh knockdown cell. Note the lack of elongated cells in the Sh knockdown. (B) Growth curve for control and Cdx2 Sh knockdown cells. (C) RT-PCR from SW480 cells for stem cell markers in control and CDX2 knockdowns. Error bars represent standard deviation from the mean expression levels of 3 independent samples. * $P < 0.05$ by Student's *t*-test.

control cell lines (Figure 36A). SW480 cells have also been shown to grow in an anchorage independent manner (Faux et al., 2004), and CDX2 deficient cell lines produced more, and larger, colonies relative to controls (Figure 36B-D). These observations support the hypothesis that loss of Cdx2 increases CRC tumorigenic potential.

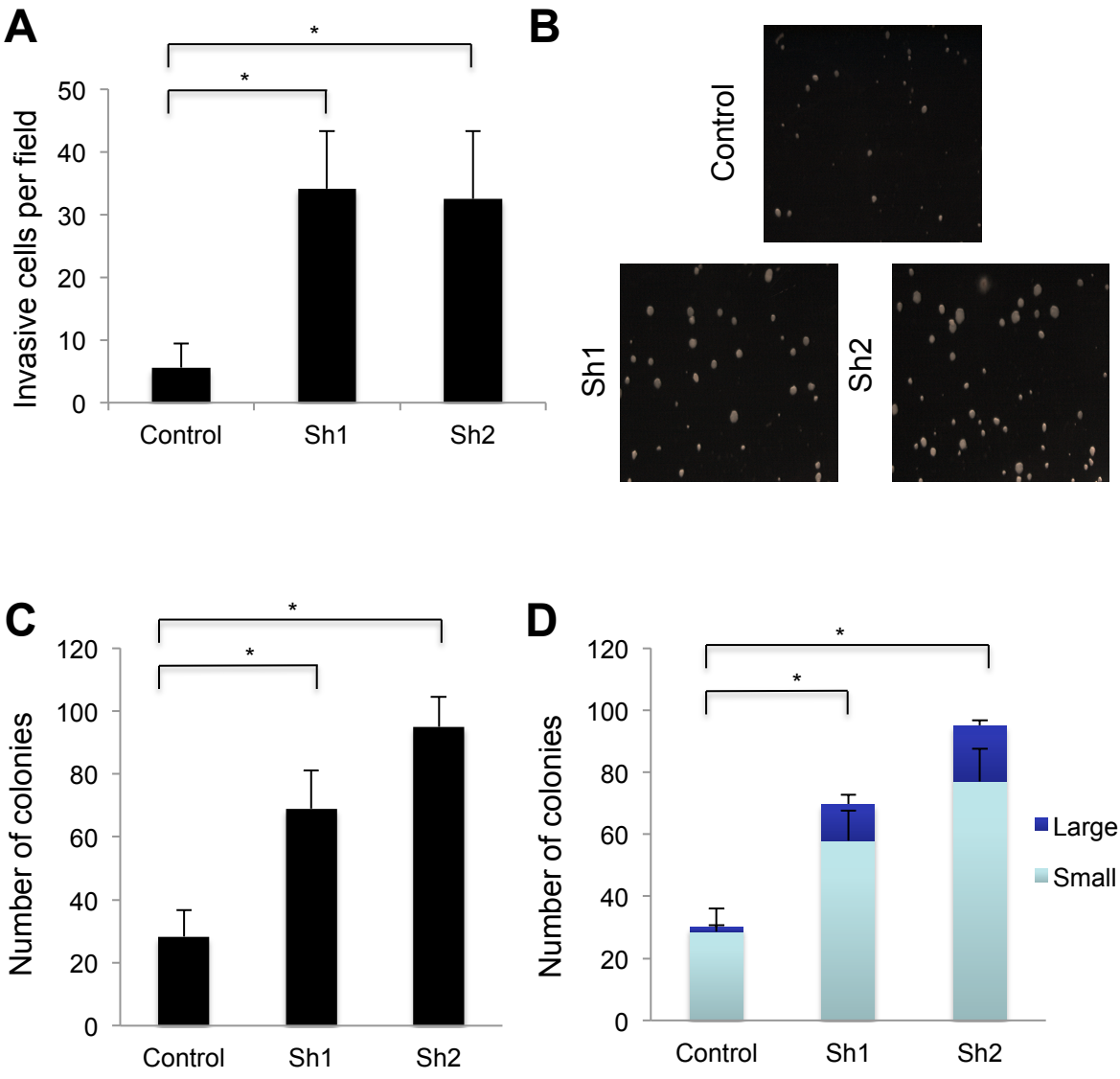

Figure 36: Cdx2 loss causes increased invasion.
(A) Quantification of cells per field that migrated through the matrigel from controls and Cdx2 knockdowns. (B) Soft agar colonies from control and Cdx2 Sh knockdowns. (C) Quantification of number of colonies in soft agar and (D) Small and large colonies formed in soft agar. Error bars represent the standard deviation of 5 fields of view from 3 independent samples. * $p<0.01$ by Student's T-test.

Discussion

Although Cdx2 has known roles in the homeostasis of the intestine, and a tumor suppressive function in some contexts, (Bonhomme et al., 2003; Hryniuk et al., 2012; Hryniuk et al., 2014; Verzi et al., 2010; Verzi et al., 2011) the mechanism by which it impacts these processes are largely unknown. To investigate this further, we developed SW480 cell lines deficient in CDX2. Consistent with prior work, we found that CDX2 loss in SW480 cells led to a loss in Notch signaling. Together with promoter analysis, we further found that Notch is a direct regulator of *ephrinB1*, offering a mechanistic basis for the loss of *ephrinB1* expression and cell sorting defects previously described in Cdx null murine polyps (Hryniuk et al., 2014). Furthermore, loss of CDX2 increased the tumorigenic potential of CRC, consistent with prior work suggesting a tumor suppressive function for CDX2 in CRC (Aoki et al., 2003; Bonhomme et al., 2003; Hryniuk et al., 2014).

Homeostasis of the intestinal epithelium is complex and requires coordinated activity and interaction between various signaling cascades and transcription factors (Richmond and Breault, 2010). Cdx2 has been linked to expression of genes in the Notch signaling pathway (Uesaka et al., 2004) while *Dll1* has been shown to be a Cdx2 target gene (Grainger et al., 2011). Consistent with these findings, loss of CDX2 also led to attenuation of *Dll1* expression concomitant with a reduction in Notch signaling (as evidenced by diminished NICD levels) in SW480 cells. Defects in Notch were also evident by the increased expression of the secretory cell marker *Math1*, in the SW480 CDX2 knockdown cell lines, congruent with Dll1 inactivation in the intestinal epithelium (Pellegrinet et al., 2011).

Notch has previously been linked to the eph-ephrin pathway in the intestine. *EPHB3* has been shown to be Notch-responsive, while the NICD can bind to an enhancer region in *EPHB2* (Rodilla et al., 2009). Consistent with this, in human CRC cells, lesions in Notch signaling impairs *EPHB3* enhancer function, while activation of Notch can induce EPHB3 expression concomitant with a tumor suppressive response (Jagle et al., 2014). In the present study, and in agreement with prior observations(Hryniuk et al., 2014), we found that CDX2 deficient SW480 cells exhibited attenuated *ephrinB1* expression. Subsequent analysis revealed that *ephrinB1* is regulated directly though Notch binding sites that could be occupied by the NICD *in vivo*. These observations evoke a novel pathway by which CDX2 impacts *ephrinB1* expression *via* regulation of Notch activity (Figure 37).

Prior studies have suggested Cdx2 has both tumor promoter (Domon-Dell et al., 2003; Salari et al., 2012; Subtil et al., 2007; Witek et al., 2005) and tumor suppressive (Baba et al., 2009; Guo et al., 2004; Pilozzi et al., 2004; Wong et al., 2004) potential. Similarly, Notch activity is critical for adenoma formation in mouse tumor models but can also impair tumor progression (Fre et al., 2009; Kim et al., 2012; Peignon et al., 2011; van Es et al., 2005). In the present study, we found that loss of CDX2 in SW480 cells increased their proliferation and invasive nature and induced stem cell markers. This is consistent with prior observations wherein loss of CDX2 in colorectal cancer cells was shown to decrease the expression of markers of differentiation concomitant with an increase in proliferation (Guo et al., 2010; Hinkel et al., 2012; Natoli et al., 2013); conversely, CDX2 overexpression decreases mobility and restricted tumor cell growth *in vivo* (Gross et al., 2008).

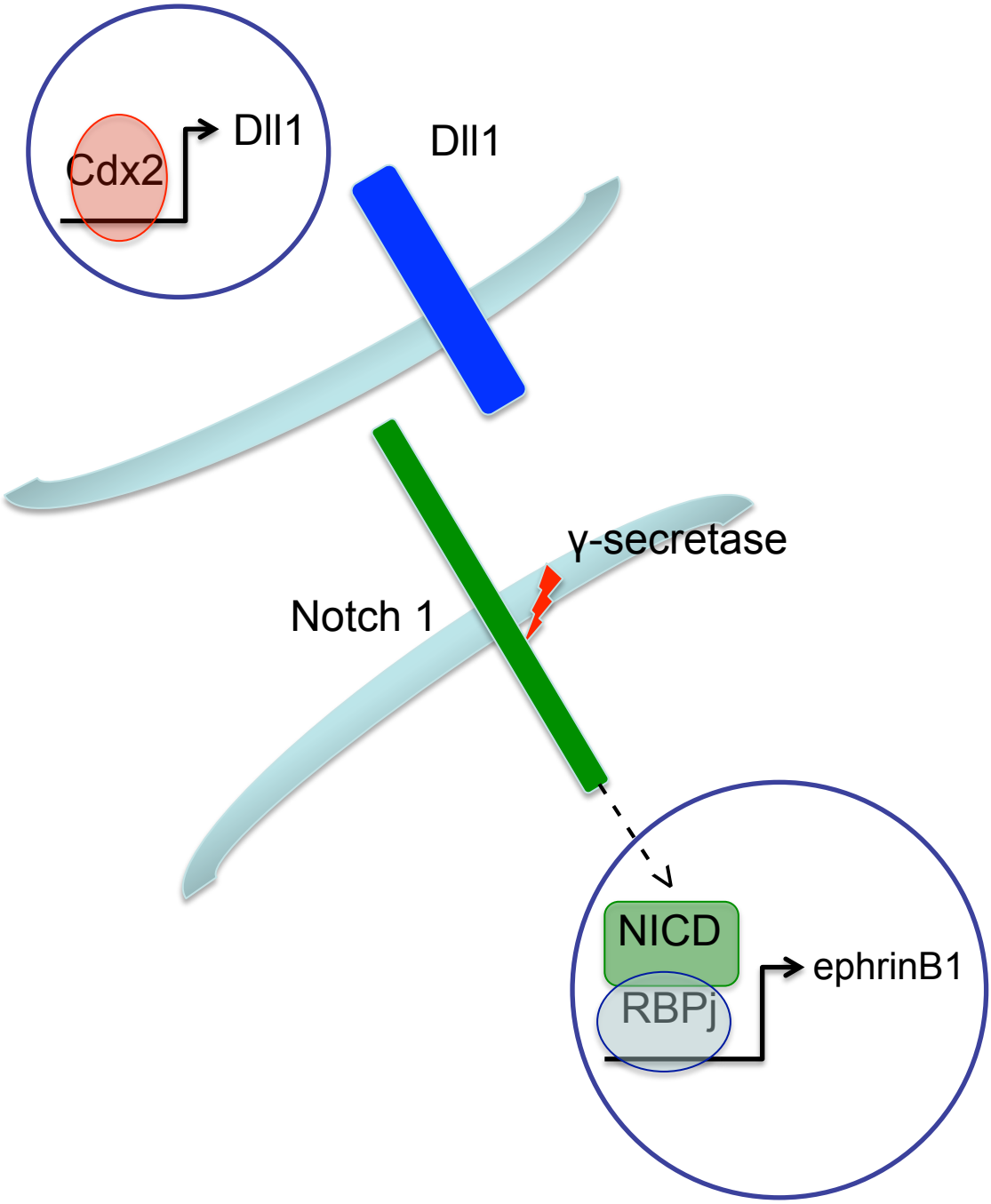

Figure 37: Schematic for Cdx2 regulation of ephrinB1 expression.
Cdx2 binds to the promoter region of Dll1 to effect transcription. Dll1 ligand can then signal to Notch1 on an adjacent cell. Activation of the Notch receptor allows for cleavage of the NICD by γ-secretase. The NICD can then translocate into the nucleus to join its DNA binding partner RBPj to allow for transcription of ephrin B1.

Our results extend the hypothesis that CDX2 signaling is involved in tumor repression, and suggests that certain effects may be mediated through regulation of Notch function. It is tempting to speculate that these effects are imparted, in part, though *ephrinB1*, which is involved in cell sorting along the crypt villous axis and serves a tumor suppressive role by compartmentalizing adenomas arising from Wnt mediated hyperproliferation of intestinal cells(Batlle et al., 2002; Cortina et al., 2007).

Acknowledgements

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CHAPTER 5
GENERAL DISCUSSION

Cdx transcription factors are important for many developmental processes including hematopoesis, neural tube development, axial elongation and gastrointestinal development (Davidson and Zon, 2006; Savory et al., 2009; Savory et al., 2011; van den Akker et al., 2002; van Nes et al., 2006; Wang et al., 2008). In the adult Cdx1 and Cdx2 are also expressed in the intestine. Cdx transcription factors have been reported to be lost in intestinal cancers and misexpressed in pathological disease states. It is therefore important to study these transcription factors in the adult to determine their role in these disease processes. To this end, I developed a conditional mutagenesis strategy to investigate the role of Cdx2, alone or in conjunction with Cdx1, in the adult mouse intestinal epithelium. I used this model to explore their role in intestinal homeostasis and in a colorectal cancer model. The main findings of these studies are summarized below.

Cdx Function is Required for Maintenance of Intestinal Identity in the Adult

I was able to circumvent the embryonic lethality inherent to *Cdx2* loss (Chawengsaksophak et al., 1997) using a previously described *Cdx2* floxed allele (Savory et al., 2009) deleted specifically in the adult intestine using a ligand-activated Cre under the control of the *villin* promoter (el Marjou et al., 2004). Rapid loss of Cdx2 throughout the intestinal tract led to death within one week most likely due to intestinal dysfunction. Villus architecture and proliferation as well as the expression of intestinal markers were all impacted following ablation of Cdx2. These indices were not further affected by subsequent loss of *Cdx1,* suggesting that Cdx1 does not have any overt function in the small intestine. Conversely, *Cdx2* ablation resulted in a transformation of the proximal colon to a cecal-like

morphology. This outcome was exacerbated with the subsequent loss of *Cdx1,* with the entire colon exhibiting cecal-like character, including altered molecular markers. This finding demonstrates a new and unsuspected role for Cdx function in maintaining colon homeostasis, and demonstrates a critical role for Cdx1 in this process.

To further elucidate the homeostasis of Cdx2 in the adult intestine, mice were treated with a low dose of tamoxifen that had no overt effect on health or longevity, but resulted in a mosaic, "patchy", knockout of Cdx2 throughout the intestine. This long-term loss resulted in epithelial dysplasias, in which all intestinal cell types were lost, and the intestine assumed a partial gastric nature, a phenotype in agreement with a concurrent study of Cdx2 function in the intestine (Verzi et al., 2011). These findings suggest that Cdx2 is important for proper differentiation of the intestinal epithelium and for homeostasis of the large intestine.

Cdx1 and Cdx2 Function as Tumor Suppressors

As Cdx members have been proposed to influence CRC, a model for intestinal tumorigenesis was used in conjunction with Cdx loss to explore this link. To this end, the Cdx2 conditional line was bred with $APC^{Min/+}$ (Min) mice and Cdx2 mosaic deletion effected. Loss of Cdx2 or Cdx1-Cdx2 in an $APC^{Min/+}$ background resulted in a significant acceleration of lethality associated with an increase in tumor incidence impacting the small intestine. Ablation of both Cdx1 and Cdx2, relative to Cdx2 alone, also resulted in a marked increase in tumor formation in the distal colon with concomitant alterations in TGF-β signaling. Finally, Cdx loss resulted in the formation of villus tumors associated with loss of ephrinB1 expression in the small

intestine. These findings underscore new roles for Cdx in suppressing CRC and reveal previously unrecognized roles for Cdx1 in intestinal tumorigenesis.

Notch signaling affects ephrinB1 in the intestine after loss of CDX2

The lack of intestinal differentiation in Cdx-deficient epithelium, along with mislocalization of Paneth cells, suggests a critical role for Cdx2 in intestinal differentiation, however, the mechanisms governed by Cdx2 to direct this differentiation are poorly understood. I chose to address this question by generating CDX2 deficient intestinal cell lines. Using this model, I found that loss of CDX2 resulted in increased expression of secretory cell markers, in agreement with prior work showing a role for Cdx2 in regulating Notch signaling (Grainger et al., 2011; van Es et al., 2005). Interestingly, ephrinB1 was also downregulated in these CDX2 deficient cell lines, as seen in Cdx2 null intestinal tumors.

In the intestine, ephrinB1 has been suggested to be a Notch target gene(Koo et al., 2009). To this end, I found that ephrinB1 was directly regulated by Notch and, in the intestine, Cdx2 affected the expression of ephrinB1 by activating the Notch signaling cascade, potentially through regulation of Dll1. Taken together, these data suggest that Cdx2 is not only involved in directing the differentiation of secretory and absorptive cells but also contributes to proper cell sorting of the intestinal epithelium during differentiation.

Cdx2 is an important regulator of adult intestinal identity

During development, activation of different gene expression programs is important for determining organ specification from the three different germ layers; ectoderm, endoderm and mesoderm. Endoderm is the precursor for the

development of the gastrointestinal tract epithelium, which is under tight regulation by transcriptional programs for appropriate development and specification. Timing and level of exposure to growth factors are important for specifying the A-P patterning of the digestive tract. For example, at the end of gastrulation, the endoderm exists as a layer of cells which is patterned by expression of regional determination factors, such as Sox2 in the anterior endoderm and Cdx2 in the posterior endoderm(Lewis and Tam, 2006; Thomas et al., 1998; Zorn and Wells, 2009).

In the case of Sox2, its expression is dominant in the anterior endoderm leading to the specification of the esophagus, trachea and stomach. Experiments in mice with modulating expressions of Sox2 have shown the importance of Sox2 in the differentiation of the esophageal epithelium as well as the morphogenesis of the esophagus and the trachea(Gontan et al., 2008; Que et al., 2007; Que et al., 2009). In humans, several congenital malformations such as anophthalmia, microphthalmia, and the anophthalmia–esophageal–genital syndrome, have been associated with heterozygote mutations in SOX2 (Williamson et al., 2006). Similarly, Sox2 expression is important for stem cell maintenance and differentiation of the stomach epithelium (Arnold et al., 2011). Ectopic expression studies of Sox2 in the developing posterior endoderm causes an anteriorization of the intestinal epithelium toward a premature gastric phenotype(Raghoebir et al., 2012). In addition, aberrant SOX2 expression may underlie the formation of gastric metaplasia in some colorectal cancers(Shaoul et al., 2000), and the formation of various human congenital anomalies of the gut, such as Meckel's diverticulum in

which ectopic gastric tissue can be present in the small intestine (Lambert et al., 2000). Cumulatively this data is suggestive of a critical role for Sox2 in anterior foregut specification and identity.

Similar to the role of Sox2 in the anterior endoderm, Cdx2 appears to have a role in posteriorization of the intestinal tract. It has been proposed from these studies that Cdx2 is necessary for proper intestinal identity as conditional loss of Cdx2 resulted in the development of characteristics of more anterior gastrointestinal structures such as the esophagus and stomach (Gao et al., 2009; Grainger et al., 2010). These results suggest that Cdx2 imposes a more posterior character on the hindgut and without it the default state of the endoderm is esophageal or gastric in nature, depending on the developmental stage investigated.

In the adult intestine, similar gene targeting studies have shown that Cdx2 plays a prominent role in proper differentiation of intestinal-specific cells (Hryniuk et al., 2012; Verzi et al., 2011). The loss of Cdx2 caused an increase in gastric markers as well as a shift of the intestinal epithelium to a more gastric identity. Of note, six months after mosaic deletion of Cdx2, areas void of Cdx2 expression are repopulated via Cdx2+ cells and re-establish a normal intestinal phenotype (Appendix Figure 1). Additionally, overexpression of Cdx2 specifically in the stomach results in differentiation to an intestinal epithelium (Mutoh et al., 2002; Mutoh et al., 2004), while in Barrett's esophagus, a condition arising due to chronic exposure to stomach acid, Cdx2 becomes ectopically expressed concurrent with a switch from an esophageal to an intestinal phenotype (Colleypriest et al., 2009;

Colleypriest et al., 2010). Taken together, these studies indicate that even in the adult Cdx2 is a critical regulator of the intestinal identity.

Plasticity of intestinal tissue

Adult tissues stem cells have the potential for plasticity and self-renewal essential for normal function and to make repairs after injury. Cell renewal is governed by highly dynamic processes. Historically, the commitment of cells to differentiate was viewed as an irreversible change of a proliferating cell to a given specific cell fate. Currently, the emerging view of self-renewal and injury repair is one of plasticity in which a stem cell state can be reacquired by dedifferentiation of already specified cells. The mediators of lineage selection within the highly dynamic cell populations promote different cell lineages and yet permit a level of plasticity to these tissues. For example, it has been demonstrated that differentiated secretory Clara cells have the ability to revert into functional stem cells of the trachea upon ablation of the normal stem cell population (Tata et al., 2013). This dedifferentiation in the adult trachea supports the notion of plasticity in an adult tissue even with damage to the stem cell population.

The intestine, due to its constant renewable epithelium, has been investigated for its ability to regenerate after injury. However, this regenerative function is not solely a function of stem cell expansion to restore homeostasis but is also aided by dedifferentiation of other cells in the intestinal epithelium (Barker et al., 2012). The ability of the intestine to overcome an acute loss of its stem cell pool may relate to the general plasticity of the TA progenitor compartment. TA cells harbour the capacity to fall back into the stem cell niche and reacquire stem cell

characteristics to assume stem cell functions (Barker et al., 2012). As an example of such plasticity, Dll1 was recently shown to mark an early daughter cell of Lrg5+ stem cells around the +5 position in the intestinal crypt. When Lgr5+ cells are killed with radiation, Dll1+ TA cells with secretory cell properties revert to an Lgr5+ stem cell during the regenerative process (van Es et al., 2012).

Ablation of Cdx2 in the developing intestinal epithelium showed loss of Dll1 expression which upon further testing revealed Dll1 to be a Cdx2 target gene (Grainger et al., 2011), a relationship that extends into the adult intestinal epithelium (Hryniuk et al., 2012). It is therefore possible that upon loss of Cdx2, TA cells that were once Dll1+ are reprogrammed to revert back to a stem cell-like fate. Consistent with this hypothesis, the stem cell niche of *Cdx2*$^{-/-}$ and *Cdx1*$^{-/-}$*Cdx2*$^{-/-}$ mice showed an expansion in the number of stem cells in the transit-amplifying zone after long-term loss of Cdx members (Hryniuk et al., 2012). Furthermore, knockdown of CDX2 in intestinal SW480 cells significantly increased the expression of Lgr5 as well as other stem cell markers concomitant with a decrease in the expression of Dll1. Taken together these result indicate a relationship between loss of Cdx2 impacting Dll1 expression in the transit-amplifying zone and a reversion to a stem cell like identity, however further studies are necessary to explore the significance of these observations.

Cdx2 and intestinal differentiation

In the intestine, TA cells terminally differentiate into the four principal epithelial cells found in the small and large intestines through specific molecular signals. Once cell fate is determined, some lineages use common signaling pathways

during terminal differentiation while others utilize parallel signals for differentiation (Appendix Figure 2, (van der Flier and Clevers, 2009)). For instance, Notch signalling provides a pivotal switch between enterocyte and secretory cell differentiation whereby expression of the Notch target gene *Hes1* represses transcription of the bHLH transcription factor Math1 directing cell fate towards the enterocyte lineage(Jensen et al., 2000). Hes1-/- mice show an increase in Paneth, goblet and enteroendocrine cells while the intestinal epithelium of Math1 mutant mice becomes populated only by enterocytes(Jensen et al., 2000; Suzuki et al., 2005; Yang et al., 2001). Expression of kruppel-like factor 4 (KLF4), Ngn3, Sox9 and E47-like factor 3 (Elf3) are required for goblet, enteroendocrine, Paneth and enterocyte cell differentiation respectively (Jenny et al., 2002; Katz et al., 2002; Mori-Akiyama et al., 2007; Ng et al., 2002). The proper sequencing and coordination of these factors during differentiation is essential for proper terminal differentiation of intestinal epithelial cells.

The impact of Cdx2 deletion has provided some novel information regarding the differentiation of intestinal cells towards their mature state. Conditional deletion of Cdx2 in the developing endoderm revealed an increase in goblet cell differentiation consistent with improper repression of Hes1 on Math1 (Grainger et al., 2011). Interestingly, upon loss of Cdx2 in the adult mouse intestinal epithelium, differentiation of all four main intestinal cell types is also absent. These results suggest that Cdx2 not only functions to help direct absorptive versus secretory cell specification but is also necessary for terminal differentiation of all intestinal cell types. Consistent with this premise, we have also observed that in *Cdx2*$^{-/-}$ mice,

expression of *Sox9* was significantly decreased compared to control (Lohnes lab, unpublished observation). ChIP analysis from murine intestines also revealed that Cdx2 was enriched at a putative CDRE in the promoter of the *Sox9* gene (Appendix Figure 3). Conditional deletion of Sox9 from the intestinal epithelium results in the complete absence of Paneth cells, indicating that its expression is necessary for proper Paneth cell differentiation(Bastide et al., 2007; Mori-Akiyama et al., 2007). Likewise, Klf4 has been shown to be necessary for maturation of goblet cells in the intestine(Katz et al., 2002). It has been suggested that Cdx2 regulates the expression of Klf4 in intestinal cell lines(Faber et al., 2013), however, it is yet to be seen whether this paradigm holds true *in vivo*. Taken together these data indicate that Cdx2 acts directly on pathways necessary for the proper specification of the terminally differentiated cells of the intestine.

While lineage specification of the intestine is important for proper function, proper positioning of differentiated cell types along the crypt-villus axis is also necessary. This positioning information is governed by signaling cues from the eph-ephrin signaling pathway. During differentiation enterocytes, goblet and enteroendocrine cells gain the expression of the ephrinB1 ligand while Paneth cells gain the expression of the EphB3 receptor. The TA and stem cells of the intestinal crypt express the EphB2 receptor. The opposition between cells expressing ligands and receptors direct the intestinal cells to migrate either upwards towards the villus tips for those cells that are ephrinB1+ or down into the crypts if they are EphB3+. Consistent with this, Paneth cells in both $EphB3^{-/-}$ and conditional ephrinB1 knockout mice are mislocalized along the crypt-villus axis (Batlle et al., 2002;

Cortina et al., 2007). Loss of ephrinB1 also results in the expansion of Eph+ receptor cells out of the crypts. Similar consequences were observed in Cdx2 mutants, which exhibited both a loss of ephrinB1 expression and Paneth cells mis-localization(Hryniuk et al., 2014). It was later determined that Cdx2 does not directly impact ephrinB1 expression but regulates Notch activation upstream of ephrinB1. Thus Cdx2 appears necessary for the expression of the ephrinB1 ligand during differentiation which will result in the proper positioning of terminally differentiated cells along the crypt villus-axis.

Lineage dependency

The close association between cell lineage and cancer phenotype has long been recognized. This raises the possibility that cellular mechanisms governing lineage proliferation and survival during development might also underlie tumorigenic mechanisms. This idea has led to the development of a lineage dependency model for human cancers, whereby master regulators that function during development in specification, proliferation and differentiation, can become deregulated and lead to cancer (Garraway and Sellers, 2006). Several tumor-suppressor genes also have lineage restricted tumor inactivation patterns. Prominent examples include APC in colon cancer (de la Chapelle, 2004), RB in retinoblastoma (Classon and Harlow, 2002) and NF1 in neurofibromatosis (Nishi and Saya, 1991). In each tissue specific example, distinct cell lineages either accumulate genetic mutations in these genes or undergo LOH.

The Cdx transcription factors may also function as lineage dependent tumor suppressors in the development of human CRC. While Cdx2 is not deemed a master

regulator in the full sense, it is very important in specification of the intestine as well as for intestinal identity as discussed above. Furthermore, it has been shown that Cdx1 and Cdx2 act as tumor suppressors in CRC progression in knockout mouse models (Aoki et al., 2003; Bonhomme et al., 2003; Hryniuk et al., 2014) and that their loss contributes to more aggressive cancers in humans (Baba et al., 2009; Hinoi et al., 2001; Lugli et al., 2008). In tissue culture models of colorectal cancer, overexpression of CDX1 and CDX2 resulted in decreased proliferation in a variety of colorectal cancer cells, along with decreasing tumor spreading in nude mice (Gross et al., 2008; Guo et al., 2004; Mallo et al., 1998). Conversely, knockdown of CDX2 in SW480 colorectal cancer cells caused an increase in the proliferative capacity along with increased invasion and metastatic properties (Appendix Figure 4). In addition, SW480 cells lacking CDX2 become composed almost exclusively of round cells instead of their normal heterogeneous epithelial and round populations. Round cells have been previously shown to grow faster, produce larger colonies in soft agar and develop into larger undifferentiated tumors in nude mice (Tomita et al., 1992). These observations suggest that CDX1 and CDX2 block pathways related to tumor expansion in colorectal cancers and that they may participate in lineage dependency during the progression of CRC, however the exact mechanism is yet to be determined.

Future Directions

While our knowledge of Cdx function in the adult intestinal tract is growing there are still many outstanding questions yet to be addressed. For example, it appears to be well established that disruption of Notch signaling leads to improper

absorptive versus secretory cell differentiation(Crosnier et al., 2005; Fre et al., 2005; van Es et al., 2005). It is also known that Cdx2 deletion affects Notch signaling phenocopying some of the outcomes of disruption of the Notch pathway(Grainger et al., 2011). However, when Cdx2 is deleted from the adult intestinal epithelium, differentiation of all intestinal cell types appear to be compromised(Hryniuk et al., 2014). Therefore, Cdx2 appears to play a fundamental role in specification of the various intestinal cell types. Preliminary data from our lab suggests that Cdx2 may directly regulate Sox9, an important specifier of Paneth cells(Mori-Akiyama et al., 2007). A non-allelic non-complementation assay between Cdx2 and Sox9 could be undertaken to further understand this relationship. This could be accomplished through the generation of *Cdx1-Cdx2-Sox9* triple heterozygotes by crossing the *Sox9*$^{f/f}$ allele(Bastide et al., 2007) into the *Cdx1*$^{-/-}$*Cdx2f*$^{/f}$ *villin* Cre-ER^{T} background. Since Cdx2 loss causes a lack of Paneth cells, as does loss of Sox9, it would be postulated that triple heterozygotes should also lead to a decrease in Paneth cell specification, which can be assessed by differential staining using Phloxine-tartrazine or immunohistochemistry for lysozyme. Other markers of mature Paneth cell function such as defensins could also be investigated. Alternatively, Cdx2 could be conditionally deleted in Paneth cells using a Paneth cell specific Cre such as *Defa6-Cre* (Adolph et al., 2013) to ascertain if Cdx2 plays a role in maintaining Paneth cell function and if Sox9 is involved in that process. Similar studies of Cdx2 in the adult intestinal epithelium along with promoter analysis could also be undertaken to investigate if Cdx2 regulates other intestinal cell differentiation factors such as Klf4 in goblet cells or Ngn3 in enteroendocrine cells.

Even though much information was gained from analysis of SW480 cells with CDX2 knockdown, residual CDX2 expression may still obscure some functional consequences. To gain more insight into the role of Cdx in tumorigenesis, intestinal cancer cells can be rendered Cdx2 null using the CRISPR-Cas9 system. Cas9 mediates genome disruption by stimulating double strand breaks (DSB) at targeted loci (Hsu and Zhang, 2012; Urnov et al., 2010). DSB are typically repaired via one of two distinct DNA repair pathways: (i) nonhomologous end joining (NHEJ) or (ii) homology-directed repair (HDR). Improper repair from the former pathway can lead to a high frequency of insertion/deletion (indel) mutations which can result in frameshift mutations and premature stop codons rendering cells null for the gene being targeted(Ran et al., 2013). Cells rendered CDX2 null could then be compared to wild type cells for differences in gene expression using various techniques such as microarray or RNA-seq, and CDX2 genome occupancy interrogated by ChIP seq of wild type cells. Convergence of these two analysis should identify putative direct target genes of CDX2 and may lead to further insight into relevant tumorigenic pathways. These target genes can then be verified with a combination of directed ChIP, EMSA and luciferase assays while in vivo transgenesis modeling can verify biological function in intestinal tumorigenesis.

Conclusion

My work has revealed a critical requirement for Cdx2 in the differentiation of all cell types of the intestine, and suggests that Cdx2 is needed to maintain a posterior identity of the adult intestinal epithelium. Loss of Cdx2 in conjunction with Cdx1 also causes anteriorization of the colon, revealing a novel role for Cdx function

in the large intestine. Cdx2 loss also impacts profoundly on Min-dependent intestinal tumorigenesis through disruption of Smad, Notch and Eph-ephrin signaling which in turn contributes to the increased tumorigenesis seen throughout the intestinal tract. I have also described a pathway wherein Cdx2 regulates ephrinB1 expression through activation of Notch signaling, offering a mechanistic basis for the altered cell sorting seen in Cdx mutant tumors. This work will help to further understand the role of Cdx and has important implications regarding our understanding of intestinal diseases, including human colorectal cancer.

REFERENCES

Adolph, T.E., Tomczak, M.F., Niederreiter, L., Ko, H.J., Bock, J., Martinez-Naves, E., Glickman, J.N., Tschurtschenthaler, M., Hartwig, J., Hosomi, S.*, et al.* (2013). Paneth cells as a site of origin for intestinal inflammation. Nature *503,* 272-276.

Agellon, L.B., Toth, M.J., and Thomson, A.B. (2002). Intracellular lipid binding proteins of the small intestine. Mol. Cell. Biochem. *239,* 79-82.

Akhurst, R.J., and Derynck, R. (2001). TGF-beta signaling in cancer--a double-edged sword. Trends Cell Biol. *11,* S44-51.

Alberici, P., Jagmohan-Changur, S., De Pater, E., Van Der Valk, M., Smits, R., Hohenstein, P., and Fodde, R. (2006). Smad4 haploinsufficiency in mouse models for intestinal cancer. Oncogene *25,* 1841-1851.

Alkhoury, F., Malo, M.S., Mozumder, M., Mostafa, G., and Hodin, R.A. (2005). Differential regulation of intestinal alkaline phosphatase gene expression by Cdx1 and Cdx2. Am. J. Physiol. Gastrointest. Liver Physiol. *289,* G285-90.

Amsen, D., Antov, A., and Flavell, R.A. (2009). The different faces of Notch in T-helper-cell differentiation. Nat. Rev. Immunol. *9,* 116-124.

Andreu, P., Colnot, S., Godard, C., Gad, S., Chafey, P., Niwa-Kawakita, M., Laurent-Puig, P., Kahn, A., Robine, S., Perret, C., and Romagnolo, B. (2005). Crypt-restricted proliferation and commitment to the Paneth cell lineage following Apc loss in the mouse intestine. Development *132,* 1443-1451.

Aoki, K., Tamai, Y., Horiike, S., Oshima, M., and Taketo, M.M. (2003). Colonic polyposis caused by mTOR-mediated chromosomal instability in Apc+/Delta716 Cdx2+/- compound mutant mice. Nat. Genet. *35,* 323-330.

Arnold, K., Sarkar, A., Yram, M.A., Polo, J.M., Bronson, R., Sengupta, S., Seandel, M., Geijsen, N., and Hochedlinger, K. (2011). Sox2(+) adult stem and progenitor cells are important for tissue regeneration and survival of mice. Cell. Stem Cell. *9,* 317-329.

Baba, Y., Nosho, K., Shima, K., Freed, E., Irahara, N., Philips, J., Meyerhardt, J.A., Hornick, J.L., Shivdasani, R.A., Fuchs, C.S., and Ogino, S. (2009). Relationship of CDX2 loss with molecular features and prognosis in colorectal cancer. Clin. Cancer Res. *15,* 4665-4673.

Bakaris, S., Cetinkaya, A., Ezberci, F., and Ekerbicer, H. (2008). Expression of homeodomain protein CDX2 in colorectal adenoma and adenocarcinoma. Histol. Histopathol. *23,* 1043-1047.

Bakkebo, M., Huse, K., Hilden, V.I., Forfang, L., Myklebust, J.H., Smeland, E.B., and Oksvold, M.P. (2012). SARA is dispensable for functional TGF-beta signaling. FEBS Lett. *586,* 3367-3372.

Barker, N., van Es, J.H., Jaks, V., Kasper, M., Snippert, H., Toftgard, R., and Clevers, H. (2008). Very long-term self-renewal of small intestine, colon, and hair follicles from cycling Lgr5+ve stem cells. Cold Spring Harb. Symp. Quant. Biol. *73,* 351-356.

Barker, N., van Es, J.H., Kuipers, J., Kujala, P., van den Born, M., Cozijnsen, M., Haegebarth, A., Korving, J., Begthel, H., Peters, P.J., and Clevers, H. (2007). Identification of stem cells in small intestine and colon by marker gene Lgr5. Nature *449,* 1003-1007.

Barker, N., van Oudenaarden, A., and Clevers, H. (2012). Identifying the stem cell of the intestinal crypt: strategies and pitfalls. Cell. Stem Cell. *11,* 452-460.

Barnard, J.A., Beauchamp, R.D., Coffey, R.J., and Moses, H.L. (1989). Regulation of intestinal epithelial cell growth by transforming growth factor type beta. Proc. Natl. Acad. Sci. U. S. A. *86,* 1578-1582.

Bastide, P., Darido, C., Pannequin, J., Kist, R., Robine, S., Marty-Double, C., Bibeau, F., Scherer, G., Joubert, D., Hollande, F., Blache, P., and Jay, P. (2007). Sox9 regulates cell proliferation and is required for Paneth cell differentiation in the intestinal epithelium. J. Cell Biol. *178,* 635-648.

Batlle, E., Bacani, J., Begthel, H., Jonkheer, S., Gregorieff, A., van de Born, M., Malats, N., Sancho, E., Boon, E., Pawson, T.*, et al.* (2005). EphB receptor activity suppresses colorectal cancer progression. Nature *435,* 1126-1130.

Batlle, E., Henderson, J.T., Beghtel, H., van den Born, M.M., Sancho, E., Huls, G., Meeldijk, J., Robertson, J., van de Wetering, M., Pawson, T., and Clevers, H. (2002). Beta-catenin and TCF mediate cell positioning in the intestinal epithelium by controlling the expression of EphB/ephrinB. Cell *111,* 251-263.

Batlle, E., Henderson, J.T., Beghtel, H., van den Born, M.M., Sancho, E., Huls, G., Meeldijk, J., Robertson, J., van de Wetering, M., Pawson, T., and Clevers, H. (2002). Beta-catenin and TCF mediate cell positioning in the intestinal epithelium by controlling the expression of EphB/ephrinB. Cell *111,* 251-263.

Baus-Loncar, M., Lubka, M., Pusch, C.M., Otto, W.R., Poulsom, R., and Blin, N. (2007). Cytokine regulation of the trefoil factor family binding protein GKN2 (GDDR/TFIZ1/blottin) in human gastrointestinal epithelial cells. Cell. Physiol. Biochem. *20,* 193-204.

Beck, F., Chawengsaksophak, K., Luckett, J., Giblett, S., Tucci, J., Brown, J., Poulsom, R., Jeffery, R., and Wright, N.A. (2003). A study of regional gut endoderm potency by analysis of Cdx2 null mutant chimaeric mice. Dev. Biol. *255,* 399-406.

Beck, F., Chawengsaksophak, K., Waring, P., Playford, R.J., and Furness, J.B. (1999). Reprogramming of intestinal differentiation and intercalary regeneration in Cdx2 mutant mice. Proc. Natl. Acad. Sci. U. S. A. *96,* 7318-7323.

Beck, F., and Stringer, E.J. (2010). The role of Cdx genes in the gut and in axial development. Biochem. Soc. Trans. *38,* 353-357.

Birkenkamp-Demtroder, K., Olesen, S.H., Sorensen, F.B., Laurberg, S., Laiho, P., Aaltonen, L.A., and Orntoft, T.F. (2005). Differential gene expression in colon cancer of the caecum versus the sigmoid and rectosigmoid. Gut *54,* 374-384.

Bjerknes, M., and Cheng, H. (1981). The stem-cell zone of the small intestinal epithelium. I. Evidence from Paneth cells in the adult mouse. Am. J. Anat. *160,* 51-63.

Bonhomme, C., Calon, A., Martin, E., Robine, S., Neuville, A., Kedinger, M., Domon-Dell, C., Duluc, I., and Freund, J.N. (2008). Cdx1, a dispensable homeobox gene for gut development with limited effect in intestinal cancer. Oncogene *27,* 4497-4502.

Bonhomme, C., Duluc, I., Martin, E., Chawengsaksophak, K., Chenard, M.P., Kedinger, M., Beck, F., Freund, J.N., and Domon-Dell, C. (2003). The Cdx2 homeobox gene has a tumour suppressor function in the distal colon in addition to a homeotic role during gut development. Gut *52,* 1465-1471.

Bonino, J.A., and Sharma, P. (2004). Barrett esophagus. Curr. Opin. Gastroenterol. *20,* 375-380.

Boudreau, F., Rings, E.H., van Wering, H.M., Kim, R.K., Swain, G.P., Krasinski, S.D., Moffett, J., Grand, R.J., Suh, E.R., and Traber, P.G. (2002). Hepatocyte nuclear factor-1 alpha, GATA-4, and caudal related homeodomain protein Cdx2 interact functionally to modulate intestinal gene transcription. Implication for the developmental regulation of the sucrase-isomaltase gene. J. Biol. Chem. *277,* 31909-31917.

Bowen, R. (2004). **Microanatomy of the Digestive Tube**.

Brabletz, T., Spaderna, S., Kolb, J., Hlubek, F., Faller, G., Bruns, C.J., Jung, A., Nentwich, J., Duluc, I., Domon-Dell, C., Kirchner, T., and Freund, J.N. (2004). Down-regulation of the homeodomain factor Cdx2 in colorectal cancer by collagen type I: an active role for the tumor environment in malignant tumor progression. Cancer Res. *64,* 6973-6977.

Brown, J.D., DiChiara, M.R., Anderson, K.R., Gimbrone, M.A.,Jr, and Topper, J.N. (1999). MEKK-1, a component of the stress (stress-activated protein kinase/c-Jun N-

terminal kinase) pathway, can selectively activate Smad2-mediated transcriptional activation in endothelial cells. J. Biol. Chem. *274,* 8797-8805.

Chawengsaksophak, K., James, R., Hammond, V.E., Kontgen, F., and Beck, F. (1997). Homeosis and intestinal tumours in Cdx2 mutant mice. Nature *386,* 84-87.

Cheng, H., and Leblond, C.P. (1974). Origin, differentiation and renewal of the four main epithelial cell types in the mouse small intestine. V. Unitarian Theory of the origin of the four epithelial cell types. Am. J. Anat. *141,* 537-561.

Cho, N.L., Javid, S.H., Carothers, A.M., Redston, M., and Bertagnolli, M.M. (2007). Estrogen receptors alpha and beta are inhibitory modifiers of Apc-dependent tumorigenesis in the proximal colon of Min/+ mice. Cancer Res. *67,* 2366-2372.

Chun, S.Y., Chen, F., Washburn, J.G., MacDonald, J.W., Innes, K.L., Zhao, R., Cruz-Correa, M.R., Dang, L.H., and Dang, D.T. (2007). CDX2 promotes anchorage-independent growth by transcriptional repression of IGFBP-3. Oncogene *26,* 4725-4729.

Chung, D.C. (2000). The genetic basis of colorectal cancer: insights into critical pathways of tumorigenesis. Gastroenterology *119,* 854-865.

Chung, L.P., Keshav, S., and Gordon, S. (1988). Cloning the human lysozyme cDNA: inverted Alu repeat in the mRNA and in situ hybridization for macrophages and Paneth cells. Proc. Natl. Acad. Sci. U. S. A. *85,* 6227-6231.

Classon, M., and Harlow, E. (2002). The retinoblastoma tumour suppressor in development and cancer. Nat. Rev. Cancer. *2,* 910-917.

Clevers, H. (2006). Wnt/beta-catenin signaling in development and disease. Cell *127,* 469-480.

Clevers, H., and Batlle, E. (2006). EphB/EphrinB receptors and Wnt signaling in colorectal cancer. Cancer Res. *66,* 2-5.

Colleypriest, B.J., Farrant, J.M., Slack, J.M., and Tosh, D. (2010). The role of Cdx2 in Barrett's metaplasia. Biochem. Soc. Trans. *38,* 364-369.

Colleypriest, B.J., Palmer, R.M., Ward, S.G., and Tosh, D. (2009). Cdx genes, inflammation and the pathogenesis of Barrett's metaplasia. Trends Mol. Med. *15,* 313-322.

Cortina, C., Palomo-Ponce, S., Iglesias, M., Fernandez-Masip, J.L., Vivancos, A., Whissell, G., Huma, M., Peiro, N., Gallego, L., Jonkheer, S*., et al.* (2007). EphB-ephrin-B interactions suppress colorectal cancer progression by compartmentalizing tumor cells. Nat. Genet. *39,* 1376-1383.

Crosnier, C., Vargesson, N., Gschmeissner, S., Ariza-McNaughton, L., Morrison, A., and Lewis, J. (2005). Delta-Notch signalling controls commitment to a secretory fate in the zebrafish intestine. Development *132,* 1093-1104.

Davidson, A.J., and Zon, L.I. (2006). The caudal-related homeobox genes cdx1a and cdx4 act redundantly to regulate hox gene expression and the formation of putative hematopoietic stem cells during zebrafish embryogenesis. Dev. Biol. *292,* 506-518.

de la Chapelle, A. (2004). Genetic predisposition to colorectal cancer. Nat. Rev. Cancer. *4,* 769-780.

de Santa Barbara, P., van den Brink, G.R., and Roberts, D.J. (2003). Development and differentiation of the intestinal epithelium. Cell Mol. Life Sci. *60,* 1322-1332.

Dearolf, C.R., Topol, J., and Parker, C.S. (1989). The caudal gene product is a direct activator of fushi tarazu transcription during Drosophila embryogenesis. Nature *341,* 340-343.

Debruyne, P.R., Witek, M., Gong, L., Birbe, R., Chervoneva, I., Jin, T., Domon-Cell, C., Palazzo, J.P., Freund, J.N., Li, P.*, et al.* (2006). Bile acids induce ectopic expression of intestinal guanylyl cyclase C Through nuclear factor-kappaB and Cdx2 in human esophageal cells. Gastroenterology *130,* 1191-1206.

Derynck, R., Akhurst, R.J., and Balmain, A. (2001). TGF-beta signaling in tumor suppression and cancer progression. Nat. Genet. *29,* 117-129.

Domon-Dell, C., Schneider, A., Moucadel, V., Guerin, E., Guenot, D., Aguillon, S., Duluc, I., Martin, E., Iovanna, J., Launay, J.F.*, et al.* (2003). Cdx1 homeobox gene during human colon cancer progression. Oncogene *22,* 7913-7921.

D'Souza, B., Miyamoto, A., and Weinmaster, G. (2008). The many facets of Notch ligands. Oncogene *27,* 5148-5167.

el Marjou, F., Janssen, K.P., Chang, B.H., Li, M., Hindie, V., Chan, L., Louvard, D., Chambon, P., Metzger, D., and Robine, S. (2004). Tissue-specific and inducible Cre-mediated recombination in the gut epithelium. Genesis *39,* 186-193.

Faber, K., Bullinger, L., Ragu, C., Garding, A., Mertens, D., Miller, C., Martin, D., Walcher, D., Dohner, K., Dohner, H.*, et al.* (2013). CDX2-driven leukemogenesis involves KLF4 repression and deregulated PPARgamma signaling. J. Clin. Invest. *123,* 299-314.

Faux, M.C., Ross, J.L., Meeker, C., Johns, T., Ji, H., Simpson, R.J., Layton, M.J., and Burgess, A.W. (2004). Restoration of full-length adenomatous polyposis coli (APC) protein in a colon cancer cell line enhances cell adhesion. J. Cell. Sci. *117,* 427-439.

Fearon, E.R., and Vogelstein, B. (1990). A genetic model for colorectal tumorigenesis. Cell *61,* 759-767.

Fevr, T., Robine, S., Louvard, D., and Huelsken, J. (2007). Wnt/beta-catenin is essential for intestinal homeostasis and maintenance of intestinal stem cells. Mol. Cell. Biol. *27,* 7551-7559.

Fodde, R., and Brabletz, T. (2007). Wnt/beta-catenin signaling in cancer stemness and malignant behavior. Curr. Opin. Cell Biol. *19,* 150-158.

Forbes, S.A., Bindal, N., Bamford, S., Cole, C., Kok, C.Y., Beare, D., Jia, M., Shepherd, R., Leung, K., Menzies, A*., et al.* (2011). COSMIC: mining complete cancer genomes in the Catalogue of Somatic Mutations in Cancer. Nucleic Acids Res. *39,* D945-50.

Fortini, M.E. (2009). Notch signaling: the core pathway and its posttranslational regulation. Dev. Cell. *16,* 633-647.

Fre, S., Huyghe, M., Mourikis, P., Robine, S., Louvard, D., and Artavanis-Tsakonas, S. (2005). Notch signals control the fate of immature progenitor cells in the intestine. Nature *435,* 964-968.

Fre, S., Pallavi, S.K., Huyghe, M., Lae, M., Janssen, K.P., Robine, S., Artavanis-Tsakonas, S., and Louvard, D. (2009). Notch and Wnt signals cooperatively control cell proliferation and tumorigenesis in the intestine. Proc. Natl. Acad. Sci. U. S. A. *106,* 6309-6314.

Funaba, M., Zimmerman, C.M., and Mathews, L.S. (2002). Modulation of Smad2-mediated signaling by extracellular signal-regulated kinase. J. Biol. Chem. *277,* 41361-41368.

Funakoshi, S., Kong, J., Crissey, M.A., Dang, L., Dang, D., and Lynch, J.P. (2010). Intestine-specific transcription factor Cdx2 induces E-cadherin function by enhancing the trafficking of E-cadherin to the cell membrane. Am. J. Physiol. Gastrointest. Liver Physiol. *299,* G1054-67.

Gao, N., and Kaestner, K.H. (2010). Cdx2 regulates endo-lysosomal function and epithelial cell polarity. Genes Dev. *24,* 1295-1305.

Gao, N., White, P., and Kaestner, K.H. (2009). Establishment of intestinal identity and epithelial-mesenchymal signaling by Cdx2. Dev. Cell. *16,* 588-599.

Gao, N., White, P., and Kaestner, K.H. (2009). Establishment of intestinal identity and epithelial-mesenchymal signaling by Cdx2. Dev. Cell. *16,* 588-599.

Garraway, L.A., and Sellers, W.R. (2006). From integrated genomics to tumor lineage dependency. Cancer Res. *66,* 2506-2508.

Gaspar, C., and Fodde, R. (2004). APC dosage effects in tumorigenesis and stem cell differentiation. Int. J. Dev. Biol. *48,* 377-386.

Gontan, C., de Munck, A., Vermeij, M., Grosveld, F., Tibboel, D., and Rottier, R. (2008). Sox2 is important for two crucial processes in lung development: branching morphogenesis and epithelial cell differentiation. Dev. Biol. *317,* 296-309.

Grady, W.M., and Markowitz, S.D. (2002). Genetic and epigenetic alterations in colon cancer. Annu. Rev. Genomics Hum. Genet. *3,* 101-128.

Grainger, S., Hryniuk, A., and Lohnes, D. (2013). Cdx1 and Cdx2 exhibit transcriptional specificity in the intestine. PLoS One *8,* e54757.

Grainger, S., Lam, J., Savory, J.G., Mears, A.J., Rijli, F.M., and Lohnes, D. (2011). Cdx regulates Dll1 in multiple lineages. Dev. Biol.

Grainger, S., Savory, J.G., and Lohnes, D. (2010). Cdx2 regulates patterning of the intestinal epithelium. Dev. Biol. *339,* 155-165.

Greenblatt, D.Y., Vaccaro, A.M., Jaskula-Sztul, R., Ning, L., Haymart, M., Kunnimalaiyaan, M., and Chen, H. (2007). Valproic acid activates notch-1 signaling and regulates the neuroendocrine phenotype in carcinoid cancer cells. Oncologist *12,* 942-951.

Gregorieff, A., and Clevers, H. (2010). In situ hybridization to identify gut stem cells. Curr. Protoc. Stem Cell. Biol. *Chapter 2,* Unit 2F.1.

Gregorieff, A., and Clevers, H. (2005). Wnt signaling in the intestinal epithelium: from endoderm to cancer. Genes Dev. *19,* 877-890.

Gross, I., Duluc, I., Benameur, T., Calon, A., Martin, E., Brabletz, T., Kedinger, M., Domon-Dell, C., and Freund, J.N. (2008). The intestine-specific homeobox gene Cdx2 decreases mobility and antagonizes dissemination of colon cancer cells. Oncogene *27,* 107-115.

Gulhati, P., Bowen, K.A., Liu, J., Stevens, P.D., Rychahou, P.G., Chen, M., Lee, E.Y., Weiss, H.L., O'Connor, K.L., Gao, T., and Evers, B.M. (2011). mTORC1 and mTORC2 regulate EMT, motility, and metastasis of colorectal cancer via RhoA and Rac1 signaling pathways. Cancer Res. *71,* 3246-3256.

Guo, R.J., Funakoshi, S., Lee, H.H., Kong, J., and Lynch, J.P. (2010). The intestine-specific transcription factor Cdx2 inhibits beta-catenin/TCF transcriptional activity by disrupting the beta-catenin-TCF protein complex. Carcinogenesis *31,* 159-166.

Guo, R.J., Huang, E., Ezaki, T., Patel, N., Sinclair, K., Wu, J., Klein, P., Suh, E.R., and Lynch, J.P. (2004). Cdx1 inhibits human colon cancer cell proliferation by reducing beta-catenin/T-cell factor transcriptional activity. J. Biol. Chem. *279,* 36865-36875.

Guo, R.J., Suh, E.R., and Lynch, J.P. (2004). The role of Cdx proteins in intestinal development and cancer. Cancer. Biol. Ther. *3,* 593-601.

Halberg, R.B., Katzung, D.S., Hoff, P.D., Moser, A.R., Cole, C.E., Lubet, R.A., Donehower, L.A., Jacoby, R.F., and Dove, W.F. (2000). Tumorigenesis in the multiple intestinal neoplasia mouse: redundancy of negative regulators and specificity of modifiers. Proc. Natl. Acad. Sci. U. S. A. *97,* 3461-3466.

Hamamoto, T., Beppu, H., Okada, H., Kawabata, M., Kitamura, T., Miyazono, K., and Kato, M. (2002). Compound disruption of smad2 accelerates malignant progression of intestinal tumors in apc knockout mice. Cancer Res. *62,* 5955-5961.

Harada, N., Tamai, Y., Ishikawa, T., Sauer, B., Takaku, K., Oshima, M., and Taketo, M.M. (1999). Intestinal polyposis in mice with a dominant stable mutation of the beta-catenin gene. EMBO J. *18,* 5931-5942.

Hasson, R.M., Briggs, A., Carothers, A.M., Davids, J.S., Wang, J., Javid, S.H., Cho, N.L., and Bertagnolli, M.M. (2014). Estrogen receptor alpha or beta loss in the colon of Min/+ mice promotes crypt expansion and impairs TGFbeta and HNF3beta signaling. Carcinogenesis *35,* 96-102.

Hierholzer, A., and Kemler, R. (2009). Cdx1::Cre allele for gene analysis in the extraembryonic ectoderm and the three germ layers of mice at mid-gastrulation. Genesis *47,* 204-209.

Himanen, J.P., Chumley, M.J., Lackmann, M., Li, C., Barton, W.A., Jeffrey, P.D., Vearing, C., Geleick, D., Feldheim, D.A., Boyd, A.W., Henkemeyer, M., and Nikolov, D.B. (2004). Repelling class discrimination: ephrin-A5 binds to and activates EphB2 receptor signaling. Nat. Neurosci. *7,* 501-509.

Hinkel, I., Duluc, I., Martin, E., Guenot, D., Freund, J.N., and Gross, I. (2012). Cdx2 controls expression of the protocadherin Mucdhl, an inhibitor of growth and beta-catenin activity in colon cancer cells. Gastroenterology *142,* 875-885.e3.

Hinoi, T., Loda, M., and Fearon, E.R. (2003). Silencing of CDX2 expression in colon cancer via a dominant repression pathway. J. Biol. Chem. *278,* 44608-44616.

Hinoi, T., Tani, M., Lucas, P.C., Caca, K., Dunn, R.L., Macri, E., Loda, M., Appelman, H.D., Cho, K.R., and Fearon, E.R. (2001). Loss of CDX2 expression and microsatellite instability are prominent features of large cell minimally differentiated carcinomas of the colon. Am. J. Pathol. *159,* 2239-2248.

Holmberg, J., Genander, M., Halford, M.M., Anneren, C., Sondell, M., Chumley, M.J., Silvany, R.E., Henkemeyer, M., and Frisen, J. (2006). EphB receptors coordinate migration and proliferation in the intestinal stem cell niche. Cell *125,* 1151-1163.

Hryniuk, A., Grainger, S., Savory, J.G., and Lohnes, D. (2014). Cdx1 and Cdx2 Function as Tumor Suppressors. J. Biol. Chem.

Hryniuk, A., Grainger, S., Savory, J.G., and Lohnes, D. (2012). Cdx function is required for maintenance of intestinal identity in the adult. Dev. Biol. *363,* 426-437.

Hsu, P.D., and Zhang, F. (2012). Dissecting neural function using targeted genome engineering technologies. ACS Chem. Neurosci. *3,* 603-610.

Itoh, F., Divecha, N., Brocks, L., Oomen, L., Janssen, H., Calafat, J., Itoh, S., and Dijke Pt, P. (2002). The FYVE domain in Smad anchor for receptor activation (SARA) is sufficient for localization of SARA in early endosomes and regulates TGF-beta/Smad signalling. Genes Cells *7,* 321-331.

Jagle, S., Ronsch, K., Timme, S., Andrlova, H., Bertrand, M., Jager, M., Proske, A., Schrempp, M., Yousaf, A., Michoel, T.*, et al.* (2014). Silencing of the EPHB3 tumor-suppressor gene in human colorectal cancer through decommissioning of a transcriptional enhancer. Proc. Natl. Acad. Sci. U. S. A. *111,* 4886-4891.

James, R., Erler, T., and Kazenwadel, J. (1994). Structure of the murine homeobox gene cdx-2. Expression in embryonic and adult intestinal epithelium. J. Biol. Chem. *269,* 15229-15237.

James, R., and Kazenwadel, J. (1991). Homeobox gene expression in the intestinal epithelium of adult mice. J. Biol. Chem. *266,* 3246-3251.

Jenny, M., Uhl, C., Roche, C., Duluc, I., Guillermin, V., Guillemot, F., Jensen, J., Kedinger, M., and Gradwohl, G. (2002). Neurogenin3 is differentially required for endocrine cell fate specification in the intestinal and gastric epithelium. EMBO J. *21,* 6338-6347.

Jensen, J., Pedersen, E.E., Galante, P., Hald, J., Heller, R.S., Ishibashi, M., Kageyama, R., Guillemot, F., Serup, P., and Madsen, O.D. (2000). Control of endodermal endocrine development by Hes-1. Nat. Genet. *24,* 36-44.

Jones, P.L. (2003). Homeobox genes in pulmonary vascular development and disease. Trends Cardiovasc. Med. *13,* 336-345.

Kaimaktchiev, V., Terracciano, L., Tornillo, L., Spichtin, H., Stoios, D., Bundi, M., Korcheva, V., Mirlacher, M., Loda, M., Sauter, G., and Corless, C.L. (2004). The homeobox intestinal differentiation factor CDX2 is selectively expressed in gastrointestinal adenocarcinomas. Mod. Pathol. *17,* 1392-1399.

Kakizaki, F., Aoki, K., Miyoshi, H., Carrasco, N., Aoki, M., and Taketo, M.M. (2010). CDX transcription factors positively regulate expression of solute carrier family 5, member 8 in the colonic epithelium. Gastroenterology *138,* 627-635.

Karam, S.M. (1999). Lineage commitment and maturation of epithelial cells in the gut. Front. Biosci. *4,* D286-98.

Katz, J.P., Perreault, N., Goldstein, B.G., Lee, C.S., Labosky, P.A., Yang, V.W., and Kaestner, K.H. (2002). The zinc-finger transcription factor Klf4 is required for terminal differentiation of goblet cells in the colon. Development *129,* 2619-2628.

Kim, B.G., Li, C., Qiao, W., Mamura, M., Kasprzak, B., Anver, M., Wolfraim, L., Hong, S., Mushinski, E., Potter, M.*, et al.* (2006). Smad4 signalling in T cells is required for suppression of gastrointestinal cancer. Nature *441,* 1015-1019.

Kim, H.A., Koo, B.K., Cho, J.H., Kim, Y.Y., Seong, J., Chang, H.J., Oh, Y.M., Stange, D.E., Park, J.G., Hwang, D., and Kong, Y.Y. (2012). Notch1 counteracts WNT/beta-catenin signaling through chromatin modification in colorectal cancer. J. Clin. Invest. *122,* 3248-3259.

Kim, T.H., Escudero, S., and Shivdasani, R.A. (2012). Intact function of Lgr5 receptor-expressing intestinal stem cells in the absence of Paneth cells. Proc. Natl. Acad. Sci. U. S. A. *109,* 3932-3937.

Kojima, M., Hosoda, H., Date, Y., Nakazato, M., Matsuo, H., and Kangawa, K. (1999). Ghrelin is a growth-hormone-releasing acylated peptide from stomach. Nature *402,* 656-660.

Kong, J., Crissey, M.A., Funakoshi, S., Kreindler, J.L., and Lynch, J.P. (2011). Ectopic Cdx2 Expression in Murine Esophagus Models an Intermediate Stage in the Emergence of Barrett's Esophagus. PLoS One *6,* e18280.

Koo, B.K., Lim, H.S., Chang, H.J., Yoon, M.J., Choi, Y., Kong, M.P., Kim, C.H., Kim, J.M., Park, J.G., and Kong, Y.Y. (2009). Notch signaling promotes the generation of EphrinB1-positive intestinal epithelial cells. Gastroenterology *137,* 145-55, 155.e1-3.

Korinek, V., Barker, N., Moerer, P., van Donselaar, E., Huls, G., Peters, P.J., and Clevers, H. (1998). Depletion of epithelial stem-cell compartments in the small intestine of mice lacking Tcf-4. Nat. Genet. *19,* 379-383.

Korinek, V., Barker, N., Morin, P.J., van Wichen, D., de Weger, R., Kinzler, K.W., Vogelstein, B., and Clevers, H. (1997). Constitutive transcriptional activation by a beta-catenin-Tcf complex in APC-/- colon carcinoma. Science *275,* 1784-1787.

Koyama, S.Y., and Podolsky, D.K. (1989). Differential expression of transforming growth factors alpha and beta in rat intestinal epithelial cells. J. Clin. Invest. *83,* 1768-1773.

Ku, J.L., Park, S.H., Yoon, K.A., Shin, Y.K., Kim, K.H., Choi, J.S., Kang, H.C., Kim, I.J., Han, I.O., and Park, J.G. (2007). Genetic alterations of the TGF-beta signaling pathway in colorectal cancer cell lines: a novel mutation in Smad3 associated with the inactivation of TGF-beta-induced transcriptional activation. Cancer Lett. *247,* 283-292.

Kuhnert, F., Davis, C.R., Wang, H.T., Chu, P., Lee, M., Yuan, J., Nusse, R., and Kuo, C.J. (2004). Essential requirement for Wnt signaling in proliferation of adult small intestine and colon revealed by adenoviral expression of Dickkopf-1. Proc. Natl. Acad. Sci. U. S. A. *101,* 266-271.

Lambert, M.P., Heller, D.S., and Bethel, C. (2000). Extensive gastric heterotopia of the small intestine resulting in massive gastrointestinal bleeding, bowel perforation, and death: report of a case and review of the literature. Pediatr. Dev. Pathol. *3,* 277-280.

Leibovitz, A., Stinson, J.C., McCombs, W.B.,3rd, McCoy, C.E., Mazur, K.C., and Mabry, N.D. (1976). Classification of human colorectal adenocarcinoma cell lines. Cancer Res. *36,* 4562-4569.

Lewis, S.L., and Tam, P.P. (2006). Definitive endoderm of the mouse embryo: formation, cell fates, and morphogenetic function. Dev. Dyn. *235,* 2315-2329.

Li, H., and Edlund, H. (2001). Persistent expression of Hlxb9 in the pancreatic epithelium impairs pancreatic development. Dev. Biol. *240,* 247-253.

Li, L., and Clevers, H. (2010). Coexistence of quiescent and active adult stem cells in mammals. Science *327,* 542-545.

Li, P., Lin, J.E., Marszlowicz, G.P., Valentino, M.A., Chang, C., Schulz, S., Pitari, G.M., and Waldman, S.A. (2009). GCC signaling in colorectal cancer: Is colorectal cancer a paracrine deficiency syndrome? Drug News. Perspect. *22,* 313-318.

Lipkin, M. (1985). Growth and development of gastrointestinal cells. Annu. Rev. Physiol. *47,* 175-197.

Liu, W., Dong, X., Mai, M., Seelan, R.S., Taniguchi, K., Krishnadath, K.K., Halling, K.C., Cunningham, J.M., Boardman, L.A., Qian, C.*, et al.* (2000). Mutations in AXIN2 cause colorectal cancer with defective mismatch repair by activating beta-catenin/TCF signalling. Nat. Genet. *26,* 146-147.

Lopez-Garcia, C., Klein, A.M., Simons, B.D., and Winton, D.J. (2010). Intestinal stem cell replacement follows a pattern of neutral drift. Science *330,* 822-825.

Lorentz, O., Duluc, I., Arcangelis, A.D., Simon-Assmann, P., Kedinger, M., and Freund, J.N. (1997). Key role of the Cdx2 homeobox gene in extracellular matrix-mediated intestinal cell differentiation. J. Cell Biol. *139,* 1553-1565.

Lugli, A., Tzankov, A., Zlobec, I., and Terracciano, L.M. (2008). Differential diagnostic and functional role of the multi-marker phenotype CDX2/CK20/CK7 in colorectal cancer stratified by mismatch repair status. Mod. Pathol. *21,* 1403-1412.

Ma, L., Juttner, M., Kullak-Ublick, G.A., and Eloranta, J.J. (2012). Regulation of the gene encoding the intestinal bile acid transporter ASBT by the caudal-type homeobox proteins CDX1 and CDX2. Am. J. Physiol. Gastrointest. Liver Physiol. *302,* G123-33.

Macdonald, P.M., and Struhl, G. (1986). A molecular gradient in early Drosophila embryos and its role in specifying the body pattern. Nature *324,* 537-545.

Mallo, G.V., Soubeyran, P., Lissitzky, J.C., Andre, F., Farnarier, C., Marvaldi, J., Dagorn, J.C., and Iovanna, J.L. (1998). Expression of the Cdx1 and Cdx2 homeotic genes leads to reduced malignancy in colon cancer-derived cells. J. Biol. Chem. *273,* 14030-14036.

Margalit, Y., Yarus, S., Shapira, E., Gruenbaum, Y., and Fainsod, A. (1993). Isolation and characterization of target sequences of the chicken CdxA homeobox gene. Nucleic Acids Res. *21,* 4915-4922.

McGinnis, W., and Krumlauf, R. (1992). Homeobox genes and axial patterning. Cell *68,* 283-302.

Mills, J.C., and Shivdasani, R.A. (2011). Gastric epithelial stem cells. Gastroenterology *140,* 412-424.

Mishra, L., Shetty, K., Tang, Y., Stuart, A., and Byers, S.W. (2005). The role of TGF-beta and Wnt signaling in gastrointestinal stem cells and cancer. Oncogene *24,* 5775-5789.

Mlodzik, M., Fjose, A., and Gehring, W.J. (1985). Isolation of caudal, a Drosophila homeo box-containing gene with maternal expression, whose transcripts form a concentration gradient at the pre-blastoderm stage. EMBO J. *4,* 2961-2969.

Mlodzik, M., and Gehring, W.J. (1987). Expression of the caudal gene in the germ line of Drosophila: formation of an RNA and protein gradient during early embryogenesis. Cell *48,* 465-478.

Moolenbeek, C., and Ruitenberg, E.J. (1981). The "Swiss roll": a simple technique for histological studies of the rodent intestine. Lab. Anim. *15,* 57-59.

Mori-Akiyama, Y., van den Born, M., van Es, J.H., Hamilton, S.R., Adams, H.P., Zhang, J., Clevers, H., and de Crombrugghe, B. (2007). SOX9 is required for the differentiation of paneth cells in the intestinal epithelium. Gastroenterology *133,* 539-546.

Morin, P.J., Sparks, A.B., Korinek, V., Barker, N., Clevers, H., Vogelstein, B., and Kinzler, K.W. (1997). Activation of beta-catenin-Tcf signaling in colon cancer by mutations in beta-catenin or APC. Science *275,* 1787-1790.

Moser, A.R., Pitot, H.C., and Dove, W.F. (1990). A dominant mutation that predisposes to multiple intestinal neoplasia in the mouse. Science *247,* 322-324.

Munoz, J., Stange, D.E., Schepers, A.G., van de Wetering, M., Koo, B.K., Itzkovitz, S., Volckmann, R., Kung, K.S., Koster, J., Radulescu, S*., et al.* (2012). The Lgr5 intestinal stem cell signature: robust expression of proposed quiescent '+4' cell markers. EMBO J. *31,* 3079-3091.

Munoz, N.M., Upton, M., Rojas, A., Washington, M.K., Lin, L., Chytil, A., Sozmen, E.G., Madison, B.B., Pozzi, A., Moon, R.T., Moses, H.L., and Grady, W.M. (2006). Transforming growth factor beta receptor type II inactivation induces the malignant transformation of intestinal neoplasms initiated by Apc mutation. Cancer Res. *66,* 9837-9844.

Mutoh, H., Hakamata, Y., Sato, K., Eda, A., Yanaka, I., Honda, S., Osawa, H., Kaneko, Y., and Sugano, K. (2002). Conversion of gastric mucosa to intestinal metaplasia in Cdx2-expressing transgenic mice. Biochem. Biophys. Res. Commun. *294,* 470-479.

Mutoh, H., Hayakawa, H., Sakamoto, H., Sashikawa, M., and Sugano, K. (2009). Transgenic Cdx2 induces endogenous Cdx1 in intestinal metaplasia of Cdx2-transgenic mouse stomach. FEBS J. *276,* 5821-5831.

Mutoh, H., Sakurai, S., Satoh, K., Osawa, H., Hakamata, Y., Takeuchi, T., and Sugano, K. (2004). Cdx1 induced intestinal metaplasia in the transgenic mouse stomach: comparative study with Cdx2 transgenic mice. Gut *53,* 1416-1423.

Mutoh, H., Sashikawa, M., and Sugano, K. (2011). Sox2 expression is maintained while gastric phenotype is completely lost in Cdx2-induced intestinal metaplastic mucosa. Differentiation *81,* 92-98.

Mutoh, H., Satoh, K., Kita, H., Sakamoto, H., Hayakawa, H., Yamamoto, H., Isoda, N., Tamada, K., Ido, K., and Sugano, K. (2005). Cdx2 specifies the differentiation of morphological as well as functional absorptive enterocytes of the small intestine. Int. J. Dev. Biol. *49,* 867-871.

Nakao, A., Afrakhte, M., Moren, A., Nakayama, T., Christian, J.L., Heuchel, R., Itoh, S., Kawabata, M., Heldin, N.E., Heldin, C.H., and ten Dijke, P. (1997). Identification of Smad7, a TGFbeta-inducible antagonist of TGF-beta signalling. Nature *389,* 631-635.

Nakao, A., Okumura, K., and Ogawa, H. (2002). Smad7: a new key player in TGF-beta-associated disease. Trends Mol. Med. *8,* 361-363.

Nathke, I.S. (2004). The adenomatous polyposis coli protein: the Achilles heel of the gut epithelium. Annu. Rev. Cell Dev. Biol. *20,* 337-366.

Natoli, M., Christensen, J., El-Gebali, S., Felsani, A., and Anderle, P. (2013). The role of CDX2 in Caco-2 cell differentiation. Eur. J. Pharm. Biopharm. *85,* 20-25.

Ng, A.Y., Waring, P., Ristevski, S., Wang, C., Wilson, T., Pritchard, M., Hertzog, P., and Kola, I. (2002). Inactivation of the transcription factor Elf3 in mice results in dysmorphogenesis and altered differentiation of intestinal epithelium. Gastroenterology *122,* 1455-1466.

Nishi, T., and Saya, H. (1991). Neurofibromatosis type 1 (NF1) gene: implication in neuroectodermal differentiation and genesis of brain tumors. Cancer Metastasis Rev. *10,* 301-310.

Oien, K.A., McGregor, F., Butler, S., Ferrier, R.K., Downie, I., Bryce, S., Burns, S., and Keith, W.N. (2004). Gastrokine 1 is abundantly and specifically expressed in superficial gastric epithelium, down-regulated in gastric carcinoma, and shows high evolutionary conservation. J. Pathol. *203,* 789-797.

Oshima, M., Oshima, H., Kitagawa, K., Kobayashi, M., Itakura, C., and Taketo, M. (1995). Loss of Apc heterozygosity and abnormal tissue building in nascent intestinal polyps in mice carrying a truncated Apc gene. Proc. Natl. Acad. Sci. U. S. A. *92,* 4482-4486.

Pardali, K., and Moustakas, A. (2007). Actions of TGF-beta as tumor suppressor and pro-metastatic factor in human cancer. Biochim. Biophys. Acta *1775,* 21-62.

Park, S.Y., Jeong, M.S., Yoo, M.A., and Jang, S.B. (2011). Caudal-related homeodomain proteins CDX1/2 bind to DNA replication-related element binding factor. Biochim. Biophys. Acta *1814,* 1891-1899.

Pasquale, E.B. (2005). Eph receptor signalling casts a wide net on cell behaviour. Nat. Rev. Mol. Cell Biol. *6,* 462-475.

Pasquale, E.B. (2004). Eph-ephrin promiscuity is now crystal clear. Nat. Neurosci. *7,* 417-418.

Peignon, G., Durand, A., Cacheux, W., Ayrault, O., Terris, B., Laurent-Puig, P., Shroyer, N.F., Van Seuningen, I., Honjo, T., Perret, C., and Romagnolo, B. (2011). Complex interplay between beta-catenin signalling and Notch effectors in intestinal tumorigenesis. Gut *60,* 166-176.

Pellegrinet, L., Rodilla, V., Liu, Z., Chen, S., Koch, U., Espinosa, L., Kaestner, K.H., Kopan, R., Lewis, J., and Radtke, F. (2011). Dll1- and dll4-mediated notch signaling are required for homeostasis of intestinal stem cells. Gastroenterology *140,* 1230-1240.e1-7.

Pilozzi, E., Onelli, M.R., Ziparo, V., Mercantini, P., and Ruco, L. (2004). CDX1 expression is reduced in colorectal carcinoma and is associated with promoter hypermethylation. J. Pathol. *204,* 289-295.

Pinto, D., and Clevers, H. (2005). Wnt, stem cells and cancer in the intestine. Biol. Cell. *97,* 185-196.

Pinto, D., Gregorieff, A., Begthel, H., and Clevers, H. (2003). Canonical Wnt signals are essential for homeostasis of the intestinal epithelium. Genes Dev. *17,* 1709-1713.

Pitulescu, M.E., and Adams, R.H. (2010). Eph/ephrin molecules--a hub for signaling and endocytosis. Genes Dev. *24,* 2480-2492.

Podolsky, D.K., Lynch-Devaney, K., Stow, J.L., Oates, P., Murgue, B., De-Beaumont, M., Sands, B.E., and Mahida, Y.R. (1993). Identification of human intestinal trefoil factor. Goblet cell-specific expression of a peptide targeted for apical secretion. J. Biol. Chem. *268,* 12230.

Polakis, P. (2007). The many ways of Wnt in cancer. Curr. Opin. Genet. Dev. *17,* 45-51.

Porsch, H., Mehic, M., Olofsson, B., Heldin, P., and Heldin, C.H. (2014). Platelet-derived growth factor beta-receptor, transforming growth factor beta type I receptor, and CD44 protein modulate each other's signaling and stability. J. Biol. Chem. *289,* 19747-19757.

Potten, C.S., Kovacs, L., and Hamilton, E. (1974). Continuous labelling studies on mouse skin and intestine. Cell Tissue Kinet. *7,* 271-283.

Powell, S.M., Zilz, N., Beazer-Barclay, Y., Bryan, T.M., Hamilton, S.R., Thibodeau, S.N., Vogelstein, B., and Kinzler, K.W. (1992). APC mutations occur early during colorectal tumorigenesis. Nature *359,* 235-237.

Que, J., Luo, X., Schwartz, R.J., and Hogan, B.L. (2009). Multiple roles for Sox2 in the developing and adult mouse trachea. Development *136,* 1899-1907.

Que, J., Okubo, T., Goldenring, J.R., Nam, K.T., Kurotani, R., Morrisey, E.E., Taranova, O., Pevny, L.H., and Hogan, B.L. (2007). Multiple dose-dependent roles for Sox2 in the patterning and differentiation of anterior foregut endoderm. Development *134,* 2521-2531.

Raghoebir, L., Bakker, E.R., Mills, J.C., Swagemakers, S., Kempen, M.B., Munck, A.B., Driegen, S., Meijer, D., Grosveld, F., Tibboel, D., Smits, R., and Rottier, R.J. (2012). SOX2 redirects the developmental fate of the intestinal epithelium toward a premature gastric phenotype. J. Mol. Cell. Biol. *4,* 377-385.

Ran, F.A., Hsu, P.D., Wright, J., Agarwala, V., Scott, D.A., and Zhang, F. (2013). Genome engineering using the CRISPR-Cas9 system. Nat. Protoc. *8,* 2281-2308.

Richmond, C.A., and Breault, D.T. (2010). Regulation of gene expression in the intestinal epithelium. Prog. Mol. Biol. Transl. Sci. *96,* 207-229.

Roberts, A.B. (1999). TGF-beta signaling from receptors to the nucleus. Microbes Infect. *1,* 1265-1273.

Rodilla, V., Villanueva, A., Obrador-Hevia, A., Robert-Moreno, A., Fernandez-Majada, V., Grilli, A., Lopez-Bigas, N., Bellora, N., Alba, M.M., Torres, F.*, et al.* (2009). Jagged1 is the pathological link between Wnt and Notch pathways in colorectal cancer. Proc. Natl. Acad. Sci. U. S. A. *106,* 6315-6320.

Rorke, E.A., Zhang, D., Choo, C.K., Eckert, R.L., and Jacobberger, J.W. (2000). TGF-beta-mediated cell cycle arrest of HPV16-immortalized human ectocervical cells correlates with decreased E6/E7 mRNA and increased p53 and p21(WAF-1) expression. Exp. Cell Res. *259,* 149-157.

Ross, M.H. (1989). Histology: a text and atlas, 1 ed. .

Rubinfeld, B., Robbins, P., El-Gamil, M., Albert, I., Porfiri, E., and Polakis, P. (1997). Stabilization of beta-catenin by genetic defects in melanoma cell lines. Science *275,* 1790-1792.

Rubio, D., Garcia, S., De la Cueva, T., Paz, M.F., Lloyd, A.C., Bernad, A., and Garcia-Castro, J. (2008). Human mesenchymal stem cell transformation is associated with a mesenchymal-epithelial transition. Exp. Cell Res. *314,* 691-698.

Runyan, C.E., Hayashida, T., Hubchak, S., Curley, J.F., and Schnaper, H.W. (2009). Role of SARA (SMAD anchor for receptor activation) in maintenance of epithelial cell phenotype. J. Biol. Chem. *284,* 25181-25189.

Salari, K., Spulak, M.E., Cuff, J., Forster, A.D., Giacomini, C.P., Huang, S., Ko, M.E., Lin, A.Y., van de Rijn, M., and Pollack, J.R. (2012). CDX2 is an amplified lineage-survival oncogene in colorectal cancer. Proc. Natl. Acad. Sci. U. S. A. *109,* E3196-205.

Samuel, S., and Naora, H. (2005). Homeobox gene expression in cancer: insights from developmental regulation and deregulation. Eur. J. Cancer *41,* 2428-2437.

Sancho, E., Batlle, E., and Clevers, H. (2004). Signaling pathways in intestinal development and cancer. Annu. Rev. Cell Dev. Biol. *20,* 695-723.

Sangiorgi, E., and Capecchi, M.R. (2008). Bmi1 is expressed in vivo in intestinal stem cells. Nat. Genet. *40,* 915-920.

Sansom, O.J., Meniel, V., Wilkins, J.A., Cole, A.M., Oien, K.A., Marsh, V., Jamieson, T.J., Guerra, C., Ashton, G.H., Barbacid, M., and Clarke, A.R. (2006). Loss of Apc allows phenotypic manifestation of the transforming properties of an endogenous K-ras oncogene in vivo. Proc. Natl. Acad. Sci. U. S. A. *103,* 14122-14127.

Sansom, O.J., Reed, K.R., Hayes, A.J., Ireland, H., Brinkmann, H., Newton, I.P., Batlle, E., Simon-Assmann, P., Clevers, H., Nathke, I.S., Clarke, A.R., and Winton, D.J. (2004). Loss of Apc in vivo immediately perturbs Wnt signaling, differentiation, and migration. Genes Dev. *18,* 1385-1390.

Sato, T., Stange, D.E., Ferrante, M., Vries, R.G., Van Es, J.H., Van den Brink, S., Van Houdt, W.J., Pronk, A., Van Gorp, J., Siersema, P.D., and Clevers, H. (2011). Long-term expansion of epithelial organoids from human colon, adenoma, adenocarcinoma, and Barrett's epithelium. Gastroenterology *141,* 1762-1772.

Sato, T., van Es, J.H., Snippert, H.J., Stange, D.E., Vries, R.G., van den Born, M., Barker, N., Shroyer, N.F., van de Wetering, M., and Clevers, H. (2011). Paneth cells constitute the niche for Lgr5 stem cells in intestinal crypts. Nature *469,* 415-418.

Sato, T., van Es, J.H., Snippert, H.J., Stange, D.E., Vries, R.G., van den Born, M., Barker, N., Shroyer, N.F., van de Wetering, M., and Clevers, H. (2011). Paneth cells constitute the niche for Lgr5 stem cells in intestinal crypts. Nature *469,* 415-418.

Sato, T., Vries, R.G., Snippert, H.J., van de Wetering, M., Barker, N., Stange, D.E., van Es, J.H., Abo, A., Kujala, P., Peters, P.J., and Clevers, H. (2009). Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. Nature *459,* 262-265.

Savory, J.G., Bouchard, N., Pierre, V., Rijli, F.M., De Repentigny, Y., Kothary, R., and Lohnes, D. (2009). Cdx2 regulation of posterior development through non-Hox targets. Development *136,* 4099-4110.

Savory, J.G., Mansfield, M., Rijli, F.M., and Lohnes, D. (2011). Cdx mediates neural tube closure through transcriptional regulation of the planar cell polarity gene Ptk7. Development *138,* 1361-1370.

Savory, J.G., Pilon, N., Grainger, S., Sylvestre, J.R., Beland, M., Houle, M., Oh, K., and Lohnes, D. (2009). Cdx1 and Cdx2 are functionally equivalent in vertebral patterning. Dev. Biol. *330,* 114-122.

Schefe, J.H., Lehmann, K.E., Buschmann, I.R., Unger, T., and Funke-Kaiser, H. (2006). Quantitative real-time RT-PCR data analysis: current concepts and the novel "gene expression's CT difference" formula. J. Mol. Med. (Berl) *84,* 901-910.

Schonhoff, S.E., Giel-Moloney, M., and Leiter, A.B. (2004). Minireview: Development and differentiation of gut endocrine cells. Endocrinology *145,* 2639-2644.

Schroder, N., and Gossler, A. (2002). Expression of Notch pathway components in fetal and adult mouse small intestine. Gene Expr. Patterns *2,* 247-250.

Schwitalla, S., Fingerle, A.A., Cammareri, P., Nebelsiek, T., Goktuna, S.I., Ziegler, P.K., Canli, O., Heijmans, J., Huels, D.J., Moreaux, G.*, et al.* (2013). Intestinal tumorigenesis initiated by dedifferentiation and acquisition of stem-cell-like properties. Cell *152,* 25-38.

Scoville, D.H., Sato, T., He, X.C., and Li, L. (2008). Current view: intestinal stem cells and signaling. Gastroenterology *134,* 849-864.

Sekiya, T., Adachi, S., Kohu, K., Yamada, T., Higuchi, O., Furukawa, Y., Nakamura, Y., Nakamura, T., Tashiro, K., Kuhara, S., Ohwada, S., and Akiyama, T. (2004). Identification of BMP and activin membrane-bound inhibitor (BAMBI), an inhibitor of transforming growth factor-beta signaling, as a target of the beta-catenin pathway in colorectal tumor cells. J. Biol. Chem. *279,* 6840-6846.

Shaoul, R., Marcon, P., Okada, Y., Cutz, E., and Forstner, G. (2000). The pathogenesis of duodenal gastric metaplasia: the role of local goblet cell transformation. Gut *46,* 632-638.

Shen, C.N., Horb, M.E., Slack, J.M., and Tosh, D. (2003). Transdifferentiation of pancreas to liver. Mech. Dev. *120,* 107-116.

Shi, Y., and Massague, J. (2003). Mechanisms of TGF-beta signaling from cell membrane to the nucleus. Cell *113,* 685-700.

Shroyer, N.F., Wallis, D., Venken, K.J., Bellen, H.J., and Zoghbi, H.Y. (2005). Gfi1 functions downstream of Math1 to control intestinal secretory cell subtype allocation and differentiation. Genes Dev. *19,* 2412-2417.

Siegel, R., DeSantis, C., Virgo, K., Stein, K., Mariotto, A., Smith, T., Cooper, D., Gansler, T., Lerro, C., Fedewa, S.*, et al.* (2012). Cancer treatment and survivorship statistics, 2012. CA Cancer. J. Clin. *62,* 220-241.

Silberg, D.G., Furth, E.E., Taylor, J.K., Schuck, T., Chiou, T., and Traber, P.G. (1997). CDX1 protein expression in normal, metaplastic, and neoplastic human alimentary tract epithelium. Gastroenterology *113,* 478-486.

Silberg, D.G., Sullivan, J., Kang, E., Swain, G.P., Moffett, J., Sund, N.J., Sackett, S.D., and Kaestner, K.H. (2002). Cdx2 ectopic expression induces gastric intestinal metaplasia in transgenic mice. Gastroenterology *122,* 689-696.

Sipos, F., and Galamb, O. (2012). Epithelial-to-mesenchymal and mesenchymal-to-epithelial transitions in the colon. World J. Gastroenterol. *18,* 601-608.

Sjoblom, T., Jones, S., Wood, L.D., Parsons, D.W., Lin, J., Barber, T.D., Mandelker, D., Leary, R.J., Ptak, J., Silliman, N.*, et al.* (2006). The consensus coding sequences of human breast and colorectal cancers. Science *314,* 268-274.

Sodir, N.M., Chen, X., Park, R., Nickel, A.E., Conti, P.S., Moats, R., Bading, J.R., Shibata, D., and Laird, P.W. (2006). Smad3 deficiency promotes tumorigenesis in the distal colon of ApcMin/+ mice. Cancer Res. *66,* 8430-8438.

Souza, R.F., Krishnan, K., and Spechler, S.J. (2008). Acid, bile, and CDX: the ABCs of making Barrett's metaplasia. Am. J. Physiol. Gastrointest. Liver Physiol. *295,* G211-8.

Sporn, M.B. (1996). The war on cancer. Lancet *347,* 1377-1381.

Stevenson, B.R., Siliciano, J.D., Mooseker, M.S., and Goodenough, D.A. (1986). Identification of ZO-1: a high molecular weight polypeptide associated with the tight junction (zonula occludens) in a variety of epithelia. J. Cell Biol. *103,* 755-766.

Stringer, E.J., Pritchard, C.A., and Beck, F. (2008). Cdx2 initiates histodifferentiation of the midgut endoderm. FEBS Lett. *582,* 2555-2560.

Su, L.K., Kinzler, K.W., Vogelstein, B., Preisinger, A.C., Moser, A.R., Luongo, C., Gould, K.A., and Dove, W.F. (1992). Multiple intestinal neoplasia caused by a mutation in the murine homolog of the APC gene. Science *256,* 668-670.

Su, L.K., Kinzler, K.W., Vogelstein, B., Preisinger, A.C., Moser, A.R., Luongo, C., Gould, K.A., and Dove, W.F. (1992). Multiple intestinal neoplasia caused by a mutation in the murine homolog of the APC gene. Science *256,* 668-670.

Subramanian, V., Meyer, B.I., and Gruss, P. (1995). Disruption of the murine homeobox gene Cdx1 affects axial skeletal identities by altering the mesodermal expression domains of Hox genes. Cell *83,* 641-653.

Subtil, C., Guerin, E., Schneider, A., Chenard, M.P., Martin, E., Domon-Dell, C., Duluc, I., Brabletz, T., Kedinger, M., Duclos, B., Gaub, M.P., and Freund, J.N. (2007). Frequent

rearrangements and amplification of the CDX2 homeobox gene in human sporadic colorectal cancers with chromosomal instability. Cancer Lett. *247,* 197-203.

Suh, E.R., Ha, C.S., Rankin, E.B., Toyota, M., and Traber, P.G. (2002). DNA methylation down-regulates CDX1 gene expression in colorectal cancer cell lines. J. Biol. Chem. *277,* 35795-35800.

Suzuki, K., Fukui, H., Kayahara, T., Sawada, M., Seno, H., Hiai, H., Kageyama, R., Okano, H., and Chiba, T. (2005). Hes1-deficient mice show precocious differentiation of Paneth cells in the small intestine. Biochem. Biophys. Res. Commun. *328,* 348-352.

Takaku, K., Oshima, M., Miyoshi, H., Matsui, M., Seldin, M.F., and Taketo, M.M. (1998). Intestinal tumorigenesis in compound mutant mice of both Dpc4 (Smad4) and Apc genes. Cell *92,* 645-656.

Takayama, T., Miyanishi, K., Hayashi, T., Sato, Y., and Niitsu, Y. (2006). Colorectal cancer: genetics of development and metastasis. J. Gastroenterol. *41,* 185-192.

Tanaka, M., Saito, H., Kusumi, T., Fukuda, S., Shimoyama, T., Sasaki, Y., Suto, K., Munakata, A., and Kudo, H. (2001). Spatial distribution and histogenesis of colorectal Paneth cell metaplasia in idiopathic inflammatory bowel disease. J. Gastroenterol. Hepatol. *16,* 1353-1359.

Tang, Y., Katuri, V., Dillner, A., Mishra, B., Deng, C.X., and Mishra, L. (2003). Disruption of transforming growth factor-beta signaling in ELF beta-spectrin-deficient mice. Science *299,* 574-577.

Tang, Y., Katuri, V., Srinivasan, R., Fogt, F., Redman, R., Anand, G., Said, A., Fishbein, T., Zasloff, M., Reddy, E.P., Mishra, B., and Mishra, L. (2005). Transforming growth factor-beta suppresses nonmetastatic colon cancer through Smad4 and adaptor protein ELF at an early stage of tumorigenesis. Cancer Res. *65,* 4228-4237.

Tata, P.R., Mou, H., Pardo-Saganta, A., Zhao, R., Prabhu, M., Law, B.M., Vinarsky, V., Cho, J.L., Breton, S., Sahay, A., Medoff, B.D., and Rajagopal, J. (2013). Dedifferentiation of committed epithelial cells into stem cells in vivo. Nature *503,* 218-223.

Thiery, J.P. (2003). Epithelial-mesenchymal transitions in development and pathologies. Curr. Opin. Cell Biol. *15,* 740-746.

Thomas, P.Q., Brown, A., and Beddington, R.S. (1998). Hex: a homeobox gene revealing peri-implantation asymmetry in the mouse embryo and an early transient marker of endothelial cell precursors. Development *125,* 85-94.

Tian, H., Biehs, B., Warming, S., Leong, K.G., Rangell, L., Klein, O.D., and de Sauvage, F.J. (2011). A reserve stem cell population in small intestine renders Lgr5-positive cells dispensable. Nature *478,* 255-259.

Tomita, N., Jiang, W., Hibshoosh, H., Warburton, D., Kahn, S.M., and Weinstein, I.B. (1992). Isolation and characterization of a highly malignant variant of the SW480 human colon cancer cell line. Cancer Res. *52,* 6840-6847.

Treuting, P.M., and Dintzis, S. (2011). Comparative Anatomy and Histology: A Mouse and Human Atlas Elsevier Science).

Tsukamoto, T., Inada, K., Tanaka, H., Mizoshita, T., Mihara, M., Ushijima, T., Yamamura, Y., Nakamura, S., and Tatematsu, M. (2004). Down-regulation of a gastric transcription factor, Sox2, and ectopic expression of intestinal homeobox genes, Cdx1 and Cdx2: inverse correlation during progression from gastric/intestinal-mixed to complete intestinal metaplasia. J. Cancer Res. Clin. Oncol. *130,* 135-145.

Uesaka, T., Kageyama, N., and Watanabe, H. (2004). Identifying target genes regulated downstream of Cdx2 by microarray analysis. J. Mol. Biol. *337,* 647-660.

Urnov, F.D., Rebar, E.J., Holmes, M.C., Zhang, H.S., and Gregory, P.D. (2010). Genome editing with engineered zinc finger nucleases. Nat. Rev. Genet. *11,* 636-646.

van den Akker, E., Forlani, S., Chawengsaksophak, K., de Graaff, W., Beck, F., Meyer, B.I., and Deschamps, J. (2002). Cdx1 and Cdx2 have overlapping functions in anteroposterior patterning and posterior axis elongation. Development *129,* 2181-2193.

van der Flier, L.G., and Clevers, H. (2009). Stem cells, self-renewal, and differentiation in the intestinal epithelium. Annu. Rev. Physiol. *71,* 241-260.

van der Flier, L.G., Haegebarth, A., Stange, D.E., van de Wetering, M., and Clevers, H. (2009). OLFM4 is a robust marker for stem cells in human intestine and marks a subset of colorectal cancer cells. Gastroenterology *137,* 15-17.

van Es, J.H., de Geest, N., van de Born, M., Clevers, H., and Hassan, B.A. (2010). Intestinal stem cells lacking the Math1 tumour suppressor are refractory to Notch inhibitors. Nat. Commun. *1,* 18.

van Es, J.H., Jay, P., Gregorieff, A., van Gijn, M.E., Jonkheer, S., Hatzis, P., Thiele, A., van den Born, M., Begthel, H., Brabletz, T., Taketo, M.M., and Clevers, H. (2005). Wnt signalling induces maturation of Paneth cells in intestinal crypts. Nat. Cell Biol. *7,* 381-386.

van Es, J.H., Sato, T., van de Wetering, M., Lyubimova, A., Nee, A.N., Gregorieff, A., Sasaki, N., Zeinstra, L., van den Born, M., Korving, J.*, et al.* (2012). Dll1+ secretory progenitor cells revert to stem cells upon crypt damage. Nat. Cell Biol. *14,* 1099-1104.

van Es, J.H., van Gijn, M.E., Riccio, O., van den Born, M., Vooijs, M., Begthel, H., Cozijnsen, M., Robine, S., Winton, D.J., Radtke, F., and Clevers, H. (2005). Notch/gamma-secretase inhibition turns proliferative cells in intestinal crypts and adenomas into goblet cells. Nature *435,* 959-963.

van Es, J.H., van Gijn, M.E., Riccio, O., van den Born, M., Vooijs, M., Begthel, H., Cozijnsen, M., Robine, S., Winton, D.J., Radtke, F., and Clevers, H. (2005). Notch/gamma-secretase inhibition turns proliferative cells in intestinal crypts and adenomas into goblet cells. Nature *435,* 959-963.

van Nes, J., de Graaff, W., Lebrin, F., Gerhard, M., Beck, F., and Deschamps, J. (2006). The Cdx4 mutation affects axial development and reveals an essential role of Cdx genes in the ontogenesis of the placental labyrinth in mice. Development *133,* 419-428.

VanDussen, K.L., Carulli, A.J., Keeley, T.M., Patel, S.R., Puthoff, B.J., Magness, S.T., Tran, I.T., Maillard, I., Siebel, C., Kolterud, A.*, et al.* (2012). Notch signaling modulates proliferation and differentiation of intestinal crypt base columnar stem cells. Development *139,* 488-497.

Verzi, M.P., Shin, H., He, H.H., Sulahian, R., Meyer, C.A., Montgomery, R.K., Fleet, J.C., Brown, M., Liu, X.S., and Shivdasani, R.A. (2010). Differentiation-specific histone modifications reveal dynamic chromatin interactions and partners for the intestinal transcription factor CDX2. Dev. Cell. *19,* 713-726.

Verzi, M.P., Shin, H., Ho, L.L., Liu, X.S., and Shivdasani, R.A. (2011). Essential and redundant functions of Caudal family proteins in activating adult intestinal genes. Mol. Cell. Biol. *10*, 2026-2039

Vider, B.Z., Zimber, A., Chastre, E., Gespach, C., Halperin, M., Mashiah, P., Yaniv, A., and Gazit, A. (2000). Deregulated expression of homeobox-containing genes, HOXB6, B8, C8, C9, and Cdx-1, in human colon cancer cell lines. Biochem. Biophys. Res. Commun. *272,* 513-518.

Wang, K., Sengupta, S., Magnani, L., Wilson, C.A., Henry, R.W., and Knott, J.G. (2010). Brg1 is required for Cdx2-mediated repression of Oct4 expression in mouse blastocysts. PLoS One *5,* e10622.

Wang, Y., Yabuuchi, A., McKinney-Freeman, S., Ducharme, D.M., Ray, M.K., Chawengsaksophak, K., Archer, T.K., and Daley, G.Q. (2008). Cdx gene deficiency compromises embryonic hematopoiesis in the mouse. Proc. Natl. Acad. Sci. U. S. A. *105,* 7756-7761.

Wells, J.M., and Melton, D.A. (1999). Vertebrate endoderm development. Annu. Rev. Cell Dev. Biol. *15,* 393-410.

Wigle, J.T., and Eisenstat, D.D. (2008). Homeobox genes in vertebrate forebrain development and disease. Clin. Genet. *73,* 212-226.

Williamson, K.A., Hever, A.M., Rainger, J., Rogers, R.C., Magee, A., Fiedler, Z., Keng, W.T., Sharkey, F.H., McGill, N., Hill, C.J.*, et al.* (2006). Mutations in SOX2 cause anophthalmia-esophageal-genital (AEG) syndrome. Hum. Mol. Genet. *15,* 1413-1422.

Witek, M.E., Nielsen, K., Walters, R., Hyslop, T., Palazzo, J., Schulz, S., and Waldman, S.A. (2005). The putative tumor suppressor Cdx2 is overexpressed by human colorectal adenocarcinomas. Clin. Cancer Res. *11,* 8549-8556.

Wong, N.A., Britton, M.P., Choi, G.S., Stanton, T.K., Bicknell, D.C., Wilding, J.L., and Bodmer, W.F. (2004). Loss of CDX1 expression in colorectal carcinoma: promoter methylation, mutation, and loss of heterozygosity analyses of 37 cell lines. Proc. Natl. Acad. Sci. U. S. A. *101,* 574-579.

Xie, Q.M., Zhang, Y., Mahmood, S., and Alpers, D.H. (1997). Rat intestinal alkaline phosphatase II messenger RNA is present in duodenal crypt and villus cells. Gastroenterology *112,* 376-386.

Yang, D.H., Tsuyama, S., and Murata, F. (2001). The expression of gastric H+-K+-ATPase mRNA and protein in developing rat fundic gland. Histochem. J. *33,* 159-166.

Yang, Q., Bermingham, N.A., Finegold, M.J., and Zoghbi, H.Y. (2001). Requirement of Math1 for secretory cell lineage commitment in the mouse intestine. Science *294,* 2155-2158.

Yoshinaga, K., Obata, H., Jurukovski, V., Mazzieri, R., Chen, Y., Zilberberg, L., Huso, D., Melamed, J., Prijatelj, P., Todorovic, V., Dabovic, B., and Rifkin, D.B. (2008). Perturbation of transforming growth factor (TGF)-beta1 association with latent TGF-beta binding protein yields inflammation and tumors. Proc. Natl. Acad. Sci. U. S. A. *105,* 18758-18763.

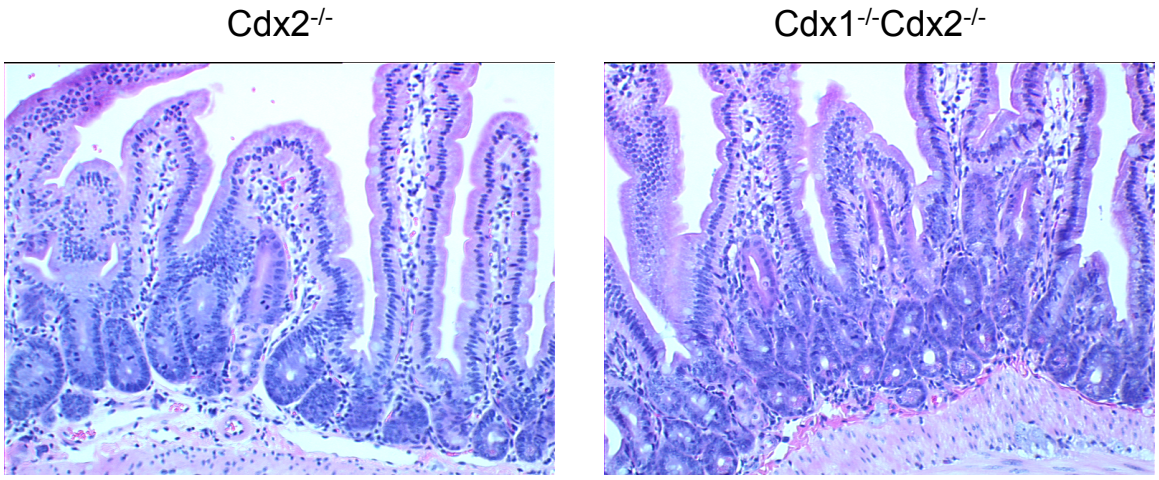
Zhu, Y., Richardson, J.A., Parada, L.F., and Graff, J.M. (1998). Smad3 mutant mice develop metastatic colorectal cancer. Cell *94,* 703-714.

Zorn, A.M., and Wells, J.M. (2009). Vertebrate endoderm development and organ formation. Annu. Rev. Cell Dev. Biol. *25,* 221-251.

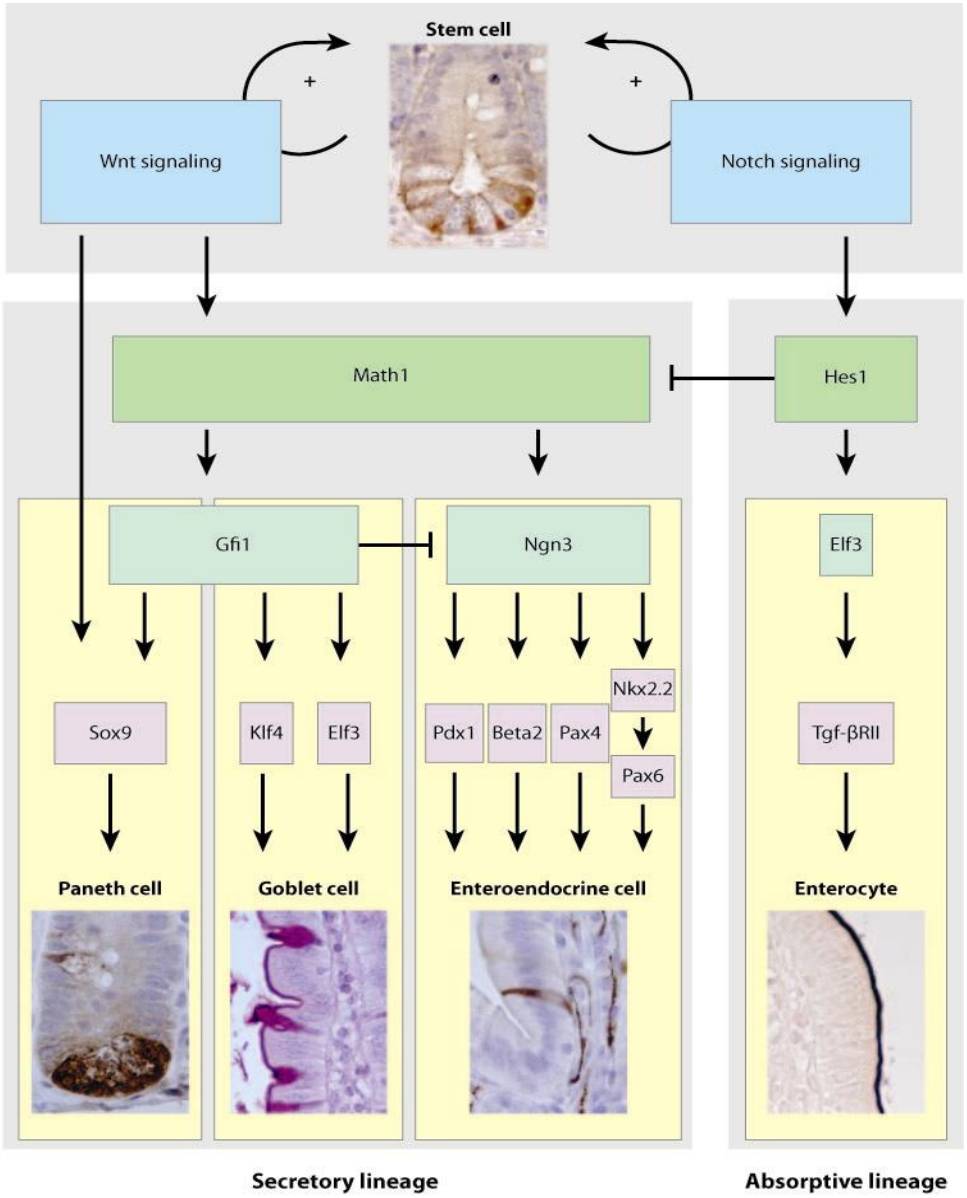
APPENDICES

Table 1: Chapter 2 PCR oligos

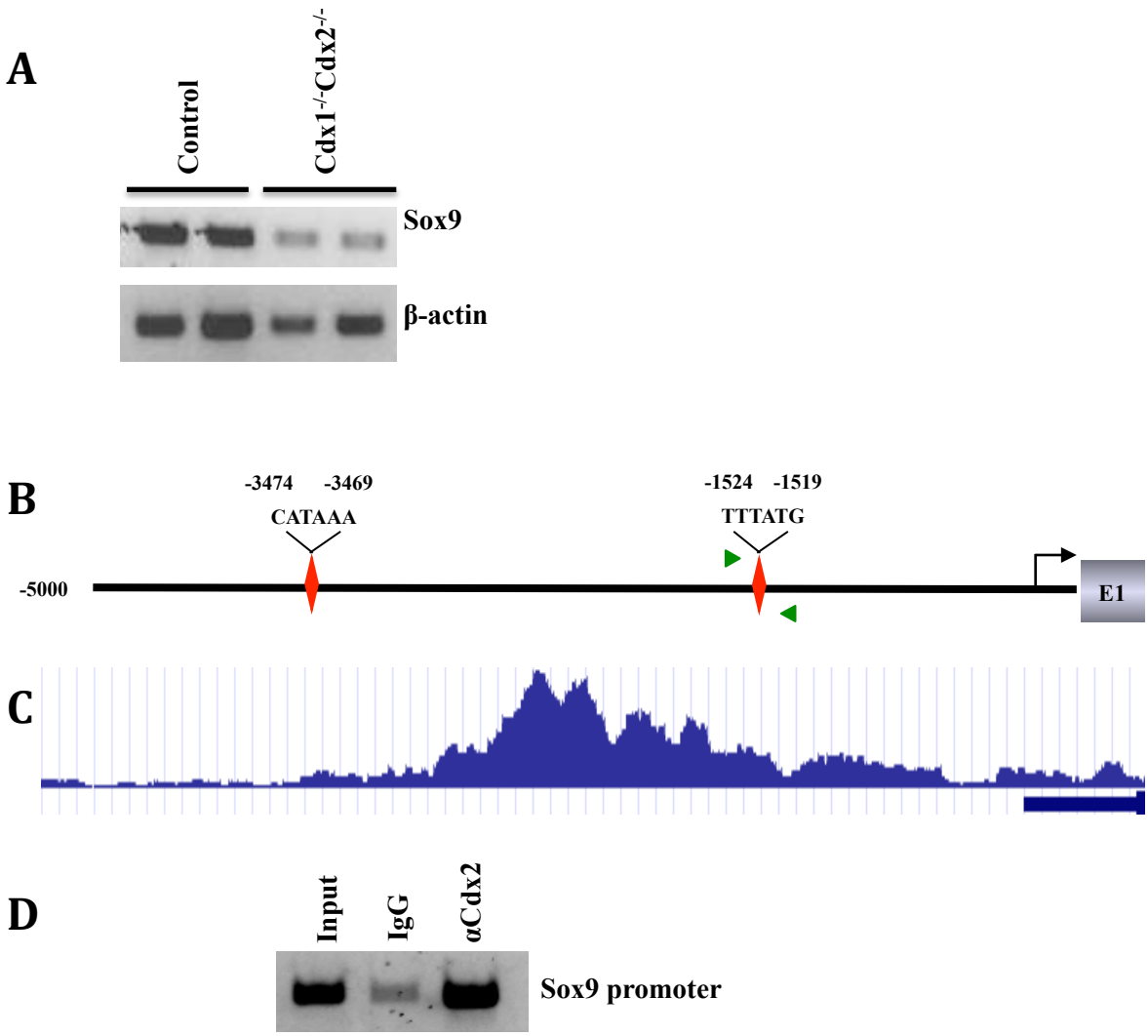
Gene	**Primer sequence**
Dll1 (sense)	TGCTGTCACGCCAGTGCTGGG
Dll1 (antisense)	CAGGGTTGCTGGCAGGTGCC
IAP (sense)	ACATACAGTGTGGACAGACAGGTTCCA
IAP (antisense)	TCCTGCACCAGATTCCTGCCATC
Gkn1 (sense)	GGGGGAAATAAGGGTGGAAGGAG
Gkn1 (antisense)	CCCCCTCACCCCTGACTATTAA
Gkn2 (sense)	CCCTCGTGGCATTTCTGGTGG
Gkn2 (antisense)	GGC GAC CCA AAC AGG AAC CAG T
Ghrl (sense)	GGTCATCTGTCCTCACCACC
Ghrl (antisense)	GCC TCT TTG ACC TCT TCC CA
IFABP (sense)	GATCATGGCATTCGACGGCA
IFABP (antisense)	GCT TTT ACT TCT TTA GCT TTG AC
SI (sense)	GTCCAACTTCAACCCCTAGTCC
SI (antisense)	CAGAGGACCAATACTGGTATCAAACAAGAC
Hoxb13 (sense)	GTTGCCAAGGTGAACAGAACCCA
Hoxb13 (antisense)	CCGCTCCAACTCCCGCAACT
Defa5 (sense)	GAGGACCAGGCTGTGTCTATCTC
Defa5 (antisense)	CAGCTGCAGCAGAATACGAAAGTTAAA
Scnn1B (sense)	AGCTCGAATATCACCCTGAGCAG
Scnn1B (antisense)	CCCCATCCAGAAGCCAAACTG
beta actin (sense)	AGCCATGTACGTAGCCATCC
beta actin (antisense)	CTCTCAGCTGTGGTGGTGAA



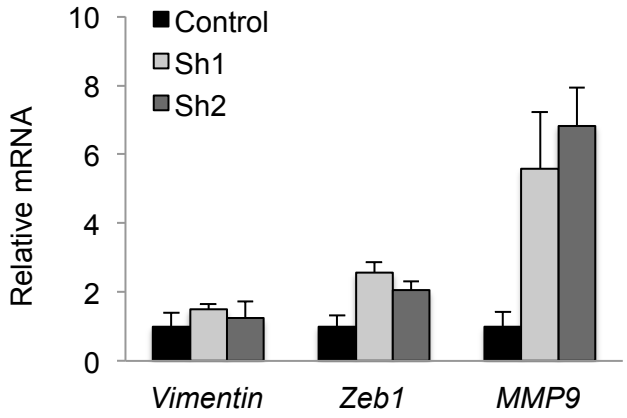
Appendix Figure 1: Reversion back to normal epithelium.
Small intestines from $Cdx2^{-/-}$ and $Cdx1^{-/-}Cdx2^{-/-}$ 6 months after chimeric Cdx2 deletion. Epithelium is reverting back to a normal intestinal epithelium.



Appendix Figure 2: Schematic overview of the genetic hierarchy of epithelial cell lineage commitment in the intestine.
Intestinal stem cells proliferate under control of both the Wnt and the Notch pathways and can differentiate into all four differentiated cell types present in the intestinal epithelium. Math1 is required for the commitment to the secretory lineage. Gfi1 and Sox9 are responsible for differentiation into Paneth cells. Gfi1, kruppel-like factor 4 (Klf4), and E47-like factor 3 (Elf3) are necessary for goblet cell development. Neurogenin3 (Ngn3) is required for endocrine cell fate specification. Hairy/Enhancer of Split 1 (Hes1), through Elf3 and the transforming growth factor β type II receptor (Tgf-βRII), is responsible for differentiation into enterocytes of the absorptive lineage. Figure and legend adapted with permission from van der Flier and Clevers 2009. (van der Flier and Clevers, 2009)



Appendix Figure 3: Cdx2 regulates expression of *Sox9* in the intestine.
(A) Semi-quantitative RT-PCR for RNA from control and $Cdx1^{-/-}Cdx2^{-/-}$ small intestines from mice 48 hours post tamoxifen treatment. (B) Schematic representation of the 5' proximal region of the *Sox9* promoter showing the relative position of putative Cdx response elements (CDRE; diamond). Numbering is relative to the transcriptional start site. (C) ChIP-seq peak diagram for Cdx2 occupancy on the *Sox9* locus. Diagram is aligned with the schematic representation in (B). (D) ChIP for Cdx2 on *Sox9* promoter, using primers flanking the CDRE at -1519 (triangles in A denote primer location).



Appendix Figure 4: Acquisition of metastatic markers upon loss of Cdx2 in SW480 cells.
Semi-quantitative RT-PCR for *Vimentin, Zeb1,* and *Mmp9* from control and Cdx2 knockdown SW480 cell lines. Error bars represent the standard deviation from the mean of 3 independent samples.