THE TRANSCRIPTIONAL REGULATION OF INTESTINAL EPITHELIAL DEVELOPMENT AND ADENOMATOUS POLYPOSIS COLI TUMOUR SUPPRESSOR GENE EXPRESSION BY DLX HOMEOBOX GENES

by

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It has recently come to my attention that doing science, and not to mention completing a Masters or PhD, can be similar to training to run a full marathon. You have dreams (ideas), set goals (hypothesis), train (experiments), get injured (failed experiments), assess the problem (troubleshoot), try again (repeat), see physiotherapist or coach (consult with advisor or committee), try again (repeat), keep training and ultimately finish the race or obtain the target time you were training for (completing degree). But to accomplish such a task is impossible to do alone.

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The work that I have accomplished so far in my project has not been achieved alone, but rather it has been a team effort. A number of people have helped me attain the goals set out for my Masters. From the very beginning when I stepped into the "DE" lab territory, I have come across challenging and yet rewarding experiences.

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Mario Fonseca,
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ABSTRACT

THE TRANSCRIPTIONAL REGULATION OF INTESTINAL EPITHELIAL DEVELOPMENT AND ADENOMATOUS POLYPOSIS COLI TUMOUR SUPPRESSOR GENE EXPRESSION BY DLX HOMEOBOX GENES

Objectives: The intestinal epithelium is a dynamic environment at both embryonic and postnatal developmental stages. A wide array of transcriptional factors are responsible for the generation of secretory and absorptive cell populations during the organism's lifetime in order to faithfully sustain tissue homeostasis. We have shown robust expression of DLX2 homeodomain transcription factor in the developing and postnatal murine small and large intestine. We are interested in determining the role *Dlx2* homeobox gene may play during development as well as during adulthood. We have shown the inverse relationship between the levels of the Adenomatous polyposis coli (*Apc*) transcript and the levels of the β-catenin protein in the *Dlx1/Dlx2* double knockout (mutant) mouse gastrointestinal tract (GIT). Therefore, we hypothesized that the homeodomain protein DLX2 is activating the *Apc* expression. This interaction implies a role for DLX2 in the canonical Wnt signalling pathway in the GIT during development and postnatally.

Methods: Qualitatively, the characterization of DLX2 protein expression pattern in the mouse GIT was conducted during embryonic development and postnatally. Co-expression analysis via immunofluorescence (IF) was performed concurrently with specific intestinal cell type markers to determine DLX2's precise cellular expression. Chromatin immunoprecipitation (ChIP) of embryonic and adult GIT was performed to identify DLX2 protein-genomic DNA complexes *in-situ*. Quantification of expression was performed on embryonic mutant GIT and wild type littermates by quantitative real time polymerase chain reaction (qRT-PCR). Electrophoretic

mobility shift assays (EMSA) and luciferase reporter assays were carried out to investigate direct binding and activity, respectively, of DLX2 on the *Apc* promoter *in-vitro*.

Results: DLX2 expression during embryonic GIT development was investigated. Immunofluorescence analysis revealed that DLX2 is present in the muscularis propria suggesting a possible role in the developing enteric nervous system. However, by E18.5, DLX2 expression was evident in the epithelium layer in both the differentiated and proliferative regions. DLX2 in the adult GIT was found to be expressed in most differentiated cell types of the intestinal epithelium. We have shown a direct relationship between the tumour suppressor gene *Apc* and DLX2. Our *in-vivo* analysis via ChIP assay has demonstrated that DLX2 protein is bound to specific regions of the *Apc* promoter *in-situ*. This result supports a direct role for DLX2 in regulating *Apc* gene expression during intestinal development. Furthermore, the expression level of the downstream mediator β-catenin, which is known to be affected by the levels of APC, is altered in the *Dlx1/Dlx2* double knockout GIT. This finding implicates the involvement of DLX2 in the canonical Wnt signalling pathway.

Conclusions: Taken together, this thesis shows that DLX2 is strongly expressed in the developing and postnatal GIT. We have also shown the direct interaction of DLX2 and the *Apc* promoter *in-vivo*. Furthermore, we have observed that DLX2 induces *Apc* transcription by directly binding to the *Apc* promoter *in-vitro*. We also showed *in-vivo* and *in-vitro*, that β-catenin protein levels are increased when DLX2 is absent or reduced. All this data are in favour of an important role for DLX2 transcription factor in the spatio-temporal expression pattern of *Apc* observed in embryonic, fetal and adult murine GIT. For the first time *Apc*, a gene that is

important in the canonical Wnt signalling pathway and in intestinal development and cell differentiation, has been shown to be a downstream target of the DLX homeodomain family of transcription factors.

LIST OF ABBREVIATIONS

AIP – Anterior intestinal portal

AP – anterior-posterior axis

Apc – Adenomatous polyposis coli gene

APC – Adenomatous polyposis coli protein

CBC – crypt base columnar

cDNA-complimentary deoxyribonucleic acid

CKO- conditional knockout

CIP – Caudal intestinal portal

CIN – chromosomal instability

ChIP – Chromatin Immunoprecipitation

CK1 – casein kinase 1

CpG – CG rich base composition

CRC – colorectal cancer

DV - dorso-ventral axis

DAB - 3,3'-Diaminobenzidine

DIF – Double immunofluorescence

DNA –deoxyribonucleic acid

DKO – Double knockout

Dlx – distaless homeobox gene

DLX - distaless homeobox protein

DMEM - Dulbecco's modified Eagle's medium

Dsh - dishevelled

E –Embryonic

EDTA - Ethylenediaminetetraacetic acid

EMSA – Electrophoretic mobility shift assay

ENS – enteric nervous system

FAP – Familial adenomatous polyposis

FBS – Fetal bovine serum

GABA - γ-Aminobutyric acid

GIT – Gastrointestinal tract

GSK3 - glycogen synthase kinase 3

HNPCC – hereditary non-polyposis colorectal cancer

HRP – Horseradish Peroxidase

IHC – Immunohistochemistry

IF – Immunofluoresence

IgG – Immunoglobulin

LEF - Lymphocyte enhancer factor

LR – left-right axis

LI – Large Intestine

MIN – microsatellite instability

MLH1 – mutL homolog 1

MMR – mismatch repair

MSH2 - mutS homolog 2

MSH6 - mutS homolog 6

NP-40 – Nonidet P-40 (octylphenoxypolyethoxyethanol)

dNTP – deoxyribonucleotide triphosphate

OCT – Optimal Cutting Temperature

ONPG - ortho-Nitrophenyl-β-galactoside

P0– postnatal day 0

PBS – Phosphate buffered saline

PBS-T - Phosphate buffered saline with 0.05% Triton X-100

PCR – Polymerase Chain Reaction

PFA – paraformaldehyde

PIC – Protein Inhbitor Cocktail

PKC – protein kinase C

PMSF – phenylmethanesulfonylfluoride

PMS1 - postmeiotic segregation increased 1

PMS2 - postmeiotic segregation increased 2

PVDF - Polyvinylidene Fluoride

RAD – radial axis

RT – room temperature

RNA-ribonucleic acid

RNAi – RNA interference

mRNA – messenger RNA

SDS-PAGE - sodium dodecyl sulfate polyacrylamide gel electrophoresis

SI – Small Intestine

shRNA – short/small hairpin RNA

siRNA – small interfering RNA

TA – transit amplifying

TBST - Tris-Buffered Saline and Tween 20

TCF – T cell factor

TE – Tris EDTA buffer

WT- Wild-type

Wnt – wingless gene

LIST OF TABLES

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INTRODUCTION

The gastrointestinal tract (GIT) is a tubular organ comprised of a highly dynamic intestinal epithelium. The GIT consists of proliferative and differentiated compartments located in the crypts of Lieberkühn and the villus, respectively. The following chapter will look at general concepts about the GIT with respect to development, structure, function and the different cell types that comprise the intestinal epithelium. As cell turnover in the intestinal epithelium occurs at a remarkably fast rate, general background on gastrointestinal stem cells will also be discussed. The Wnt signalling pathway is a key regulator of homeostasis in the GIT and de-regulation of the Wnt cascade may transform the tissue into a malignant state. The adenomatous polyposis coli (APC) protein, a crucial player in the Wnt cascade, is highly mutated in human Familial Adenomatous Polyposis (FAP) and sporadic colon cancer cases. Colorectal cancer, resulting from aberrant Wnt signalling as a result of *Apc* mutation/inactivation, will be covered. A general background section on *Dlx* homeobox genes and their developmental importance in various organ systems will also be provided. Finally, the limited knowledge regarding of *Dlx* gene expression in the developing gut will be discussed.

The Gastrointestinal Tract

The mammalian gastrointestinal tract, which extends from mouth to anus, is one of the most dynamic and complex organs. Although the entire GIT includes the luminal digestive system of the pharynx, esophagus, stomach, small intestine and large intestine, this report will present data only relating to the small and large intestine, which will be designated as 'gut'. The gut is composed of the three germ layers: the endoderm (epithelial lining of the lumen), mesoderm (smooth muscle layers) and ectoderm (enteric nervous system, as well as the most anterior and

posterior luminal digestive structures) (P. de Santa Barbara et al. 2003). In order to perform its primary function of digestion, absorption, and excretion, different cell types are assembled to form the elaborate structure and architecture of the adult small intestine. The epithelial cells in the adult large intestine are required for secretion and absorption. In addition, to battle the daily exposure of infection or foreign agents to the epithelial environment, the adult GIT contains protective mucosal immune cells. The mammalian intestinal epithelium continually renews itself every 3-5 days from a small pool of intestinal stem cells localized in the crypt region (Giles et al. 2003).

Development of the Gastrointestinal Tract

The formation and regionalization of the primitive vertebrate gut tube is remarkably complex. Early in gastrulation, Nodal signalling is important during the initial endoderm specification in the primitive intestinal epithelium. The production of a number of crucial transcription factors is generated as a result of Nodal gradient activity. As development proceeds, endodermal cells are combined to form definitive endoderm during gastrulation. Ultimately, this complex multi-layer arrangement gives rise to the three germ layers (Figure 1A,B). Between embryonic day (E)7.5 and E9.5, the formation of the gut begins in the mouse. The endoderm invaginates at its anterior and posterior ends which gives rise to two ventral pockets, or portals. These portals are known as the anterior intestinal portal (AIP) and the caudal intestinal portal (CIP). These invaginations move reciprocally with respect to each other to elongate the endodermal layer until they fuse in the midline of the embryo (Figure 1C) (Heath 2010). In the mouse, the lumen is created by E9.0 from a proportion of invaginated cells that have also migrated dorsally and is laterally surrounded by a tube of endoderm and lateral plate-derived splanchnic (visceral) mesoderm (Heath 2010). At approximately E14.5 the multistratified epithelium begins to converge into a

single layer as the endoderm invaginates. As development progresses, neural-crest derived cells begin to migrate and colonize the gut tube, which will give rise to the enteric nervous system (ENS). The ENS is formed as the neural crest cells de-laminate from the dorsal region of the neural tube, colonizing the entire gut, which will subsequently establish the innervation of the GIT (Heath 2010).

The formation of the gut tube in many vertebrates is evolutionarily conserved (Wells & Melton 1999). The endoderm undergoes an extraordinary metamorphosis from a two-dimensional structure into a three-dimensional tube. After gastrulation, the visceral endoderm appears uniformly stratified and the embryonic gut becomes patterned into a three-dimensional structure consisting of an anterior-posterior (AP) axis, dorso-ventral (DV) axis, left-right (LR) axis and subsequently the radial (RAD) axis (P de Santa Barbara et al. 2003). Morphological differentiation along the AP axis will give rise to the formation of the three regions: the foregut, the midgut, and the hindgut.

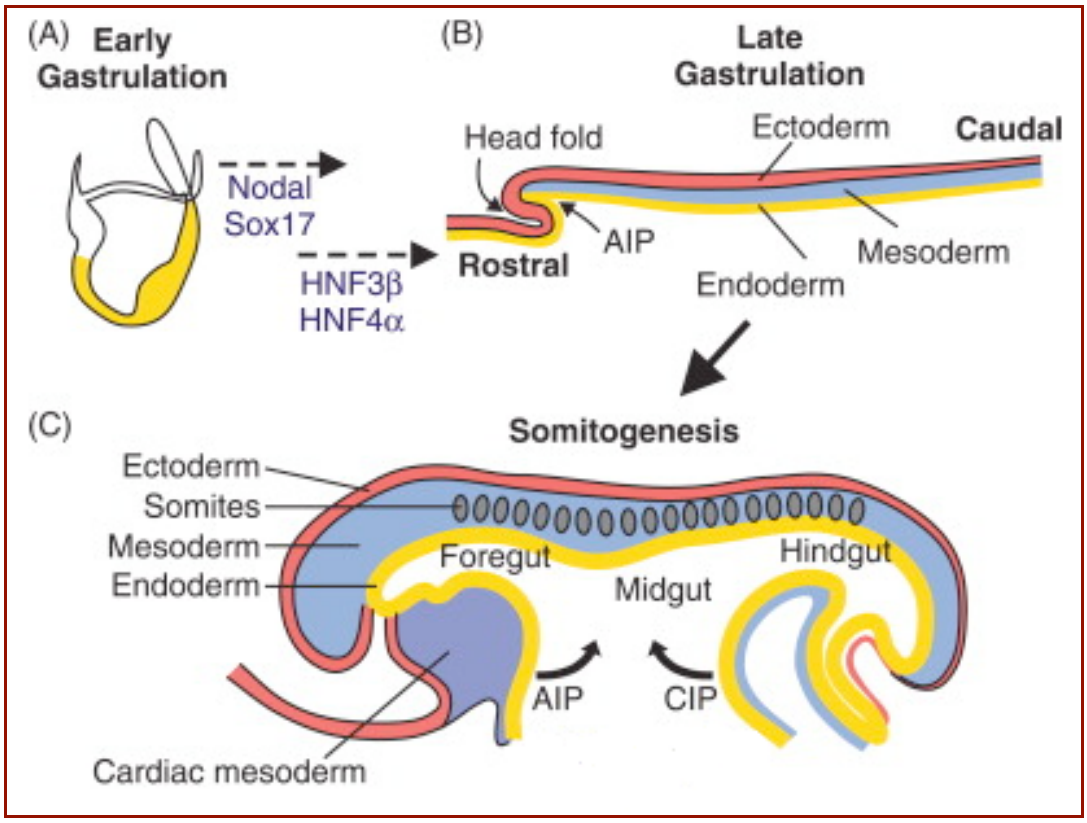

Figure 1: Key events during intestinal organogenesis

(A) During early gastrulation Nodal signalling induces Sox17 to specify endodermal cells. (B) Late gastrulation is initiated by activity from HNF3β and HNF2α to form the definitive ribbon endoderm and the anterior intestinal portal (AIP). (C) During somatogenesis tube formation begins by directed movements of the AIP and caudal intestinal portal (CIP). Molecular players such as Bmp, Shh/Ihh, and Wnt/PCP pathways are key during this process (Taken from Heath 2010).

Consequently, these structures will form the adult gut: pharynx, esophagus, and stomach (from the foregut), the small intestine (midgut-derived), and the large intestine (hindgut-derived) (Heath 2010). The left-right axis is established early as well, where the stomach is positioned on the left side of the organism and the gut rotates in a counter clockwise orientation. In most vertebrates, the endoderm remains and is composed of stratified cuboidal cells throughout all the axes of the gut until approximately the midgestation period of the fetus (Figure 2A). Signalling between the epithelial-mesenchymal compartments is crucial at this point since these interactions

will determine endodermal differentiation with respect to AP and DV regionalization. As the gut proceeds through development, the endoderm becomes more differentiated in that all four axes start to become more phenotypically specific. By E18.5, all of the morphogenetic changes (anterior to posterior) required to distinguish between the small and large intestine are completed. By this stage, the fetal small intestine consists of the finger-like protrusions known as the villi (Figure 2B). Each villus is made up of differentiated cells, while the region between the villi consists mainly of undifferentiated cells with the exception of the Paneth cell population. The anatomical features of the fetal large intestine or colon is different from that of the small intestine since the large intestine does not contain villi and the crypts grow deeper into the intestinal submucosa. Similar to the small intestine, the differentiated compartment is localized at the surface of the epithelium and the proliferative cells reside at the bottom of the crypts. Postnatally, the gut continues to be reshaped. For instance, at approximately postnatal day (P)7, crypts are formed in the small intestine and in the large intestine the crypts continue to grow deeper into the intestinal submucosa. Ultimately, the adult gut has a morphologic and functional pattern that is distinct in all four axes (Figure 2C) (P de Santa Barbara et al. 2003).

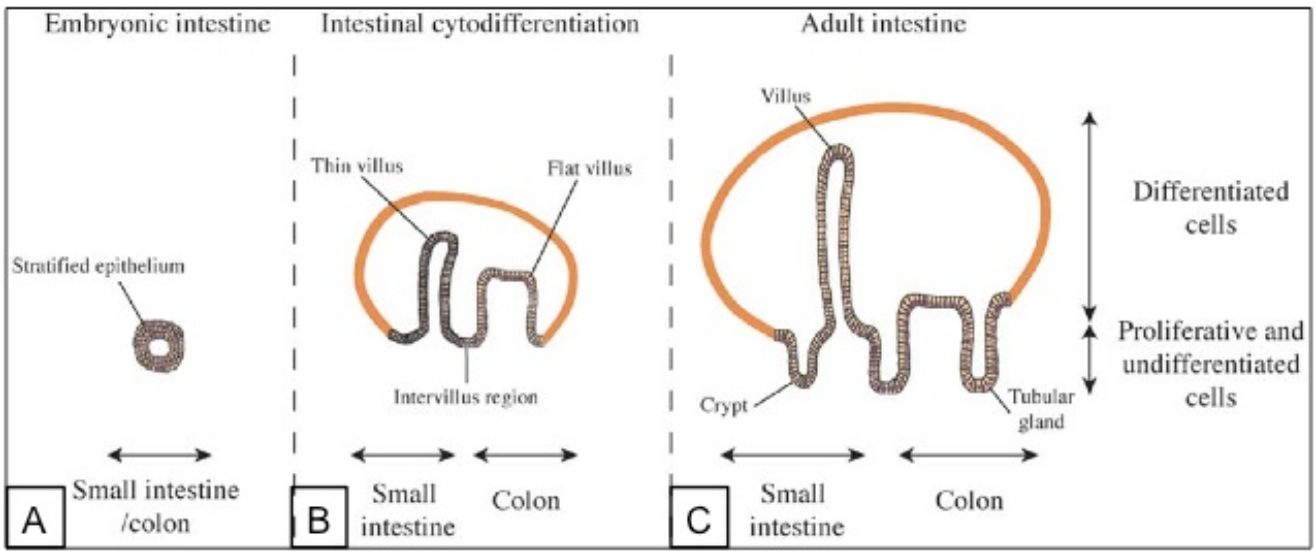

Figure 2: Differentiation of the intestinal epithelium in most vertebrates.

(A)Undifferentiated stratified cuboidal endoderm cells remain uniform in its morphology throughout all axes. (B) Endoderm differentiation into anterior-posterior (AP) and dorsal-ventral (DV). (C) Endoderm becomes phenotypically specific in AP, DV and radial axes (mature gut) (Adapted from P de Santa Barbara et al. 2003).

Structure and Function of the Gastrointestinal Tract

Once established, the physiological function of the mature intestinal tract is to absorb and digest nutrients that have been ingested. Subsequently, the intestinal tract must dispose of the ingested material that is not of any nutritional benefit to the organism. For example, as the ingested nutrients pass through the small intestine, absorption of most of the water, electrolytes, dietary organic molecules and fatty acids occurs. Once the digested material reaches the large intestine, further water absorption occurs, but the main function of the large intestine is to form and store feces. It is in the large intestine where bacterial microflora are found (Sears 2005).

Anatomically in the adult mouse, the small intestine can be subdivided into three regions: the duodenum, the jejunum, and the ileum. Throughout the small intestine, the same basic anatomic structure is observed, and at the microscopic level it is difficult to distinguish the three regions.

However, the region that connects the duodenum and the jejunum is anatomically differentiated by the ligament of Treitz (a ligament connecting the diaphragm to the small intestine and aids in the passage of material through the small intestine as it expands and contracts) (Rubin 2003). Furthermore, Peyer's patches (important in the immune surveillance of the intestinal lumen) and goblet cells are more abundant in the distal region of the small intestine (ileum) (Stevens & Lowe 2002). The principal anatomical difference between the duodenum on the one hand and jejunum and ileum is that Brunner's glands are most abundant in the duodenum (Stevens & Lowe 2002). Similarly, the adult mouse large intestine is subdivided into the cecum, colon, rectum and anal canal. However, histologically, each region is indistinguishable from one another. It is in the large intestine where goblet cells are more abundant than in the small intestine (Stevens & Lowe 2002).

The mature intestine comprises of four layers: an inner mucosa, the submucosa, muscularis propria and outer serosal layers. The inner mucosa contains the intestinal epithelium as well as a supporting lamina propria and the muscularis mucosae, where several layers of smooth muscle fibres are found. In the submucosal layer, mesenchymal tissue plays an important role in connecting the mucosa to the muscle wall via collagenous extracellular matrix where blood, lymphatic vessels and immune cells reside. The muscularis propria contains several layers of smooth muscle arranged as inner circular and outer longitudinal muscle fibres. Enteric neuron ganglia can be found between the two muscle layers (arranged at right angles to each other), which are responsible for the peristaltic movement that moves food along the gut. Finally, the outer serosal layer consists of loose supporting tissue, which carries major vessels and nerves supported by the simple squamous epithelium. Once the mature structure of the intestinal tract is established, differentiation along the radial axis takes place (Figure 3) (Heath 2010). This radial

differentiation in the intestinal epithelium is crucial for tissue homeostasis and remodelling (Nathke 2004). In the mature intestinal epithelium, cells are constantly exposed to gut contents and cells at the surface epithelium are repeatedly sloughed off as they undergo apoptosis.

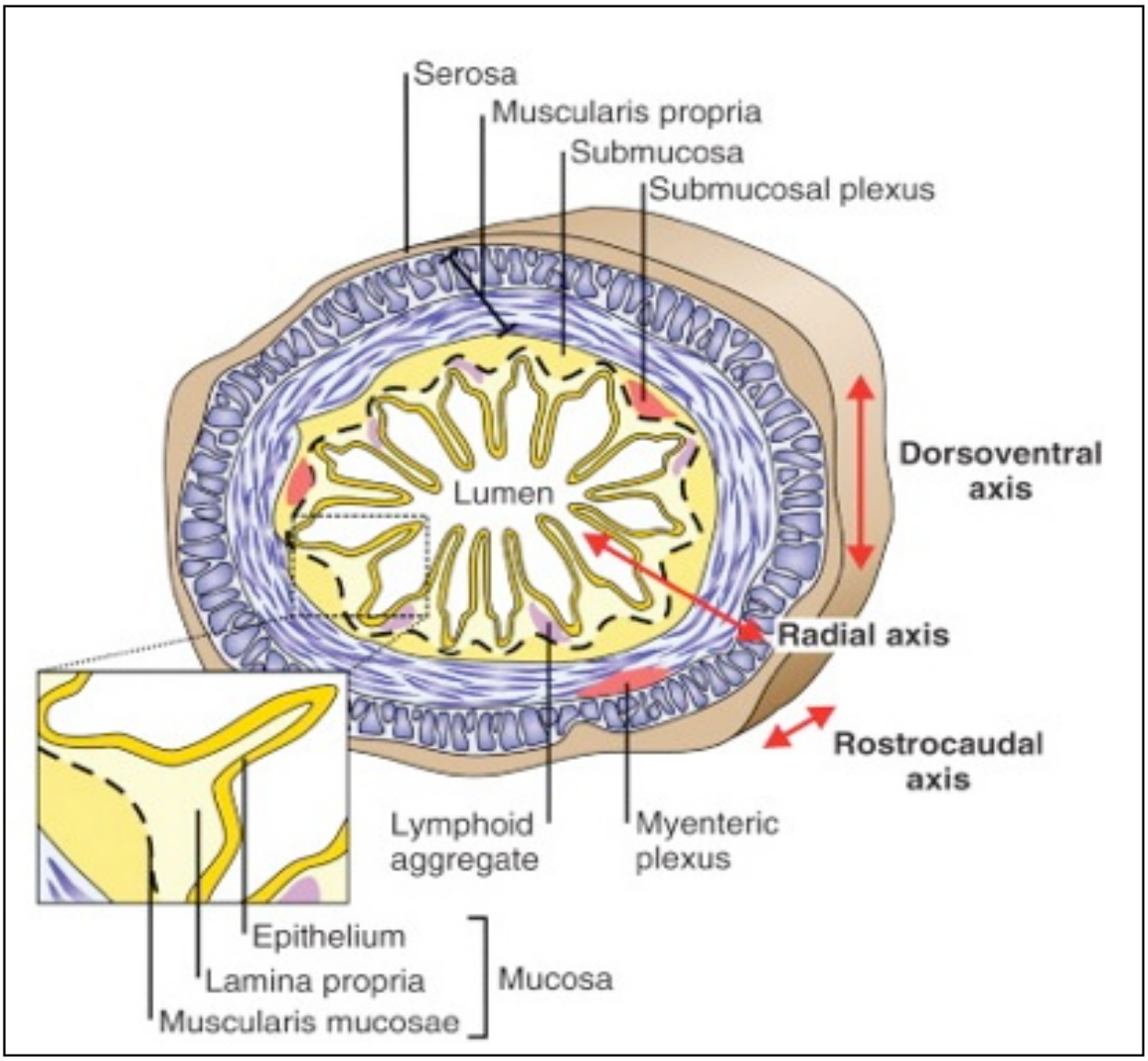

Figure 3: The mature gastrointestinal tract exhibiting three different aspects of organization: dorsoventral, radial and rostrocaudal axes.

The mature intestine comprises four concentric layers of tissue: the mucosa, submucosa, muscularis propria, and serosa. The inset shows the inner lining of the tube consisting of the mucosa (epithelium, lamina propria, muscularis mucosae, and a supportive connective tissue containing lymphoid aggregates) (Taken from Heath 2010)

Therefore, the epithelium must renew itself continually in order to maintain homeostasis. In the mouse, cells normally take two to seven days to make the journey from the site of their final division in the crypt to the point of their exfoliation from the villus tip (Crosnier et al. 2006). During the process of cell differentiation, four distinct cell types are generated: enterocytes, enteroendocrine, goblet and Paneth cells. These cell types can be further classified as absorptive (enterocytes or columnar) or secretory (goblet, enteroendocrine and Paneth cells). Enterocytes, which absorb nutrients, are the most abundant cells in the mature villus (Crosnier et al. 2006). Following absorption, nutrients are then transported across the epithelium to allow uptake into capillaries. The ultrastructure of the enterocytes clearly demonstrates polarization, as these cells display extensive microvilli at their apical surface facing the luminal area. Conversely, cell-cell junctions are evident along the crypt-villus axis (Nathke 2004). The secretory goblets are dispersed between the columnar cells and are responsible for secreting mucous polysaccharides (Nathke 2004). This mucous secretion is important for efficient transport of luminal contents along the gut. Furthermore, the mucous also provides protection against the frictional damage that may occur during content transport. The secretion of hormones is also an important feature of the adult intestinal tract. Enteroendocrine cells, which are scattered among the enterocytes, are important in releasing hormones including serotonin, substance P, and secretin to regulate the secretion of digestive enzymes, bile, and bicarbonate fluids from the pancreas and liver (Höcker & Wiedenmann 1998). Localized at the bottom of the crypt are the Paneth cells. Paneth cells play an important role in secreting protective antimicrobial agents. This protective characteristic of Paneth cells is extremely important. Since gastrointestinal stem cells give rise to all four cell types in the gut, the stem cell niche must be well protected, and the proximity of Paneth cells can

provide one type of protection by secreting antimicrobial peptides in their immediate vicinity (Nathke 2004).

Gastrointestinal Stem Cells

In the mouse and human GIT, the epithelium must be generated throughout the lifetime of the organism since the surface of the intestinal epithelium is constantly being exposed to one of the most chemically toxic and mechanically stressful environments in the body. The consequence of this constant abuse is the fast cell turnover in the gastrointestinal tract. Usually, differentiated cells in the gut have a lifespan of about 3-5 days. The current understanding of intestinal epithelial generation is that stem cells carry out a dual task, in that intestinal stem cells generate all the mature cell types in the intestinal epithelium and at the same time also re-generate themselves. This is accomplished through asymmetric cell division. The intestinal stem cell compartment is located near the crypt bottom and they produce the rapidly dividing cell population known as the transit-amplifying cells (TA) (T. Sato et al. 2009). TA cells are located above the stem cell and Paneth cell compartments and this group of rapidly dividing cells is partially differentiated (progenitors). Furthermore, once TA cells are destined to become any of the aforementioned cell types, an upward migration ensues in order to find their final position along the villus axis. The exception is found in the fourth cell type: the Paneth cell. Paneth cells do not migrate upward and instead, they differentiate during migration downward to the bottom of the crypt.

Over the last 50 years the identification of gastrointestinal stem cells using definitive molecular markers has proven elusive and has become a dynamic topic and is still under fierce debate. However, with the advent of mouse transgenic technology by means of functional and/or genetic tracing, *Lgr*5 and *Bmi*1 have recently been identified as novel gastrointestinal stem cell markers

(Nick Barker et al. 2007; Sangiorgi & Capecchi 2008). Presently, there are two models regarding the exact position of the gastrointestinal stem cell: the +4 position and the crypt bottom position known as the crypt base columnar (CBC) cells (T. Sato et al. 2009).

Colorectal Cancer and the Wnt/β-catenin pathway

Generally, tissues that are known to possess proliferative activity are more prone to undergoing genetic alterations over time than tissues that are static in nature. The gastrointestinal tract epithelium provides an exemplary model of a dynamic self-renewal system, similar to the skin and the hematopoietic system (Radtke et al. 2006). When the homeostasis of the intestinal epithelium is disturbed, it can give rise to tumorigenesis.

Intestinal neoplasia can be manifested in two different forms: hereditary and sporadic. Hereditary intestinal neoplasia can be subdivided based on the presence of polyposis as demonstrated by familial adenomatous polyposis (FAP) and hereditary nonpolyposis colorectal cancer (HNPCC) or Lynch syndrome (Sancho et al. 2004). Although both FAP and HNPCC develop colonic adenomas, the number of polyps manifested in FAP cases is significantly more than in HNPCC. Adenomas and the high number of polyps precede colorectal cancer carcinomas, which normally result in 100% of cohorts by approximately age 40 years (Sancho et al. 2004). FAP syndromes are known to display a number of molecular abnormalities such as mutations in suppressor genes and oncogenes such as *APC*, K-*ras*, *p53* and chromosomal instability (CIN) (Sancho et al. 2004). Reports have indicated that mutations in the tumour suppressor gene *APC* cause these adenomas to behave aggressively in terms of invasiveness and metastasis. The 5' half of the last exon is often a mutational target. Solomon and colleagues have shown that a relatively high proportion of sporadic colorectal carcinomas lose all or part of one chromosome 5, presumably because of the FAP gene function favours tumour progression

(Solomon et al. 1987). Other forms of *APC* mutations have been reported. The attenuated form of FAP renders a much lower number of polyps and causes manifestation of CRC at a later age than the more usual FAP patients (Leppert et al. 1990).

The force driving HNPCC is microsatellite instability (MIN) suggesting that colorectal cancers may arise through a mechanism that does not necessarily involve loss of heterozygosity (Thibodeau et al. 1993). Microsatellites are defined as genomic regions or single nucleotides that are highly repetitive. The repetitive nature of these genomic regions can be the cause of contraction or elongation during DNA replication. Five different mismatch repair (MMR) genes are known to be involved in HNPCC cohorts and these are: *MSH2, MLH1, MSH6, PMS1, PMS2* (Sancho et al. 2004). Of these MMR genes, *MLH1* and *MSH2* account 90% of all identified mutations (Sancho et al. 2004). In addition to the mutation in mismatch repair genes, β-*catenin*, transforming growth factor beta-receptor II, and the proapoptotic gene *Bax* have been implicated in HNPCC tumours caused by MIN (Rampino et al. 1997).

In sporadic colorectal cancer, there is no genetic or familial predisposition linked to the disease. The frequency of sporadic cancer is approximately 75-89% (Poulogiannis et al. 2010). Mismatch repair defects are not only restricted to HNPCC tumours. Somatic changes are known to occur in at least one of the MMR genes, which account to approximately 15-20% of sporadic colorectal cancer (Poulogiannis et al. 2010). Although genomic instability, via MMR genes, is observed in both hereditary and sporadic colorectal cancers, the mode of action in each is different. In HNPPC cases, one of the alleles (MMR genes) is already mutated at the germ-line level, and the subsequent acquired mutation of the second allele will give rise to a mutated MMR phenotype (Poulogiannis et al. 2010). Conversely, in sporadic colonic cancers, the inactivation of the MMR genes (*MLH1*) is caused almost entirely by hypermethylation of the *MLH1*

promoter, which results in silencing both copies of the *MLH1* gene at the transcriptional level (Cunningham et al. 1998).

In the last two decades, important signalling pathways that are responsible for the maintenance of the intestinal epithelium have been elucidated. A substantial amount of research has been conducted to determine that when certain molecular players are either mutated or inactivated, it can lead to tumorigenesis which can escalate into malignant transformation of tissue mass. Signalling pathways play an important role during embryogenesis as they control key events during development. Several signalling molecules have been identified within the last two to three decades such as the Hedgehogs, Wnts, bone morphogenetic proteins and the fibroblast growth factors (FGFs) (Inke S Näthke 2004).

Many types of cancers have an underlying signalling pathway that is responsible for the neoplastic condition, which may support that cancer is a faulty form of development. The Wnt signalling pathway was initially discovered in the fruit fly *Drosophila melanogaster* (*Wingless* gene), and subsequently *Wnt* homologues have been found in vertebrates as well. Wnts are cell-cell signalling proteins that are involved in a wide range of developmental processes and also play an important role in adult tissue homeostasis (Nusse 2008).

The canonical Wnt pathway recruits a number of players that are found in all three compartments of the cell: membrane, cytoplasm and nucleus. In the absence of Wnt stimulation, the cytoplasmic protein complex consisting of Dishevelled (Dsh), glycogen synthase kinase -3-β (GSK-3), Axin, and APC tags available cytoplasmic β-catenin for degradation by the proteosome (Gregorieff & Hans Clevers 2005). The phosphorylation of β-catenin by GSK-3 allows it to be ubiquitinated and targeted for rapid destruction by the proteosome. As a consequence, β-catenin

levels are low and this allows repression of the T cell factor (TCF) via the interaction with transcriptional repressors such as Groucho, hence no transcription of *Wnt* target genes ensues. Conversely, upon Wnt stimulation, the protein complex is disabled resulting in the stabilization of free cytoplasmic β-catenin. When free stabilized β-catenin levels increase, it is translocated into the nucleus where it binds TCF and activates the transcription of *Wnt* target genes (Figure 4).

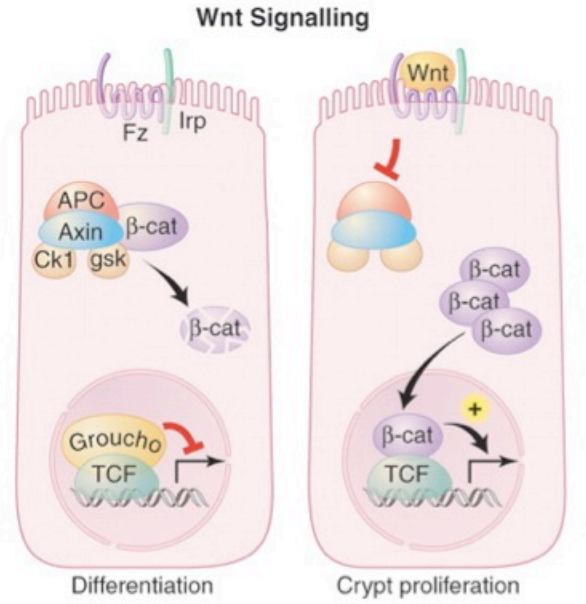

Figure 4: Schematic of the canonical Wnt signalling pathway

Cells that are responsive to the Wnt signals express the Frizzled receptor (Fz) and Lrp5 or Lrp6. In the absence of the Wnt signal (cell on the left), degradation of the β-catenin occurs via the destruction complex preventing the translocation of β-catenin into the nucleus and thereby repressing specific Wnt target genes. When the Frizzled receptor interacts with a Wnt molecule (cell on right), the destruction complex is inactivated allowing β-catenin to be translocated into the nucleus and activating Wnt target genes. (F. Radtke, H. Clevers Science 2005)

The Wnt pathway can be manifested in two different developmental scenarios: the canonical Wnt pathway and the non-canonical Wnt pathway. The non-canonical pathway can be further subdivided into two pathways. The first one is the Frizzled activated pathway which is also known as the planar polarity pathway. The planar polarity pathway was first identified in the *D. melanogaster*, and is important in the development of different cell types (Strutt 2003). For instance, hairs and feathers must be oriented in a certain direction in the right place at the right time. These polarization events have been best studied in *D. melanogaster.* The second non-canonical pathway is known to involve intracellular calcium. The membrane bound protein Frizzled is also involved, but it is known to interact with intracellular heterotrimeric G-proteins (α, β, γ subunits) in order to transport intracellular $Ca^{2+,}$ as well as to stimulate protein kinase C (PKC) (Strutt 2003). This report will only focus on the canonical Wnt pathway (β-catenin dependent).

The canonical Wnt signalling pathway plays a crucial role in maintaining a tight balance between proliferation, differentiation, migration and cell death in the intestinal epithelium. The beginning of tumour formation takes place when the homeostasis in the intestinal epithelium is uncoupled resulting in hyperproliferation. The outcome, with respect to the pathological manifestation, is normally observed as colorectal cancer (Giles et al. 2003).

The Wnt signalling pathway has been implicated in a number of human cancers (Giles et al. 2003). When the Wnt signalling pathway is activated because of a known genetic alteration, aberrant crypt foci and small polyps (early lesions) develop (Powell et al. 1992). Both APC and β-catenin have been central to understanding colorectal carcinogenesis as well as intestinal development and homeostasis. Molecular studies have elucidated activating mutations in the molecular components of the Wnt signalling pathway. Approximately 90% occurrence of

colorectal cancer has been strongly linked with activation mutations in the Wnt pathway as *Apc* mutation has a higher prevalence in colorectal neoplasia (Giles et al. 2003). In sporadic adenomas and cancers, over 80% are attributed to an inactive or mutated form of the APC protein. Furthermore, in over 85% of human colorectal cases, namely Familial adenomatous polyposis (FAP) cases, the *Apc* gene is mutated or inactivated (Kinzler & Vogelstein 1996). The level of β-catenin increases as a result of an inactive APC protein, and this will render the β-catenin destruction complex inactive and will turn the cell into a proliferative state. Robust evidence clearly indicates that the β-catenin/TCF mode of transcription is responsible for promoting cellular proliferation and repressing differentiation. This is the case during embryogenesis and postnatally in the murine intestinal epithelium proliferative compartment, the crypts (Giles et al. 2003). Mutations in the β-catenin gene are also found in approximately 10% of CRC tumours (Giles et al. 2003). Normally the mutation occurs in or near exon 3 of the β-catenin gene, which renders it resistant to phosphorylation by GSK-3, thereby refractory to degradation by the proteosome (Morin et al. 1997). Although APC is the primary culprit behind the formation of polyposis in the intestinal epithelium, other components belonging to the canonical Wnt pathway have been found to be mutated. Mutations in the scaffolding Axin/Axin2 gene have also been responsible for elevated levels of intracellular β-catenin, hence activating TCF signalling but these mutations only result in the predisposition of individuals to colorectal cancer (W. Liu et al. 2000).

Dlx **Homeobox Genes**

Dlx (distalless homeobox) genes generate homeodomain proteins that are important during embryonic development in both invertebrates and vertebrates (Cohen & Jürgens 1989; McGuinness et al. 1996). Originally the *Dlx* genes were first discovered in *D.melanogaster*

(Cohen & Jürgens 1989). Subsequently, vertebrate orthologues of the *Drosophila* gene *distal-less* were also discovered (McGuinness et al. 1996). Six known *Dlx* genes are found in mice and humans (Grace Panganiban & John L R Rubenstein 2002a). The organization of mammalian *Dlx* genes is such that they are arranged in bigenic clusters as three pairs (10-30 kb apart) and each pair is linked to one of the four *Hox* clusters (Stock et al. 1996).

Vertebrate homeobox genes encode transcription factors containing a highly conserved 60 amino acids DNA-binding homeodomain with the *Drosophila distal-less* gene (Merlo et al. 2000). The homeodomain is made up of four helices: three α-helices and a fourth helix. Through the second and third helices, these homeodomain proteins discriminately bind to ATTA/TAAT DNA sequences found on gene regulatory regions, which will subsequently regulate the expression of the target genes that are involved in development and differentiation (J T Wigle & D D Eisenstat 2008).

Loss-of-function analyses of *Dlx* genes have shown that they are important in a number of organ systems. In the developing central nervous system, *Dlx* genes are important for the tangential migration of subpallial-derived GABAergic interneurons to the neocortex in the mouse (Anderson et al. 1997a). In the developing retinal epithelium, *Dlx* genes have also been shown to impart functional roles in terminal differentiation and survival of late-born retinal ganglion cells in the developing mouse retina (de Melo et al. 2005a). In the *Dlx1/Dlx2* double knockout mouse, which dies at birth, the retina displayed a reduced ganglion cell layer due to apoptosis hence the thinning of the optic nerve (de Melo et al. 2005a). In our laboratory, it has been shown that the developing pancreas also expresses *Dlx1* and *Dlx2* genes. This is of interest since the pancreas originates from the same embryonic germ layer as the intestine (endoderm), and more interestingly the fact that important pancreatic hormones were found to be co-expressed with

Dlx1 and *Dlx2* gene products (A. Ho, D. Eisenstat, unpublished). *Dlx* genes have also been demonstrated to be important in limb and craniofacial development (G Panganiban 2000; M. Qiu et al. 1997).

Dlx **Genes in the Gastrointestinal Tract**

The complexity that is involved during embryonic organogenesis is remarkable. This complexity requires the cross talk between tissue-restricted transcription factors. A vast array of transcription factors are involved during gut organogenesis (Choi et al. 2006; Vohra et al. 2006; Heanue & Pachnis 2007). Amongst the several transcription factors that have been reported, the homeodomain transcription factors are well represented in the developing gut (Choi et al. 2006). Furthermore, in a prior publication it was found that both *Dlx1* and *Dlx2* genes are actively expressed in the murine enteric nervous system during midgestation (M. Qiu et al. 1995). Thus far, there is little information about the functional role that *Dlx* genes play in the developing GIT. Similarly, information on the functional role of *Dlx* genes in the postnatal gut remains to be elucidated.

Hypotheses and Research Aims

Hypothesis 1

Dlx genes are expressed in transit-amplifying cells as well as in several differentiated cells types in the small intestine and large intestine. Loss of *Dlx1/Dlx2* may result in increased proliferation, apoptosis and/or alternative cell-fate specification.

Specific aim 1: Determine the role of *Dlx* genes in intestinal development and epithelial self-renewal.

Dlx2 expression will be assessed by immunohistochemistry and/or immunofluorescence throughout intestinal development using high affinity DLX2 antibodies. Co-expression analysis using various cell-specific markers and DLX2 antibodies will be used by immunohistochemistry and/or immunofluorescence to identify which cells express *Dlx* genes at various stages during development. Cell cycle and cell death will be assessed in *Dlx*1/*Dlx*2 double knockouts. *Dlx2* expression will be explored in the gastrointestinal stem cell population using recently established gastrointestinal stem cell markers.

Hypothesis 2

We predict that DLX2 directly activates *Apc* gene transcription; hence affecting Wnt pathway signalling by decreasing APC levels and increasing β-catenin mediated transcription in the *Dlx1/Dlx2* mutant.

Specific aim 2: Characterize the transcriptional regulation of the *Apc* gene by DLX2 and subsequently measure levels of its downstream targets.

Using specific antibodies against murine APC and β-catenin, immunohistochemistry and/or immunofluorescence will be performed on E18.5 wild type and *Dlx*1/*Dlx*2 knockout small and

large intestine. *In-vivo* semi quantitative analysis will be performed by Western immunoblotting by extracting total protein from E18.5 small and large intestine and from wild type and *Dlx1/Dlx2* knockout mice. *In-vivo* quantitative analysis will be performed by extracting RNA from E18.5 small and large intestine and subjected to qRT-PCR using specific primers for *Apc* and β-*catenin*.

Chromatin immunoprecipitation (ChIP) analysis will determine whether the DLX2 protein localizes to the proximal *Apc* promoter regions. Following ChIP analysis, electrophoretic mobility shift assay (EMSA) will be performed to assess specificity of DLX2 binding on the *Apc* promoter regions by using affinity purified recombinant DLX2 protein and intestinal nuclear extracts.

Gene reporter assays will be performed to investigate the functional significance of the DLX2 interaction on the *Apc* promoter regions. This will be conducted by performing luciferase reporter gene assay using the human embryonic kidney (HEK293) cell line.

MATERIALS AND METHODS

Animals and tissue preparation

The animal tissues used for these studies were obtained from the mouse (*Mus musculus*). The wild type CD-1 [CD-1 (ICR)BR.Swiss strain of albino mice] strain was used for postnatal and adult studies. *Dlx*1/*Dlx*2 knockout (KO) mice used were generated at the laboratory of Dr. J. Rubenstein (Qiu et al., 1997; Anderson et al., 1997a), University of San Francisco. For comparative studies, mutant mice were compared to wild type littermate controls. All animals were sacrificed by performing cervical dislocation. Small intestine (SI) and large intestine (LI) were harvested from adult mice and placed in cold 1x PBS. Small and large intestines were gently flushed with cold 1xPBS to clear out residual fecal matter. Embryonic animals were sacrificed by decapitation prior to dissection. Small and large intestines taken from animals that were older than E16.5 were dissected out of the embryo or newborn. However, for animals that were younger than E16.5, the gastrointestinal tract tissue was left *in-situ* in the embryo. All tissue collected was obtained from timed-pregnant mice. Timed pregnancy was determined by visual assessment of a vaginal plug after breeding, for which this day became 0.5. All animals protocols were conducted in accordance with guidelines set by the Canadian Council on Animal Care and were approved by the University of Manitoba animal care committee.

Tails from collected embryos were used to determine *Dlx*1/2 DKO from wild-type littermates. Genotyping was accomplished by isolating genomic DNA and PCR amplification using the Extract-N-Amp kit (Sigma Catalogue# XNAT2R) with primer pairs specific for the *Dlx*1/2 gene. Collected small and large intestines were washed in cold 1XPBS and were fixed in freshly made 4% paraformaldehyde (PFA) overnight 4˚C with rotation. Tissues were then passed through a sucrose gradient (10%, 15% and 20%) for cryopreservation. Prior to embedding, tissue was

incubated in a 1:1 solution of 20% sucrose and optimal cutting temperature (OCT) (Tissue-Tek) for 30 mins with rotation at room temperature. The tissue was oriented both in longitudinal and cross-sectional planes and using dry ice, was frozen in OCT.

Frozen blocks were cut at 12 micron thickness using a ThermoShandon cryostat and collected on SuperFrost Plus slides (Fisher) with every 10^{th} slide was kept for reference staining. Sectioned tissue was kept at -80˚C until use.

Immunostaining

Immunofluorescence is an approach to detect antigens using specific antibodies directly on biological tissue by employing fluorescent agents. The method is based on the principle that a specific antibody is raised against a specific epitope on a molecule that is localized *in-situ*. A secondary antibody is then applied to detect the primary antibody. In order to detect the primary antibody, the species of origin of the secondary antibody will be dependent on where the primary antibody was raised (rabbit, goat, mouse, etc). Ultimately, the detection is mediated via a fluorescent conjugated IgG using a properly equipped fluorescent microscope. If necessary, a tertiary fluorescent molecule may be added that will recognize a biotinylated secondary antibody. In some cases, non-fluorescence detection may be performed. This form of detection is enzyme-mediated via a peroxidase reaction (J. C. Adams 1981).

Designated slides were removed from -80˚C and left at room temperature for 5 mins to air dry. Slides were blocked with prepared blocking solution (0.1% BSA, 0.2% Triton-X 100, 0.2% Sodium Azide, 5% Horse/Goat Serum in 1XPBS pH 7.4) for 2 hrs at room temperature. Primary antibody was diluted in prepared blocking buffer and applied to the slides. Slides were incubated overnight at 4˚C. The following day, slides were washed in 1XPBS-T three times for 5 mins

each at room temperature. For immunohistochemistry (IHC) staining, slides were treated with a biotinylated secondary antibody (diluted in blocking buffer), applied to slides and incubated at room temperature for 2 hrs. Slides were again washed, and were then treated with 0.5% hydrogen peroxide in PBS-T for 30 mins at room temperature. Slides were then washed followed by 30 min incubation with Elite ABC solution (Vector) at room temperature. Following another round of washing, slides were developed using DAB (Vector) and was stopped with a wash in water for 5 mins. Slides were dehydrated through an ethanol gradient (50%, 75%, 85%, 95%, 100%, xylene) and mounted with coverslips (Fisher) and Mounting medium (Richard-Allan Scientific).

For slides undergoing immunofluorescence (IF) staining, a fluorochrome-conjugated secondary antibody was diluted in blocking buffer and applied. Slides were incubated at room temperature for 2 hrs in dark. Following incubating, slides were washed as previously described and mounted with VectaShield with DAPI and coverslips (Vector Labs).

Double immunofluorescence (DIF) staining procedures followed the protocol for single IF with 2 hour room temperature blocking and a overnight 4˚C incubation with the primary antibody. On Day 2, primary antibody was washed followed by incubation with a fluorochrome-conjugated secondary antibody as described. After incubation, slides were washed and incubated with the second primary antibody overnight at 4˚C. Day 3 begins with washing the slides followed by a 2 hr-room temperature incubation with the second secondary antibody. This secondary antibody is biotinylated. Slides are washed and are then incubated in the dark for 2 hours at room temperature with a streptavadin-conjugated fluorochrome that fluoresces at a different

wavelength than the first secondary antibody to avoid overlapping emissions. Following washing, slides were mounted with VectaShield with DAPI (Vector) and coverslips.

Western Blotting

SDS-PAGE

Protein lysates from GIT tissues were extracted by grinding with mortar and pestle in liquid nitrogen. Disintegrated tissue was then dissolved in protein lysis buffer (RIPA solution [1% triton, 158 mM NaCl, 10 mM Tris-HCl ph 7.6, 0.1% SDS, 1% Na-deoxycholate, 1 mM EDTA], 1X protease inhibitor cocktail, 50 mM NaF, 1 mM sodium vanadate, 1 mM PMSF). Tissue was then incubated in the lysis buffer on ice for 30 min with vortexing every 5 min. Protein lysate was then centrifuged at high speed on a table top microfuge for 15 min at 4˚C. Supernatant was then removed as this fraction contains the total protein content. Total protein from cell lines was obtained by washing cells in flasks twice with cold 1XPBS, and then cell cultures were trypsinised. Trypsinised cells were then centrifuged at 1200 rpm for 5 min to remove trypsin. Pelleted cells were then washed twice in 1XPBS and lysis buffer was then added and incubated for 30 min in ice with vortexing every 5 min. Protein lysate was then centrifuged at high speed over in a table top microfuge for 15 min at 4C. Supernatant was then removed. Protein concentrations were measured using a BCA Protein Assay Reagent (ThermoScientific) with protocol described in Prod No.23225 Doc No.1296.

Proteins were prepared by boiling samples in 1X SDS Lysis Buffer (4X: 0.25M Tris-HCl pH 6.8, 8% SDS, 10% 2-Mercapto-βethanol, 30% Glycerol, 0.02% Bromophenol blue) for 5 mins to denature proteins. Samples were spun and were run on a 12% SDS acrylamide gel for 1.5hr at 145-160 V at room temperature. Samples were transferred onto a PVDF membrane (Perkin

Elmer) by using a membrane-transferring cassette (BioRad), with stirring, for 1.5hrs at 100V at 4˚C.

Western Blotting

Transferred membranes were then prepared for blotting by incubating with blocking buffer (5% powdered milk solution in 1xTBST) for 1 hr with rotation at RT. Primary antibody was diluted in blocking buffer and incubated overnight at 4°C with rotation. The following day, blots were washed with 1XTBST three times for 5 min followed by one 15 min wash. A secondary horse-radish peroxidase (HRP) conjugated antibody was diluted in blocking buffer and incubated with the membrane for 1hr at RT. Wash steps were repeated and the membrane was developed using ECL Kit (GE Healthcare Life Sciences) and ECL Plus film (GE Healthcare Life Sciences) as described in Protocol RPN2132 / RPN2133 (GE Healthcare Life Sciences).

Table 1: Primary antibodies used in immunostaining

Primary Antibody	Dilution	Source
Rabbit polyclonal anti-DLX2	1:300	Purified in-house by Dr. Eisenstat
Rabbit polyclonal anti-Lysozyme	1:800	DakoCytomation
Rabbit monoclonal anti-alkaline phosphatase	1:100	Groovy Blue Genes
Goat polyclonal anti-NeuroD	1:1000	Santa Cruz
Goat polyclonal anti-Mucin 2 (R-12)	1:200	Santa Cruz
Goat polyclonal anti-Indian Hedgehog (c-15)	1:200	Santa Cruz
Rabbit polyclonal anti-Bmi1	1:100	abCam
Rabbit polyclonal anti-Sox9		Millipore
Rabbit polyclonal anti-Math1		Millipore
Rabbit polyclonal anti-β-catenin	1:50	Cell Signalling Technologies
Rabbit polyclonal anti-cleaved caspase-3 (Asp 175)	1:100	Cell Signalling Technologies
Rabbit polyclonal anti-APC (C-20)	1:1000	Santa Cruz
Rabbit polyclonal anti-c-myc	1:200	Santa Cruz
Mouse monoclonal anti-cyclin D1	1:100	Cell Signalling Techonolgies

Table 2: Secondary antibodies used in immunostaining

Secondary Antibody	Dilution	Source
Biotinylated goat anti-rabbit	1:200	Vector Labs
Biotinylated rabbit anti-goat	1:200	Vector Labs
FITC-conjugated donkey anti-rabbit	1:200	Jackson Immunoresearch
FITC-conjugated donkey anti-goat	1:200	Jackson Immunoresearch
Alexa-594 conjugated donkey anti-rabbit	1:200	Invitrogen
Alexa-594 conjugated donkey anti-goat	1:200	Invitrogen

Table 3: Primary antibodies used for Western blotting

Primary Antibody	Dilution	Source
Rabbit polyclonal anti-DLX2	1:1000	Purified in-house by Dr. Eisenstat
Rabbit polyclonal anti-β-catenin	1:1000	Cell Signalling Technologies
Rabbit polyclonal anti-c-myc	1:1000	Santa Cruz
Mouse monoclonal anti-β-actin	1:1000	Sigma

Table 4: Secondary antibodies used for Western blotting

Secondary Antibody	Dilution	Source
goat anti-rabbit HRP	1:10,000	Jackson Immunoresearch
goat anti-mouse HRP	1:10,000	Jackson Immunoresearch

Chromatin Immunoprecipitation (ChIP)

ChIP was performed on both adult and E18.5 small intestine (SI) and large intestine (LI). After harvesting the SI and LI, both were gently flushed with cold 1XPBS to clear out residual fecal mater. Both adult SI and LI were gently cut along its longitudinal axis and the top three surface layers (muscularis [outer and inner], submucosa and mucosa) were gently scraped off from its supporting outermost connective tissue (adventitia) to avoid obtaining collagen-rich tissue. The scraped-off tissue was then washed twice in cold 1XPBS by centrifuging at 2000 rpm at 4 ˚C for 5 min (swing bucket). The tissue was then gently dissociated into single cells by pipetting and/or passing tissue through a 20 gauge needle and cells were then pelleted by centrifuging at 2000 rpm at 4 ˚C for 5 min. Pelleted cells were then fixed in freshly made 1% paraformaldehyde (PFA) + 1X protease inhibitor cocktail (PIC) for 30 min at room temperature with rotation. Cells were then washed 2x in cold 1XPBS. At this point cells can be frozen at -80˚C if needed. Sixty

μL Sepharose A/G beads (Pierce UltraLink #53132) were primed by adding 1mL dilute buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl pH 8.1, 167 mM NaCl)+ 1xPIC to total amount of beads. Beads were gently resuspended and centrifuged at 2000 rpm for 2min at 4˚C (swing bucket). Supernatant was removed and beads were washed once more with dilute buffer + 1xPIC. After removing supernatant from second wash, an equal volume of dilute buffer + 1xPIC was added (60 μL) to make 50% beads. Primed beads were left on ice until sonicated chromatin was ready. Cells were washed twice with cold 1xPBS to remove PFA. Once supernatant was removed from second wash, 400 μL of freshly made lysis buffer (1% SDS, 10 mM Tris-HCl ph 8.1, 10 mM EDTA) + 1xPIC was added. Cells were then sonicated on ice for 15-20x at 15 seconds intervals with 30 seconds rest periods. About 1-3 μL of sonicated chromatin was then analyzed by agarose electrophoresis (1% agarose) to determine proper size of chromatin shearing (~300-700 bp). Once proper chromatin size was determined, the sonicated chromatin was divided into two 200μL tubes (+antibody and –antibody) and 800 μL of dilute buffer was added to each tube. In order to pre-clear the sonicated chromatin from excess non-specific IgGs that may interfere with bead binding to the desired antibody (DLX2 IgG) in subsequent steps, 60μL of primed beads was added to each of the diluted sonicated chromatin. The cells with primed beads mixture was then incubated with rotation for 1hr at 4˚C. The mixture was then centrifuged at 2000rpm at 4˚C for 5 min. Supernatant was then transferred to fresh tubes and beads were discarded. To the collected supernatant, 500 μg/mL each of BSA and tRNA was added. About 1-2 μg of DLX2 IgG was added only to tubes that were designated +antibody. To the second tube an irrelevant rabbit IgG was added. This mixture was incubated at 4˚C overnight with rotation. Another set of beads were primed as previously performed and incubated at 4˚C overnight with rotation. The next day 60 μL of primed beads was added to each

tube and incubated at overnight at 4˚C with rotation. The following day, beads were centrifuged at 2500 rpm at 4˚C for 5 min. Supernatant was then transferred to a fresh tube (this represents the total input sample). Beads were then washed in 1 mL of the following buffers: 5 min in "low salt buffer" (0.1% SDS, 1% triton X-100, 2mM EDTA, 20mM Tris-HCl pH 8.1, 150 mM NaCl); 30 min in "high salt buffer" (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20mM Tris-HCl pH 8.1, 500 mM NaCl); 30 min in Lithium Chloride buffer (250 mM LiCl, 1% deoxycholate, 1mM EDTA, 10mM Tris-HCl pH 8.1, 1% NP-40); 5 min in TE buffer pH 8.0. Each wash was done with rotation at 4˚C, beads were resuspended in the corresponding buffer, centrifuged to pellet beads, supernatant was removed and then proceeded to add the next buffer. After the last wash, 250 μL of pre-heated and freshly made elution buffer (1% SDS, 0.1M $NaHCO_3$) was added to beads and were incubated for 15 min at room temperature with agitation. Beads were then centrifuged at high speed in a tabletop micro-centrifuge for 5 min at 4˚C. Supernatants were transferred to a fresh tube and the elution step was repeated once more. After the second elution, supernatants were pooled (~500 mL total) and 1 mL of RNaseA and 25 mL of 5.0M NaCl was added and incubated at 68˚C overnight. Then next day, 10 mL of 0.5 M EDTA, 20 mL 1M Tris-HCl pH 6.5 and 2 mL Proteinase K was added and incubated at 65˚C (temperature dependent on Proteinase K optimal activity) for 2 hours. The mixture was then cleaned up using Qiagen PCR purification kit (cat # 28104). Subsequently, the ChIPped DNA was then subjected to polymerase chain reaction using specific primers (See Table #5) to the designated regions of interest on the promoter region.

Table 5: APC Primers for ChIP

Region	Name	Sequence (5'->3')
I	APC 1F	CAGACGCGTTAGAGCAGACAACCACAGC
	APC 1R	GACCTCGAGACTTTGCCTCCCAACCCTC
II	APC 2F	CAGACGCGTCCATGTAGGTACCTAAGGC
	APC 2R	GACCTCGAGTTCTGTCTAGATCCAGACG
III	APC 3F	CAGACGCGTTGAACCTCCGAATTGTAAGC
	APC 3R	GACCTCGAGAGGCCATAGGGCTCTGGC
VI	APC 4FA	CAGACGCGTTCAGCCTGGTCTACAGAGTGAG
	APC 4RA	GACCTCGAGCCTGAACCACTGTCCATCCTAT
V	APC 5F	CAGACGCGTAGCTGAGTGCCAAAGACG
	APC 5R	GACCTCGAGAAGAGCAGGACGATCTTTC
	APC 5FA	CAGACGCGTTCTACCTACTGATCCATTGC
	APC 5RA	GACCTCGAGCACCTCAGATCGTCTTGCT
VI	APC 6F	CAGACGCGTCTCTTAGGGAGTTTACTGCT
	APC 6R	GACCTCGAGAAGTTGACTTGCCACAAGAC
	APC 6FA	CAGACGCGTGGTGCATCTGAGACAGGC
	APC 6RA	GACCTCGAGCAGGCCAGGGTATGAGGC
VII	APC 7FA	CAGACGCGTGCGTTCATTTTCTCTTCCTTGT
	APC 7RA	GACCTCGAGCCGGTGGAGAAGACAGAAATC
VIII	APC 8F	CAGACGCGTGTCTGATAGCTGTTTGAGAC
	APC 8R	GACCTCGAGTGCCTCTGCGGTATTTCG

Electrophoretic Mobility Shift Assays (EMSA)

EMSA is a common technique that is used to study DNA-protein interactions based on electrophoretic separation of a protein-DNA mixture on a gel matrix (Garner & Revzin 1981; Fried & Crothers 1981). Normally the gel matrix consists of components that render the gel non-denaturing. In non-denaturing EMSA gels the activity and structure of the polypeptide molecule interacting with the nucleotide probe are retained. When an electric current is applied, different molecules move through the gel at specific rates dictated by their size. In EMSAs, DNA probes are labeled with a radioactive isotope (Phosphorous 32). When the labeled probe associates with proteins, the migration of this double complex will be slower in the gel matrix. Furthermore,

when the recombinant protein that is complexed with the radioactive probe interacts with an IgG against the recombinant protein, this triple complex will travel even slower in the gel matrix. The procedure to generate and affinity purify the recombinant DLX2 protein was performed by employing the nickel (Ni2+)-charged HisTrap FF crude Kit column chromatography from GE Healthcare. The DLX2 IgG was affinity purified as previously described in Porteus et al. (1994). Oligonucleotides (oligos) of 20-25 bp were designed to include the sites of promoter regions where DLX2 was shown to occupy by ChIP (TABLE #6). Forward and reverse oligos were annealed with annealing buffer as described by Sigma-Aldrich protocol for annealing oligos. Annealed oligos were subsequently radiolabeled using $P^{32}\gamma$ (Perkin Elmer) by T4 Polynucleotide Kinase (Invitrogen) and purified using GE HealthCare Illustra Micro-spin G-25 spin columns. Radioactivity was measured using a Beckman Coulter scintillation counter. Samples were prepared as previously described (Zhou et al., 2004) and incubated for 30 mins without the radiolabelled oligos, then for another 20 mins after addition of the oligos. Samples were run on a 4% non-denaturing acrylamide gel for 45 mins at 280V and vacuum-dried for 1.5 hours using a gel drier (BioRad). The gel was exposed to autoradiography film (Kodak) overnight at -80^{o}C.

Table 6: EMSA Oligonucleotides designed for *Apc* ChIP-positive region I

Oligo Name	Sequence Detail (**potential binding site**)
RI MOTIF1EMSA SENSE	aaaacacctttct**taat**gtggctcttcaaa
RI MOTIF1EMSA COMPLEM	ttttgtggaaaga**atta**caccgagaagttt
RI MOTIF2EMSA SENSE	caggtgggcttgc**atta**tccttctgctggac
RI MOTIF2EMSA COMPLEM	gtccacccgaacg**taat**aggaagacgacctg

Gene Reporter Assay (Luciferase)

Molecular cloning of Apc Region 1 and the full length Apc promoter

Region I of the *Apc* promoter (accession AF534124) was cloned into the pGL3 reporter vector. Similarly, the full length of the *Apc* promoter was also cloned into the pGL3 vector. Both (region 1 and full length) DNA sequences were cloned using *Mlu*1 and *Xho*1 at their 5' and 3' ends, respectively. Using primers for region 1, the ChIPped PCR product was used as a template to amplify region 1 in order to clone it into pGL3. Genomic DNA was used as a template to amplify the full length *Apc* promoter sequence. Primers were designed with an extension at their 5' end. This extension included the *Mlu*1 restriction site for the forward primers and *Xho*1 restriction site for the reverse primers. The primers were designed as such in order to facilitate direct cloning into the reporter vector pGL3. After extracting the PCR product from region 1 using an agarose gel extracting kit (Qiagen), the pure PCR product was measured using a spectrophotometer. The resulting PCR product (5μg) and the pGL3 vector (3μg) were digested concurrently with measured amounts of *Mlu*1 and *Xho*1 restriction enzymes (New England Biolabs) for 2 hrs at 37 °C. The resulting digested products were purified using Qiagen's Gel Extraction kit and measured. Proper ratios of insert to vector were ligated using T4 Ligase (New England Biolabs) at 16 °C overnight. Then next day, the resulting ligated products were used to directly transform chemically competent DH5α *E.coli* cells (Invitrogen). After performing the standardized $CaCl_2$ mediated *E.coli* transformation protocol (Dagert & Ehrlich 1979), transformed bacteria were spread-plated onto pre-warmed ampicillin/carbenicillin agar plates and incubated at 37 °C overnight. Successfully transformed *E.coli* that were transformed will grow on the ampicillin/carbenicillin agar plates. The following day 3-5 colonies were picked and inoculated liquid LB-broth with ampicillin/carbenicillin and incubated at 37 °C with

shaking. After 24 hr, the tubes containing bacterial growth were chosen to perform plasmid purification using a mini-prep kit (Sigma). Purified plasmids were digested with *Mlu*1 and *Xho*1 to verify successful insert ligation. The plasmids from the successful clones were sequenced confirmed and glycerol stocks from the *E.coli* cultures containing the correct constructs were made and stored at -80 C.

Luciferase reporter assays

The luciferase reporter gene assay is a useful method to study promoter activation *in-vitro* (Fan & Wood 2007). The principle of this assay is such that the promoter sequence of interest is inserted upstream of the coding sequence for the gene *luciferase* in the pGL3 transfection plasmid construct. The intensity of the *luciferase* gene product will luminesce according to the action of the transcription factor that targets the promoter sequence inserted upstream. The chemiluminescence intensity will subsequently be detected by a luminometer and quantified accordingly. The HEK293 cells were grown in $75cm^2$ cell culture flasks (Nalgene) to 80% confluence. Cells were then trypsinized (Gibco Invitrogen) and stained with Trypan Blue for counting using a hemocytometer. 10^5 cells were plated into each well of two 12-well culture plates (Nalgene) with 1 ml of DMEM+10%FBS media (Invitrogen). Cells were cultured overnight at 37°C at 5% CO_2 (Fisher Scientific IsoTemp Incubator) to reach 70% confluence. Cells were then washed with warmed 1xPBS and then incubated with 1ml of warmed OPTIMEM media (Gibco-Invitrogen) at 37°C until the transfection mixture was ready. Transfection mixture per well was prepared as follows: 0.5µg of designed reporter pGL3 plasmids (empty pGL3 vector was used as a control), 0.5µg of pcDNA$_3$-DLX2 (empty pcDNA$_3$

as control) and 0.1μg of β-galactosidase to measure transfection efficiency in 125ml of OPTIMEM. 2.5μl of Lipofectamine 2000 reagent (Invitrogen) was incubated with 125ml OPTIMEM for 5 mins at RT in a separate tube. The two mixtures were combined and allowed to incubate at RT for 20 mins before adding 250ml of the mixture to each well. After a 48 hr incubation, the transfected cells were lysed using 100ml of 1x Lysis Reporter Buffer (Protégé).

Luciferase activity measurements were performed using an Lmax Luminometer (Molecular Devices) by combining cell lysates (10ml) with Luciferase assay substrate buffer (Promega). β-galactosidase activity was measured to determine transfection efficiency at 420 nm using the SpectraMax 190 instrument (Molecular Devices) and after addition of ONPG buffer (4mg/ml).

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

RNA Isolation

Small and large intestine were harvested from E18.5 WT and *Dlx1/2*$^{-/-}$ embryos and kept in 750 μL of RNA*later* (Qiagen) at -20˚C. RNA*later* prevents RNA degradation in the tissue. Samples were kept at -20˚C until RNA extraction was performed. RNA extraction was performed by removing RNA*later* and then freezing tissue in liquid nitrogen then by grinding to a powder. Total pure RNA was isolated from the powderized tissue by using QiaShredders and RNeasy (Qiagen) mini protocol. Total RNA concentrations were measured by using a Biochrom Ultraspec 3100 *pro* spectrophotometer.

cDNA Synthesis

To 1µg of total RNA, 1 µL of oligo $(dT)_{50}$, 0.5 µL 20 mM dNTP and molecular grade water to a final volume of 13 µL were added. Components in the tube were mixed well and heated to 65˚C for 5 minutes followed by incubation in ice for 1 min. After a brief centrifugation, 4 µL of 5X first strand buffer, 1 µL 0.1 M DDT, 1 µL RNaseOUT, 1 µL of Superscript III reverse transcriptase were added. Samples were gently mixed by pipetting up and down and incubated at 50˚C for 60 min. The reaction was inactivated by heating at 70˚C for 15 min. All reagents were acquired from Invitrogen.

qRT-PCR

For the *Apc* mRNA expression, the forward and reverse oligonucleotides were designed within the last exon of its coding sequence. For the *Apc* gene, agarose electrophoresis analysis imaging was performed to confirm the correct size of its reverse transcription reaction. Synthesized cDNA was submitted to standard polymerase chain reaction, but utilizing the Qiagen QuantiTech SYBR Green RT-PCR Kit and run on an Applied Biosystem 7500 Real Time PCR System as per the manufacturer's instructions. Cycling conditions consisted of an initial 15 min at 95˚C followed by 40 cycles of: 15 sec denaturation at 94˚C, 30 sec annealing at 55˚C, 30 sec extension at 72˚C. Analysis was performed using the Applied Biosystems Sequence Detection Software Version 1.4.

Table 7: Designed Primers for *Apc* coding sequence for qRT-PCR

Primers	Sequence (5' →3')
APC forward	aggcagagtccctcacagaa
APC reverse	taggtcaaggggaaccagtg

RNA Interference (RNAi) Assays on Colo320 cell lines

Colo320 cell lines were maintained at 37 ˚C in a 5% CO_2 humidified incubator in RPMI-1640 medium with 10% fetal bovine serum. Duplex siRNA was designed and ordered from Invitrogen. The siRNA was targeted to the *Dlx2* coding sequence (TABLE 8). Once cells were grown to approximately 90% confluence, $2x10^5$ Colo320 cells were seeded in a 6 well plate and cultured. One microgram siRNA-*Dlx2* and siRNA-control were added to corresponding wells in Opti-MEM and lipofectamine-2000 (Invitrogen) to Colo320 cells. The following day the growth medium was changed. 48 hrs after transfection, cells were collected and whole cells lysates were prepared for β-catenin, c-Myc and DLX2 Western immunoblotting detection.

Table 8: Designed siRNA Oligos for *Dlx*2 RNA silencing

siRNA Oligos duplex	Sequence (5' → 3')
Dlx2 sense	GGAAGAACCUUGAGCCUGAAdTdT
Dlx2 antisense	UUCAGGCUCAAGGUCUUCCdTdT
Control sense	UUCUCCGAACGUGUCACGUdTdT
Control antisense	ACGUGACACGUUCGGAGAAdTdT

RESULTS

DLX2 Expression pattern in the developing GIT

Although *Dlx* gene expression was first discovered in the ventral forebrain (Porteus et al. 1991; Porteus et al. 1992), subsequent studies determined that *Dlx* genes were also expressed in the primitive gut epithelium by radioactive and non-radioactive RNA *in-situ* hybridization (M. Qiu et al. 1995; Heanue & Pachnis 2007; Vohra et al. 2006). However, the expression pattern of *Dlx* genes in the developing and postnatal gastrointestinal tract has not been fully explored. Using a high affinity purified rabbit polyclonal DLX2 antibody, DLX2 expression in the gut was explored in detail from embryonic stage (E13.5) to adulthood via immuno-detection.

Our findings show that at E13.5, DLX2 expression was observed in the enteric neuromuscular system during midgestation (Figure 5A). The same expression pattern was observed during later stages in development at E14.5 and E16.5 (Figure 5B,C). However, at E18.5 or P0, DLX2 immunopositivity was observed in both the enteric neuromuscular system and the intestinal epithelium (Figure 5D). Postnatally, some DLX2 expression was still observed in the enteric neuromuscular system, suggesting an important role *Dlx*2 may be playing in the enteric nervous system, but a more robust expression is now evident in the epithelial layer (Figure 6A,B). In the intestinal epithelium, DLX2 positive cells were observed to be present in both the differentiated (villus) and the proliferative (crypt) compartments, although more expression was abundant in the villus (Figure 6A,B).

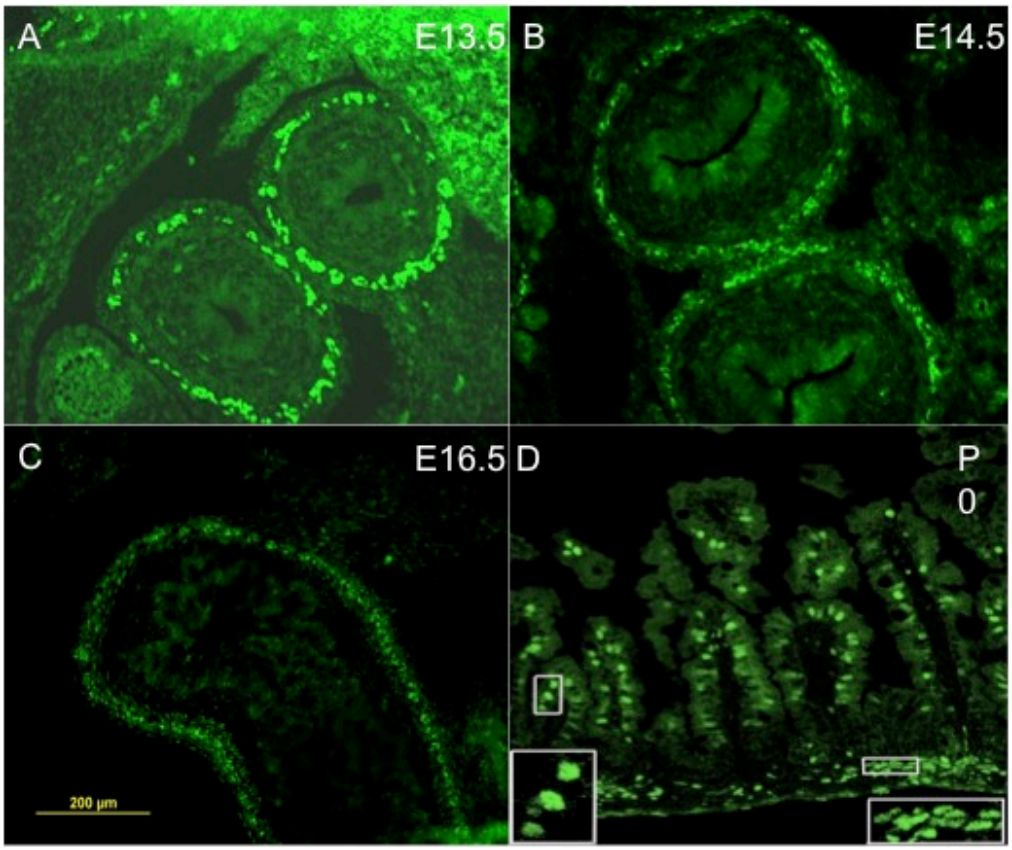

Figure 5: DLX2 expression during embryogenesis in the mouse gastrointestinal tract.

DLX2 expression is confined to the muscularis propria up to E16.5 (A, B, C). However, at P0 DLX2 expression is found in the epithelium layer (D) as well as in the muscularis propria. Inset in (D) shows expression in both muscularis propria and epithelial compartments.

Of the four different post-mitotic cell types present in the adult intestinal epithelium (enterocytes, enteroendocrine, goblets and Paneth cells), we then sought to determine which cells were DLX2 positive using corresponding markers (Table 1). The hydrolytic enzyme alkaline phosphatase is an enterocyte marker (Real et al. 1992). In our double immunofluorescence analysis, we observed that DLX2 and alkaline phosphatase were co-expressed in the same cell (Figure 7A). The basic helix loop helix transcription factor NeuroD is known to be a marker for the enteroendocrine cell population (Naya et al. 1997).

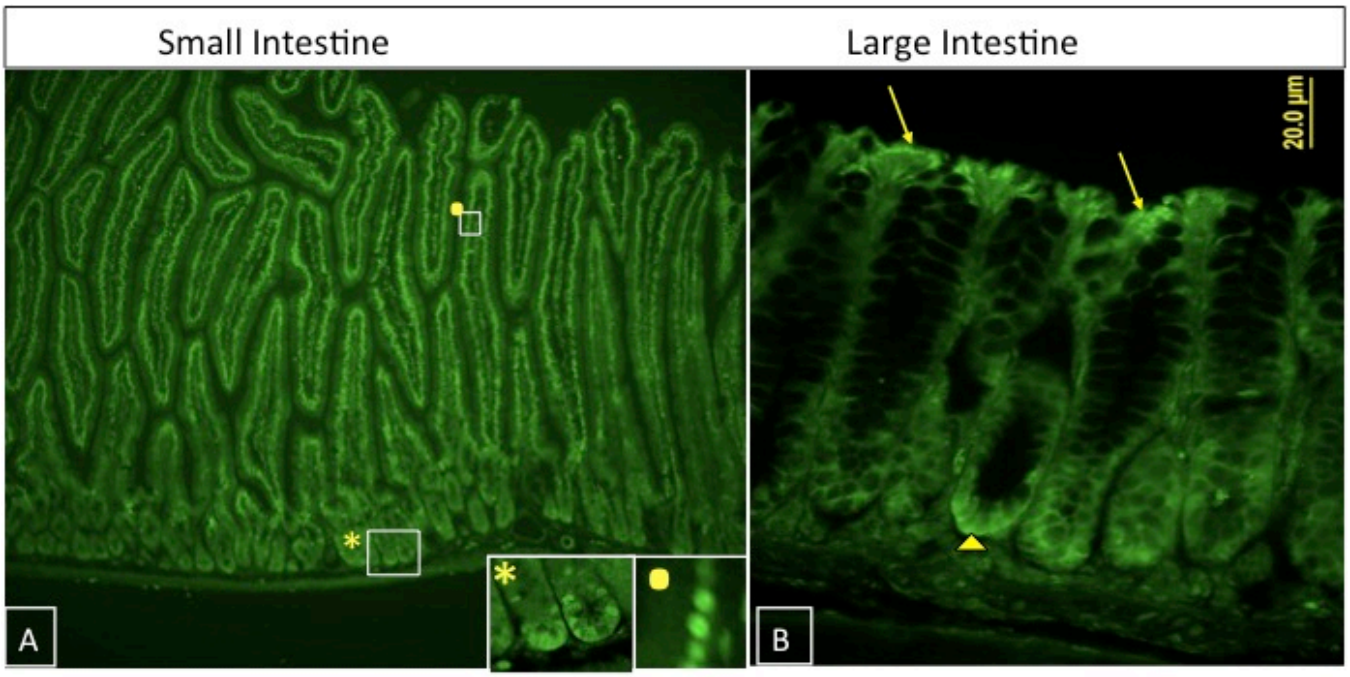

Figure 6: DLX2 expression in the adult mouse gastrointestinal tract.

(A) DLX2 is strongly expressed in the small intestine in both the differentiated (•) and proliferative (*) compartments. (B) DLX2 expression in the large intestine at the surface epithelium (arrows) and at the bottom of the crypts (arrowhead).

We observed the co-expression of DLX2 and NeuroD in our double immunofluorescence (Figure 7C). To identify goblet cells, we employed a histochemical staining approach known as Alcian blue staining. The goblet cells stain blue via Alcian blue staining due to their ability to produce acid mucopolyssaccharides (Gambús et al. 1993). Therefore, we performed DLX2 immunohistochemistry by means of the DAB (3,3'-diaminobenzidine) approach, followed immediately by the histochemical staining (Alcian blue) (Figure 7D). DLX2 expression was also observed in the crypt region of the small intestine. Therefore, we sought to determine the possible expression of DLX2 in the Paneth cell population. In the crypt region the Paneth cells are known to express lysozyme (Batlle et al. 2002). Our double immunofluorescence analysis

with DLX2 and lysozyme revealed that lysozyme expression coincides with DLX2 expression (Figure 7B).

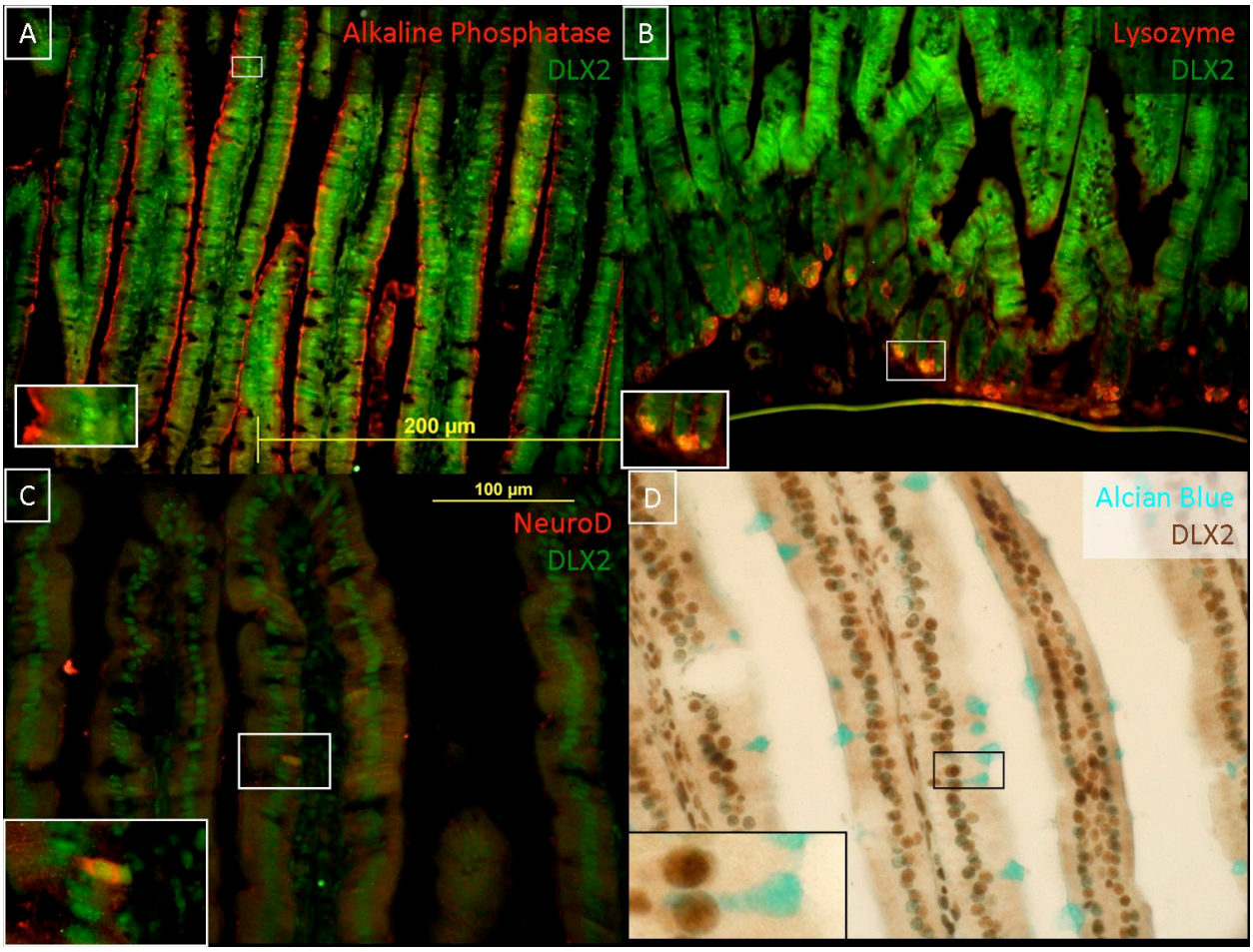

Figure 7: DLX2 co-expression in the four small intestine cell types.

(A) Colonocytes, (B) Paneth, (C) enteroendocrine, and (D) goblet cells are immunopositive for DLX2.

The two cell types that are abundant in the large intestine are colonocytes and the goblet cells. Our immunofluorescence staining showed that DLX2 is co-expressed in the same cell with the Indian hedgehog (IHH) protein, a marker for the adult colonocytes (Figure 8B). For this particular image, single staining on consecutive slides had to be performed. The reason for this approach is because during the double immunofluorescence experiment (DLX2/IHH), the DLX2

fluorescent signal was substantially compromised, and a merged image that demonstrated co-expression could not be generated.

The goblet cell population in the large intestine was stained with the Mucin2 antibody. Our double immunofluorescence demonstrated that MUCIN2 expression does not coincide with the DLX2 staining (Figure 8A). This result poses an interesting observation in that the goblets present in the small intestine may be distinct from the goblet cells found in the large intestine.

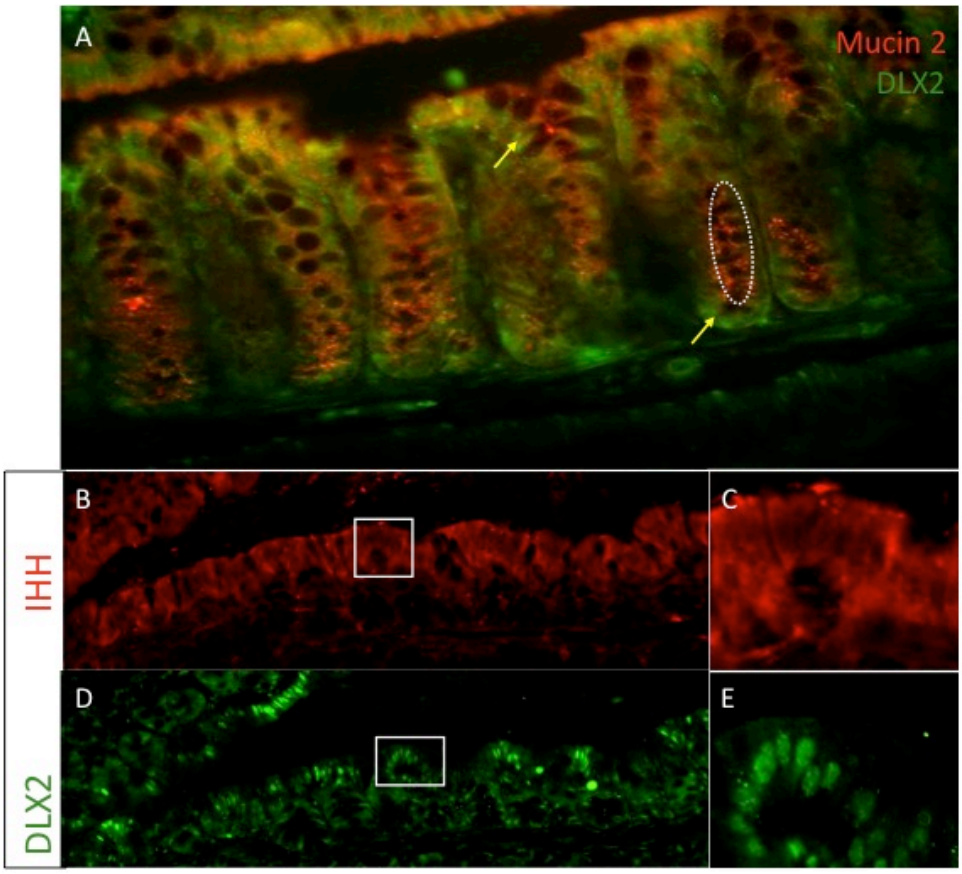

Figure 8: DLX2 co-expression in the large intestine.

(A) DLX2 expression does not appear to be co-expressed in the goblet cells. (B) Cytoplasmic Indian-Hedge hog (IHH), a marker for colonocytes is co-expressed with nuclear DLX2.

In the crypt region where lysozyme and DLX2 were co-expressed, we observed a small number of cells that lacked lysozyme immunopositivity but were *Dlx*2 positive (Figure 7B inset). The

lack of lysozyme staining indicated that these cells were not Paneth cells. Since stem cells are also found in the crypt region, we sought to determine if these lysozyme negative cells were gastrointestinal stem cells. Definitive markers for gastrointestinal stem cells have remained elusive for the last decade; however, through elegant knock-in and lineage tracing studies in mice, *Bmi*1 and *Lgr*5 have been identified as markers for the stem cell population in the intestinal epithelium (Sangiorgi & Capecchi 2008; Nick Barker et al. 2007).

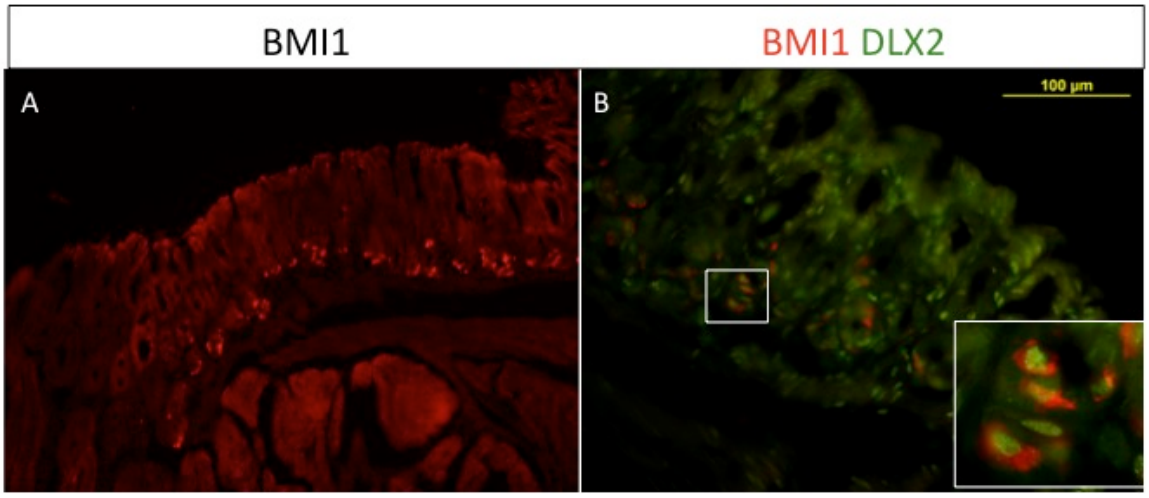

Figure 9: Gastrointestinal stem cell marker BMI1 expression in the colon.

(A) BMI1 is strongly expressed in the crypt bottom where the stem cells reside. (B) BMI1 and DLX2 are co-expressed in the crypt bottom of the colon. Inset shows DLX2 (nucleus) expression in the same cells expressing BMI1.

Our BMI1 immunofluorescence results on the adult mouse small and large intestine show distinct expression in the crypt region (Figure 9A). Sangiorgi and Capecchi have shown that the *Bmi*1 transcript is present in cells located at the bottom of the crypt, above and sporadically among the Paneth cells (Sangiorgi & Capecchi 2008). Interestingly, their results only represent BMI1 expression in the small intestine. However, we have shown that in addition to the sporadic BMI1 expression via immunofluorescence in the small intestine, there is significantly more BMI1 expression in the crypts of the large intestine (Figure 9A). We have shown that in the

large intestine, a number of BMI1 expressing cells are also DLX2 positive (Figure 9B). Therefore, we have shown that in the developing GIT, DLX2 positive cells are found in the muscularis propria region, but not in the primitive intestinal epithelium. Also, we have shown DLX2 expression postnatally in the intestinal epithelium in the proliferative and differentiated compartments.

DLX2 Binds to the Murine *Apc* Promoter *in-vivo* and *in-vitro*

In-vivo analysis

The murine *Apc* gene proximal promoter is approximately 4kb in size (Karagianni et al. 2005). Upon examination of the promoter sequence, we discovered that the sequence contained 23 putative homeodomain binding sites represented by the tetranucleotide sequence ATTA/TAAT (Figure 10). This observation led us to examine the possible interaction between the DLX2 protein and the *Apc* promoter by performing chromatin immunoprecipitation (ChIP). In ChIP, the tissue of interest is treated with a fixative (paraformaldehyde), which cross-links molecules *in-situ*. This allows us to study spatial and temporal interactions and chromatin dynamics with respect to a given protein binding to a specific DNA sequence *in-vivo*. Chromatin fractions containing the specific protein of interest can be enriched by using antibodies that are specific against the proteins that are present at one or more locations in the genome.

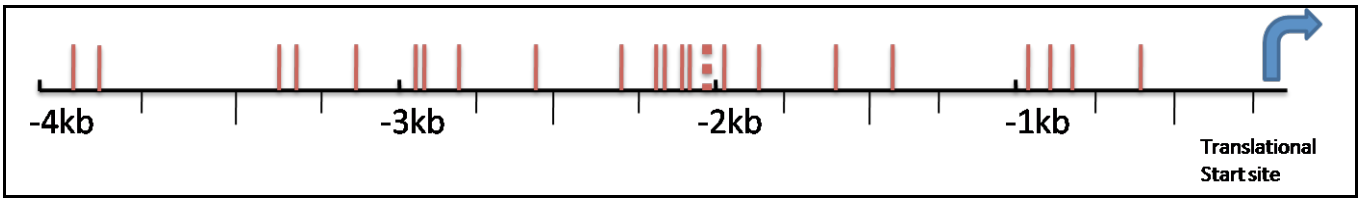

Figure 10: Diagrammatic representation of the *Apc* proximal promoter (accession number AF534124) with putative homeodomain binding sites.

Solid vertical red lines denote single putative binding DLX2 motifs. Dashed vertical line represents doublet (ATTAAT) DLX2 binding motifs (M.Fonseca and V.Pinto).

The immunoprecipitated chromatin fragments are then submitted to reverse cross-linking. The remaining DNA fragments are then analyzed by PCR. Positive results (presence of bands) signify the presence of our protein of interest on a particular region of the genome. Chromatin immunoprecipitation (ChIP) experiments revealed that the DLX2 protein was found to be interacting with the *Apc* promoter sequence (Figure 11A,B). The nature of the length of this promoter sequence, coupled to the numerous putative binding sites, led us to arbitrarily divide the sequence into 10 regions for our polymerase chain (PCR) reaction analysis. Each set of primer sets was designed to cover at least one putative homeodomain-binding site. Both adult small and large intestine tissue were treated and fixed to conduct ChIP analysis. The results obtained were similar in both regions of the GIT. The only difference was observed in region seven where DLX2 does not bind in the LI but binds in the SI (Table 9). ChIP analysis was also performed on E18.5 GIT tissue. The binding pattern observed was significantly different from that in the adult GIT tissue, and yet both tissues had similar binding pattern at this embryonic stage (Table 9). The major difference between both time-stages was that DLX2 did not interact with regions 2 to 9 at E18.5, where at the adult stage DLX2 interacted with regions 2, 6, 7 and 8 in the SI and LI (except for region 7 in the LI and inconclusive results for region 4 in the SI) (Table 9).

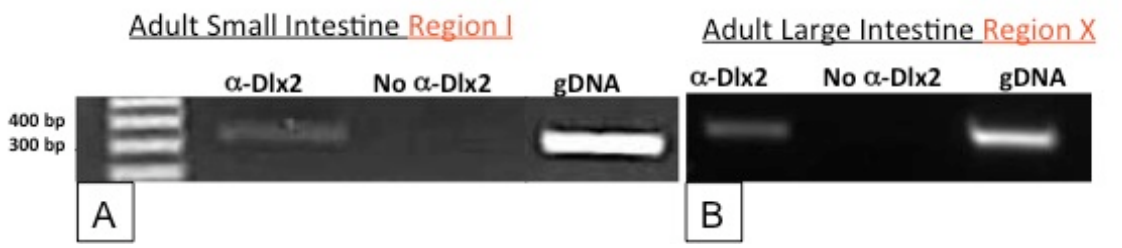

Figure 11: Representation of *Apc* promoter ChIP assay on the small and large intestine on two different designated regions.

(A) Localization of DLX2 protein on region I of the *Apc* promoter in the small intestine. (B) Region X (or Region 10) of the *Apc* promoter in the large intestine was observed to be occupied by DLX2 protein. All ChIP assays were performed using a DLX2 high affinity purified immunoglobulin G (IgG). For negative controls, no DLX2 IgG was used. Genomic DNA was utilized as a positive control.

Table 9: Designated Regions on the *Apc* promoter Identified as ChIP Positive for DLX2 at two developmental stages. Unshaded regions represent adult GIT. Shaded regions represent E18.5 GIT. *Denotes regions that have been cloned into the pGL3 reporter plasmid. ?-denotes inconclusive results

Regions	1*	2	3	4	5	6	7	8	9	10*
SI	+	+	-	?	-	+	+	+	-	+
LI	+	+	-	+	-	+	-	+	-	+
SI	+	-	?	-	-	-	-	-	-	+?
LI	+	-	?	-	-	-	-	-	-	+?

In-vitro analysis

To investigate and to confirm specific DLX2 interaction with the *Apc* promoter, we performed electrophoretic mobility shift assays (EMSA). Our EMSA results showed that the recombinant DLX2 protein specifically interacts with region 1 of the *Apc* promoter. This was demonstrated by the shift in migration rate in the *Apc* region 1 lane when DLX2 recombinant was added. In

order to verify the specificity of this shift, three additional experiments were performed: cold competition assay, antibody supershift detection and irrelevant antibody addition. In the cold competition assay, 100X excess of unlabeled cold oligos were mixed with the recombinant protein. This effect out-competed the labeled oligos. In the second experiment, DLX2 binding was analyzed by combining the DLX2-probe mixture with the DLX2 high affinity antibody. This triple complex (Protein-Antibody-DNA) mixture traveled much slower in the gel creating a "supershift" effect. Finally, the addition of an irrelevant non-specific antibody should result in the same gel shift observed in the original DLX2-oligo scenario (Figure 12). These experiments validated the direct DLX2 protein interaction on the *Apc* region 1 promoter *in vitro*.

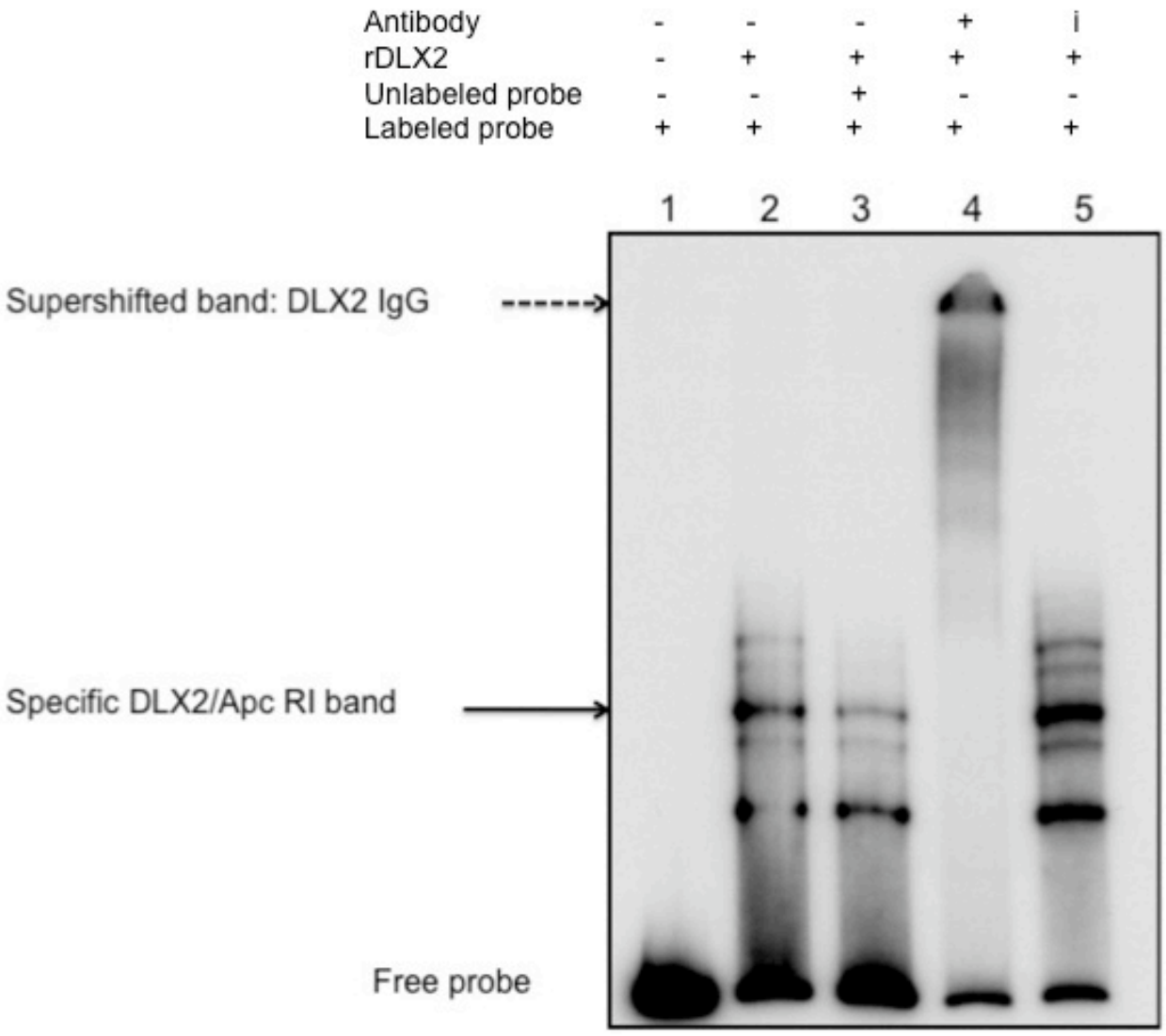

Figure 12: Electrophoretic mobility shift assay (EMSA) demonstrates recombinant DLX2 bind to the *Apc* promoter region I *in-vitro*.

EMSA was performed using recombinant DLX2 and a radiolabeled *Apc*RI (region I) oligonucleotide probe, with cold competition (lane 3) and specific DLX2 antibody 'supershift' assay (lane 4). "i" denotes irrelevant antibody. (M. Eshraghi and M. Fonseca).

APC and β-catenin levels are altered in the *Dlx*1/*Dlx*2 double knockout GIT

To investigate APC levels in the GIT of the *Dlx*1/*Dlx*2 mutant at E18.5, APC immunofluorescence analysis was performed. Due to the multi-functional nature of the APC protein and its presence in different compartments (membrane, cytoplasm and nucleus) (I S Näthke et al. 1996; Neufeld & White 1997), APC was detected throughout the cell by immunofluorescence in both the wild type and mutant small intestine (Figure 13A,B). Interestingly, our immunofluorescence results indicated the absence of nuclear APC in the *Dlx*1/*Dlx*2 double knockout small intestine (Figure 13B).

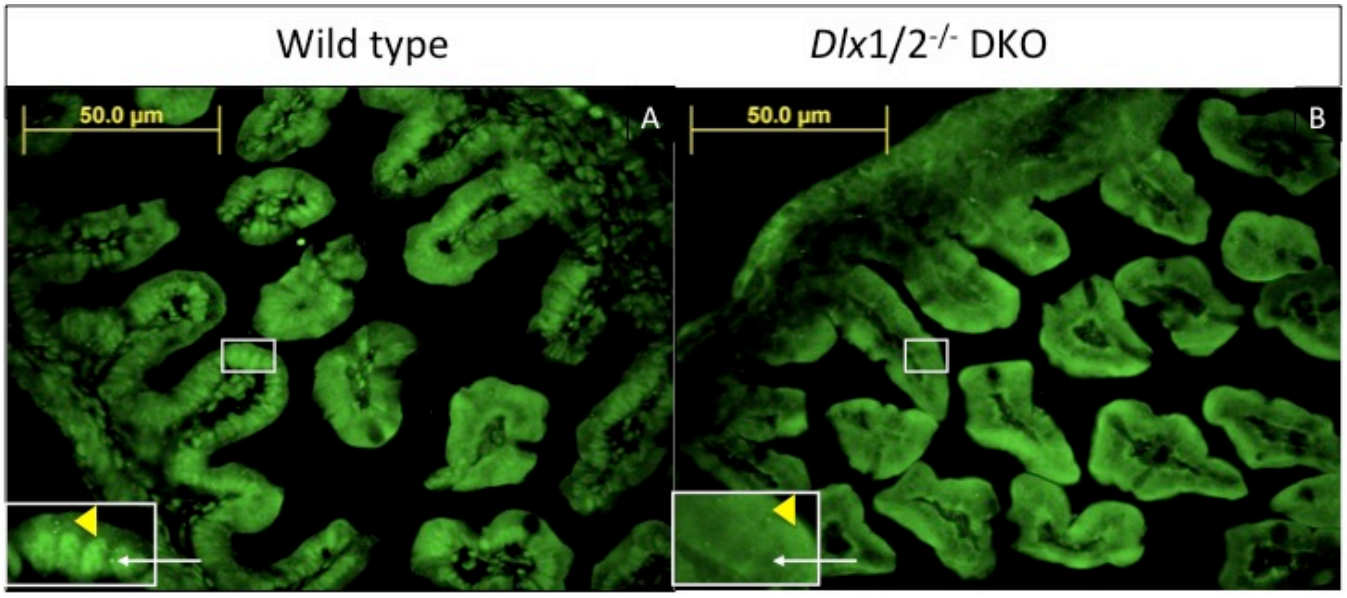

Figure 13: APC expression in E18.5 mouse small intestine.

(A) In the wild type small intestine APC appears to be expressed in the nucleus and also in the cytoplasm. (B) In the *Dlx*1/2 double knockout small intestine APC was still observed in the cytoplasm; however, nuclear APC was absent. White arrows and yellow arrowheads are pointing at nucleus and cytoplasm, respectively.

If the levels of APC were lowered in the *Dlx*1/*Dlx*2 double knockout GIT, then it would be expected to see higher levels of the β-catenin protein in the cell. It was evident that in our immunofluorescence results, β-catenin levels were increased in the *Dlx*1/*Dlx*2 double knockout small intestine (Figure 14B).

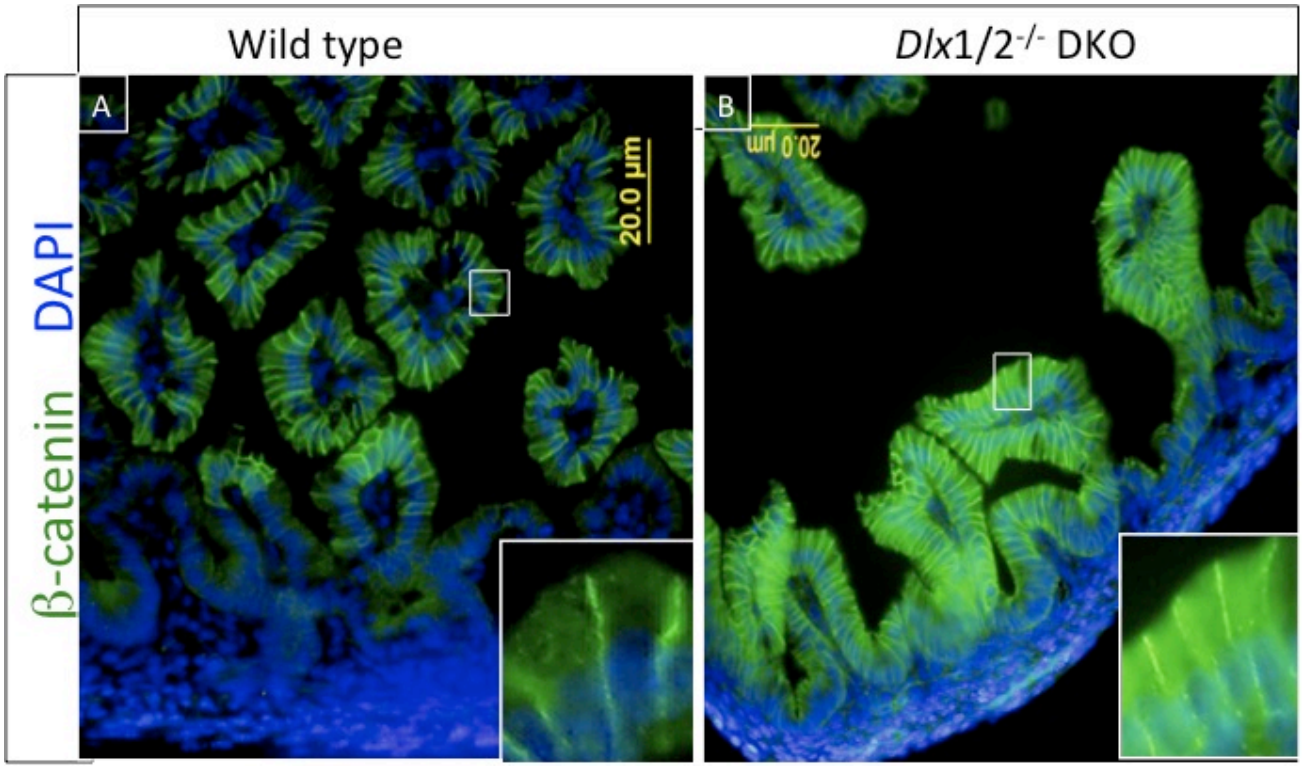

Figure 14: β-catenin expression in the wild type and *Dlx1/2* DKO GIT.

(A) In the wild type GIT, β-catenin appears to be localized mainly at the membrane compartment with some cytoplasmic localization. (B) In the *Dlx*1/2 DKO GIT, β-catenin protein levels appear to be upregulated with a more diffuse localization in the cytoplasm and at the plasma membrane.

These immunofluorescence results for APC and β-catenin expression in the *Dlx*1/*Dlx*2 double knockout tissue are only qualitative. In order to assess the levels of APC and β-catenin in our E18.5 mutant tissue from a more quantitative perspective, we performed Western immunoblotting (semi-quantitative) and qRT-PCR (quantitative) analyses. Our Western

immunoblotting results clearly showed that in both the small and large intestine, the levels of β-catenin were upregulated in the *Dlx*1/*Dlx*2 double knockout GIT (Figure 15A). Conversely, although not significantly decreased, when the transcript levels for *Apc* gene expression were investigated in the *Dlx*1/*Dlx*2 double knockout tissue, the levels of *Apc* transcripts appeared to be reduced in the *Dlx* DKO tissue (Figure 16).

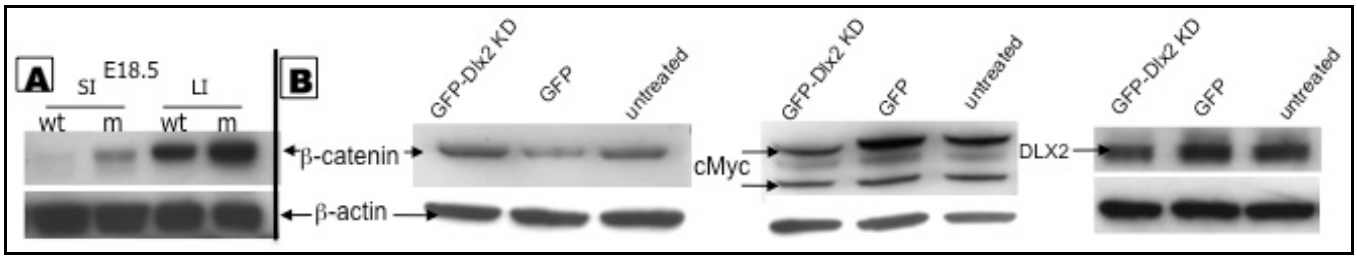

Figure 15: Protein expression

(A) β-catenin western immunoblotting of E18.5 wild type and *Dlx*1/2 DKO GIT total protein lysates. β-catenin protein levels are upregulated in the *Dlx*1/2 DKO tissue in both small and large intestine. (B) *In-vitro* analysis of β-catenin levels upon *Dlx*2 RNA interference on Colo320 colon carcinoma cell line. In the first panel β-catenin protein levels were increased in the Colo320 cell lines when the *Dlx*2 message was silenced. The middle panel shows c*Myc* expression, a downstream target of the Wnt signalling cascade, was not upregulated upon *Dlx*2 silencing, but appeared to be downregulated. The third panel shows the confirmation that *Dlx*2 expression was reduced. Although *Dlx*2 was not entirely silenced, it did however appear to be downregulated when detected by western immunoblotting (C.Jiang and M.Fonseca)

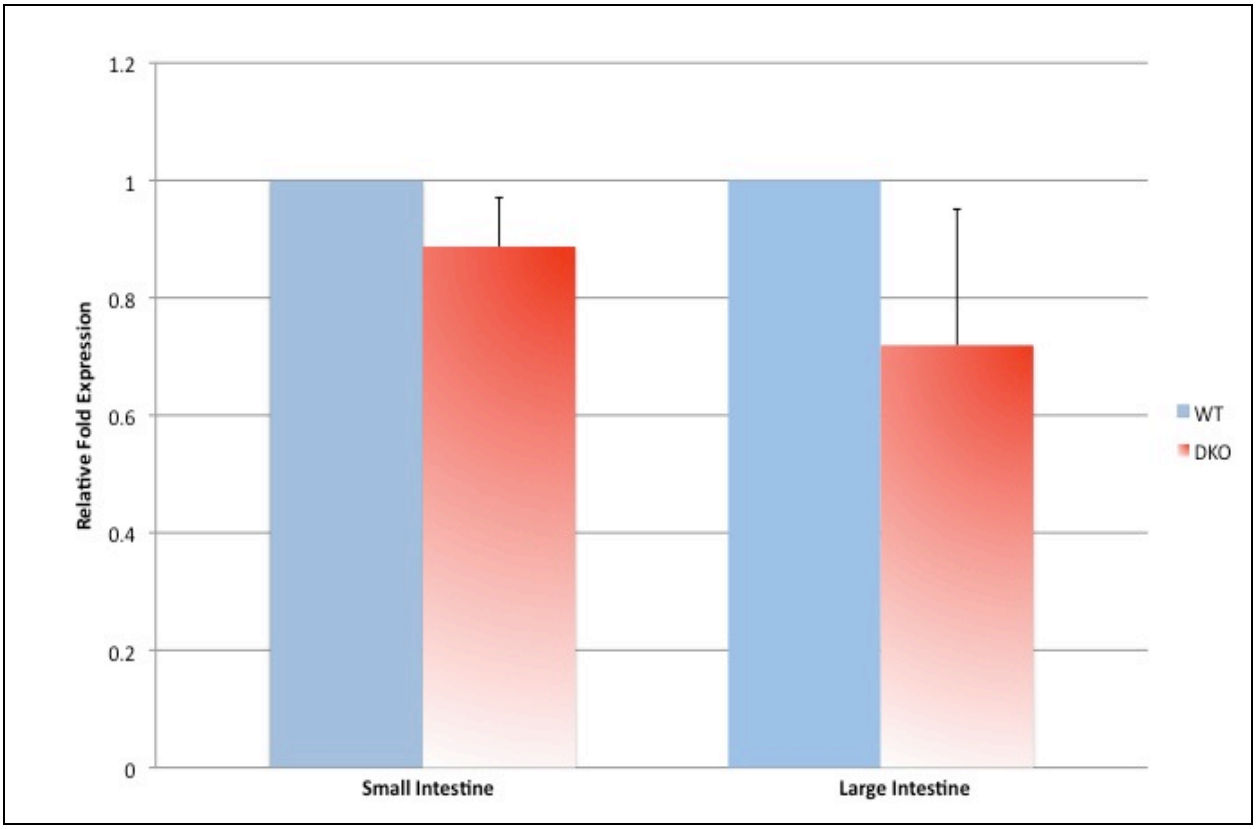

Figure 16: RT-PCR analysis of *Apc* mRNA expression in the small and large intestine of wild type and *Dlx* DKO E18.5 mice. Bar graph representing the fold changes of *Apc* mRNA levels quantified by normalization to *Gapdh* as an internal control. In each of the three different litters (n=3) total RNA was isolated for each wild type and *Dlx* DKO GIT.
Small intestine p=0.2942; Large intestine p=0.3802

β-catenin levels are altered in-vitro when *Dlx*2 is inactivated

To further consolidate our findings obtained *in-vivo*, we then conducted *in-vitro* assays to mimic the *Dlx*1/*Dlx*2 double knockout tissue. RNA knockdown assays were performed using the human colon carcinoma cell line Colo320. RNA silencing is a powerful way for cells to regulate gene expression at the transcriptional level (L. He & Hannon 2004). This form of regulation is made possible by small RNA molecules which downregulate protein production via inhibition of protein translation or promoting the degradation of mRNA (Bruneau 2005). Previously, we

determined that Colo320 cell lines endogenously express DLX2 (data not shown). In order to silence the *Dlx*2 message in the Colo320 cell lines, transfection with a short-hairpin (sh) *Dlx*2 RNA Green Fluorescent Protein (GFP) construct was performed, and after the appropriate incubation time the extracted total protein lysate was analyzed via Western immunoblotting. To ensure correct interpretation of the results, the assay was set up with two negative controls: GFP construct without sh*Dlx*2 and untreated cells. The immunoblotting experiment from the transfected Colo320 cell lines reproduced similar results for our β-catenin detection as in our *Dlx*1/*Dlx*2 double knockout tissue (Figure 14B). The cells that were knocked down with the sh*Dlx*2 RNA had higher levels of β-catenin than the corresponding controls (Figure 15B left panel). Since c-*Myc* is known to be a downstream target of the activated Wnt signalling pathway (T. C. He et al. 1998), we also performed immunoblotting to investigate levels of the c-MYC protein in the Colo320 cells with knocked down expression of *Dlx*2. Our immunoblotting results showed that the c-MYC protein levels were not increased in the Colo320 cells that were knocked-down (Figure 15 middle panel). Although this finding was somewhat unexpected, it was not surprising since the β-catenin immunostaining results did not show nuclear β-catenin in our *Dlx*1/*Dlx*2 double knockout tissue (Figure 14B).

DLX2 regulates *Apc* expression *in-vitro*

To investigate the functional aspects of the relationship between DLX2 and the *Apc* promoter we conducted gene reporter assays using Human Embryonic Kidney 293 (HEK293) cell lines that do not express DLX2 endogenously. As mentioned previously, the *Apc* promoter was arbitrarily divided into 10 regions. Thus far, of the regions that were pulled out by the ChIP assay, region 1 and the full length of the *Apc* promoter were subcloned upstream of the luciferase gene into the

pGL3 reporter construct. The cells co-transfected with the *Apc*RI-pGL3/pCDNA-*Dlx*2 and *Apc*-FL-pGL3/pCDNA-*Dlx*2 constructs experienced a significant increase in gene reporter activity by approximately two-fold and 1.6 fold activation, respectively (Figure 17A,B). DLX2 protein activated *Apc* region 1 more so when compared to the increase in activation that occurred on the *Apc* full-length promoter (Figure 17A,B).

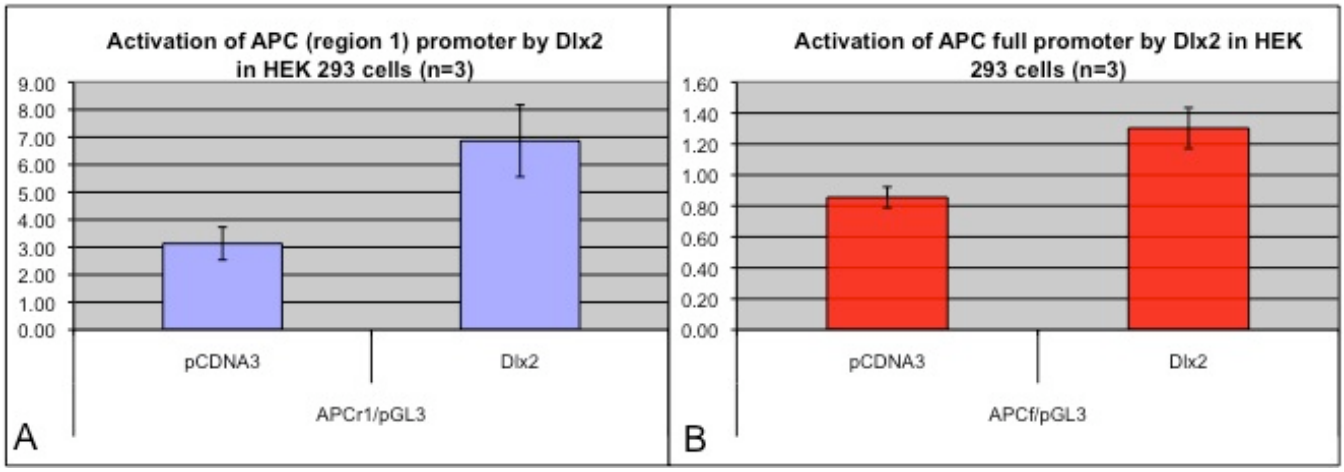

Figure 17: Gene reporter assay (Luciferase) with *Apc* region I (ChIP positive) and *Apc* full-length both cloned into the pGL3 reporter construct..

(A) APCr1/pGL3 (region I) activity was increased in the presence of *Dlx*2 co-expression ($p<0.05$). (B) APCf/pGL3 (full-length) activity was also increased upon *Dlx*2 co-expression ($p<0.05$). All results were normalized to β-galactosidase (M. Eshraghi and M. Fonseca).

DISCUSSION

Remarkably, certain genes possess the ability to carry out central developmental functions in different organ systems. Several reviews have cited the developmental importance of *Dlx* genes in various organs (Grace Panganiban & John L R Rubenstein 2002b; Merlo et al. 2000; J T Wigle & D D Eisenstat 2008). As mentioned previously, this is the first full report where the expression pattern and importance of *Dlx* genes is explored in yet another organ: the gastrointestinal tract.

First, this section will discuss the expression pattern and potential role *Dlx2* may have in the developing gut. The expression pattern of *Dlx2* was investigated from E13.5 to adulthood in the mouse GIT. Embryonically, we have demonstrated the expression of DLX2 in the muscularis propria of the GIT in the enteric neuromuscular system. We have elucidated the pattern of DLX2 expression in the adult GIT, particularly in the differentiated cell types in both the small and large intestine. In addition, we have also observed the expression of DLX2 in the gastrointestinal stem cell population. It will be interesting to explore the potential role that DLX2 may be playing in the gastrointestinal stem cell. Second, the involvement of DLX2 in the Wnt signalling pathway with respect to *Apc* transcriptional regulation will be discussed. We have determined that DLX2 binds to the *Apc* promoter *in-vivo* and *in-vitro*. In addition, we have observed that in the absence or reduced expression of DLX2 (*in-vivo* and *in-vitro*, respectively), the levels of *Apc* transcripts are decreased, and conversely, β-catenin protein levels are increased. Although the *Apc* transcript levels are not significantly decreased, a trend that supports our hypothesis has been observed. Increasing the sample size may yield significant results.

DLX2 Expression Pattern in the Developing Gastrointestinal Tract

DLX2 expression is restricted to the muscularis propria in the embryonic gut

Previous studies have shown that *Dlx1* and *Dlx*2 expression are restricted to the muscularis propria in the developing murine GIT, specifically in the enteric neuromuscular system (M. Qiu et al. 1995; Vohra et al. 2006; Heanue & Pachnis 2007).

By employing immunofluorescence, we investigated the DLX2 expression pattern at three different stages in the mouse GIT: E13.5, E16.5 and E18.5. At these particular developmental stages, the presence of DLX2 expression was obvious in the muscularis propria region (Figure 5 A-C). Embryonically, it is difficult to identify at the cellular level which cell types are DLX2 positive. However, the morphologic location of the signal suggests that the presence of DLX2 corresponds to the developing myenteric parasympathetic ganglia (Auerbach ganglia). Developmentally, it is important to note that both the central nervous system and enteric nervous system are derived from the same embryonic germ layer, the ectoderm. The ectoderm gives rise to the neural plate which folds to form the neural tube. After neural tube takes shape neural crest cells are formed, which subsequently migrate to form dorsal root ganglia, chromaffin cells of the adrenal medulla, melanocytes of skin and the enteric plexi (Sanes et al. 2006). Therefore, it is not surprising to see *Dlx* gene expression on both arms of the nervous system (central and peripheral) since they share the same germ layer of origin. It will be interesting to elucidate the role that *Dlx* genes may potentially be playing during ENS development and adulthood since the myenteric ganglia are formed by the neural crest-derived cells.

DLX2 *expression in the postnatal small and large intestine*

Further into development at E18.5, DLX2 expression is still present in the enteric neuromuscular region; however, some DLX2 positive cells are now present in the epithelial region (mucosal layer) (Figure 5D). At this particular stage, not all cells in the villus are DLX2 positive (Figure 5D). It is worth mentioning that at P0, both the *Dlx*2 single and *Dlx1*/*Dlx*2 double knockout mice, massive distention of their proximal gastrointestinal tract develops in the newborns (P0) approximately within 1-2 hr after birth (M. Qiu et al. 1995). In addition, less peristalsis movement was also observed which led to swallowing of excessive air (M. Qiu et al. 1995). The latter observation implies a defect in the enteric neuromuscular system. This particular phenotype is not observed in *Dlx1* single mutants, which die within the first month of life (M. Qiu et al. 1995; Anderson, M. Qiu et al. 1997a).

Several homeobox genes are expressed during vertebrate intestinal development and adulthood (R. James & Kazenwadel 1991; Walters et al. 1997; Freund et al. 1998; Beck et al. 2000; Silberg et al. 2000), and the presence of *Dlx* genes in the epithelium layer may be contributing to a larger regulatory network in the GIT postnatally.

In the small intestine at the adult stage, there is no particular distinction with respect to *Dlx*2 expression amongst the four postmitotic differentiated cell types. DLX2 expression is sustained during adulthood with a distinct expression pattern along the crypt-villus axis, which implies a functional role with the status of differentiation of the cells. It is highly expressed on the tips of the villi and its expression decreases as we go downward towards the crypt (Figure 6A).

Homeobox genes play important roles embryonically and postnatally during GIT development (R. James & Kazenwadel 1991; Beck et al. 2000; Beck 2002). For instance, the homeobox genes *Cdx*1 and *Cdx*2 have been reported to be expressed at high levels during adulthood in the mouse intestinal epithelium and are involved in cell differentiation (R. James et al. 1994). Another family of transcription factors, the GATA zinc-finger transcription factors also play important role in the regulation of key genes such as *Mucin*2 in the adult murine GIT (van der Sluis et al. 2004). The robust expression of DLX2 in all four differentiated cell types in the small intestine may be indicative of a possible role with respect to differentiation or maintaining the cell in a terminally differentiated state, potentially regulating intestine-specific genes (Figure 7A-D). Interestingly, DLX2 is not expressed in the adult large intestine goblet cells (Figure 8A). This is an interesting observation as the goblet cells in the small intestine were found to be DLX2 positive. Although goblet cells are found in both regions of the GIT, they may not be functionally or biochemically identical. However, the abundant colonocytes (enterocytes in the small intestine) in the large intestine were DLX2 positive (Figure 8B).

DLX2 expression was not confined solely to the differentiated compartment along the crypt-villus axis in the adult gut, but was also observed in the immature proliferative zone in the crypt region (Figure 6 A,B). The crypts of Lieberkühn also showed strong DLX2 immunopositivity (Figure 6A*). Of the four postmitotic cell types that exist in the vertebrate GIT, the Paneth cells do not journey upwards along the crypt-villus axis, but instead migrate downwards into the crypt region. As demonstrated, Paneth cells are positive for DLX2 expression (Figure 7B); however we observed *Dlx*2 positive cells that were not lysozyme positive. In the murine small intestinal crypt, approximately 250 cells are found, of these, approximately 150 are actively dividing and half of which are in S phase at any given time (Bach et al. 2000). These actively dividing cells

belong to two cell populations: stem cell and transit amplifying cells. It is important to note that *Dlx* genes belong to the homeobox family of transcriptions factors. Both the murine and human *Dlx* genes are arranged in three convergently transcribed pairs, where each pair is linked to a Hox cluster. For instance, *Dlx1* and *Dlx*2 are linked to *Hoxd*; *Dlx*3 and *Dlx*4 linked to *Hoxb*; and *Dlx*5 and *Dlx*6 are linked to *Hoxa* (Ozçelik et al. 1992; McGuinness et al. 1996; Simeone et al. 1994; Stock et al. 1996). *Hox* genes are known to be involved in the complex regulatory network of transcription factors that control human T-cell development (Taghon et al. 2003). Similarly, developmental processes such as patterning and cell lineage specification have been shown to be under the control of a specific *Hox* genetic code affecting all three germ layers. For instance, the combined loss of *Hoxa*1 and *Hoxb*1 has an effect in the differentiation of embryonic stem cells which subsequently has an effect on the patterning of the vertebrate hindbrain. In the same way, targeted disruption of *Hoxa*3 yields defects of lineage specification resulting in the loss of the thymus (Gouti & Gavalas 2008). We observed that the recently established gastrointestinal stem cell marker *Bmi*1 (Sangiorgi & Capecchi 2008) was found to be co-expressed in the same cell as DLX2 (Figure 9A and B). With the emerging knowledge that *Hox* genes may possess an important role in regulating specific genetic pathways during adult stem cell maturation, interest has been generated in our laboratory with regards to the role of *Dlx2* within that network of transcription factors.

The role of *Dlx*2 in the Canonical Wnt Signalling pathway

Wnt/β-catenin signalling is not only fundamental during embryogenesis but also plays an important role in adult tissue homeostasis (Hans Clevers 2006). At the heart of this pathway the cytoplasmic β-catenin destruction complex is found, which consists of Axin, GSK-3, CKI and APC. Free cytoplasmic β-catenin is known to act as a co-transcriptional activator of

proliferative genes. The levels of free β-catenin in the cytoplasm will be dependent upon the stability of this protein complex. Unaffected or stabilized β-catenin in the cytoplasm accumulates in the nucleus and associates with the T cell factor/lymphoid enhancer factor (TCF/LEF) family of transcription factors to augment gene expression (He Huang & X. He 2008).

The rapid self-renewing tissue of the GIT (3-5 days in the mouse) requires a dominant pathway that closely monitors the cell turnover rate. Present evidence indicates that the Wnt/β-catenin signalling cascade is the principal force controlling cell fate in the adult murine intestinal epithelium (crypt-villus axis) (Hans Clevers 2006). Furthermore, Wnt signalling activation in the colonic epithelium appears to be one of the main events in the formation of polyps. The association between β-catenin and APC has been well documented (Su et al. 1993; Rubinfeld et al. 1993). APC is a critical component of the β-catenin destruction complex. Two important studies have demonstrated (*in-vitro* and *in-vivo*) that upon APC mutation, β-catenin levels become altered (Munemitsu et al. 1995; Sansom et al. 2004). In addition, it was shown *in-vivo* that not only does the level of β-catenin increase, it also becomes re-localized into the nucleus (Sansom et al. 2004). This is critical as it has been established that the APC protein possesses a significant role in colon cancer progression. In the human population, truncation mutations in the gene encoding for APC protein are found in over 80% of sporadic colonic tumours and are also responsible for Familial Adenomatous Polyposis (FAP), an inherited form of colon cancer (Fodde 2002; Su et al. 1992)). A wealth of knowledge exists about APC mutations as the leading cause of polyposis formation, a precursor to adenoma. A number of elegant experiments have generated mouse models of gastrointestinal tumours by genetically engineering knockout mice with various *Apc* mutations. These genetically engineered mice have proven that Wnt signalling activation via a crippled APC protein is the main driver of intestinal tumours (Taketo

2006).

Although knowledge about *Apc* mutations leading to polyposis formation is useful in studying colon cancer progression, limited efforts have been put forth to elucidate the regulation of *Apc* transcription especially during development. Commendable efforts have shed light into understanding *Apc* transcription by investigating epigenetic changes with respect to gene inactivation via CpG island methylator phenotype. It has been found that the aberrant methylation of cytosines that are present in CpG islands in the *Apc* proximal promoter sequence leads to gene silencing (gene inactivation) (Hiltunen et al. 1997; Jin et al. 2001; Jing et al. 2010; Tsuchiya et al. 2000). The activity of transcription factors in concert with the transcription machinery is an alternate way in which *Apc* gene expression can be modulated. However, little is known about transcription factors that affect *Apc* gene expression.

Herein, utilizing chromatin immunoprecipitation (ChIP) of adult and embryonic gastrointestinal tissue, we have demonstrated that DLX2 binds to homeodomain binding motifs within the murine *Apc* promoter. Upon analysis of the distal *Apc* promoter, we found 23 putative homeodomain binding motifs. Due to the large size of the promoter (3.9 kb), it was divided into 10 different arbitrary regions where PCR oligos were designed to cover at least one candidate-binding motif for each region (Figure 10). The DLX2 ChIP analysis on both the adult small and large intestine showed similar binding patterns with the exception of region seven (Table 9). This data indicates that despite the difference in the anatomical regions, the DLX2 localization on the *Apc* promoter remains somewhat consistent in the GIT at the adult stage. However, the DLX2 ChIP analysis on the embryonic GIT (E18.5) revealed that the DLX2 binding pattern on the *Apc* promoter differs from the adult (Table 9). The discrepancy observed in DLX2 localization on the *Apc* promoter between the two temporal stages of development may be

indicative of a functional role the APC protein may have embryonically versus postnatally. It is also possible that the *Apc* gene may be under the control of different transcription factors at different stages in development. The ChIP results clearly indicate that DLX2 is localized at the *Apc* promoter at two different time stages. This is in accordance with our hypothesis that DLX2 binds to the *Apc* promoter *in-vivo*. To assess this interaction further, specific binding was confirmed by EMSA (*in-vitro* analysis) (Figure 12) and to test our hypothesis that DLX2 is activating *Apc* gene expression, we performed functional analyses in the form of gene reporter assays. This *in-vitro* experiment showed that DLX2 activated both region-I and the full-length promoter regions (Figure 17A,B). This *in vitro* data supports our hypothesis that DLX2 is activating *Apc* gene transcription *in vivo*.

Loss of *Dlx*2 has an effect on *Apc* and β-catenin levels *in-vivo*

Unfortunately, our *Dlx1*/*Dlx*2 double mutant mice die shortly after birth, which makes it difficult to analyze the consequence of the absence of *Dlx1* or *Dlx*2 gene postnatally. Therefore, the latest stage that we can study the effects of the absence of *Dlx1* and *Dlx*2 gene function is at E18.5 or P0. *Apc* levels were analyzed qualitatively and quantitatively *in-situ*. By qualitative analysis via immunofluorescence, we discovered that at E18.5 APC localization appeared unchanged in the wild type and *Dlx1*/*Dlx*2 mutant GIT in the cytoplasm (Figure 13A,B). However, the difference in APC localization was observed to be in the nucleus (Figure 13A,B). APC appeared to be absent in the nucleus of the *Dlx1*/*Dlx*2 mutant small intestine when compared to the nuclear APC staining of the wild type small intestine. This observation will merit further investigation into the possible cause of the absence of APC expression in the nucleus in the *Dlx1*/*Dlx*2 mutant mice. We quantified *Apc* gene expression by quantitative real time polymerase chain reaction (qRT-PCR). We discovered that in the small and large intestine at E18.5, the *Apc* transcripts are

lower in the *Dlx1*/*Dlx*2 mutant GIT than in the wild type, but these results did not reach statistical significance (Figure 16). Following the established knowledge of the canonical Wnt cascade, we decided to assess the levels of β-catenin protein in the *Dlx1*/*Dlx*2 double mutants versus their wild type littermates. First, a qualitative analysis was performed via immunofluorescence analysis. Our immunostaining analysis showed that β-catenin levels were higher in the *Dlx1*/*Dlx*2 mutant GIT when compared to the wild type intestinal tissue (Figure 14). At this particular time-stage, the β-catenin appeared to be diffusely expressed throughout the cell, with apparent higher levels of β-catenin localized at the cellular membrane compartment, likely via E-cadherin interactions. Although, this observation coincides with our hypothesis, we did not observe β-catenin nuclear re-localization in our *Dlx1*/*Dlx*2 mutant intestinal tissue. This unexpected result will merit further investigation into why β-catenin does not re-localize into the nucleus at this particular stage. It is possible that a combination of temporal factor(s) could be involved such as the recruitment of oncogenes or tumour supressor genes. Fearon and Vogelstein have proposed a model where genetic alterations at different stages are required for the development of malignancy. In their model, they suggest that colorectal tumours occur as a result of the mutational activation of oncogenes coupled with the inactivation of tumour-suppressor genes (Fearon & Vogelstein 1990); (Kinzler & Vogelstein 1996). It has been reported that β-catenin is almost negligible in the nucleus during the early stages of tumour initiation; however, β-catenin re-localizes into the nucleus as genetic changes associated with colorectal tumorigenesis progress (Giles et al. 2003). If *Apc* inactivation due to the loss of *Dlx*2 is occurring, the accumulation of β-catenin in the *Dlx1*/*Dlx*2 mutant intestinal tissue could be one of the events found during the initial stages of the model proposed by Fearon and Vogelstein about 20 years ago; hence the lack of observation of β-catenin in the nucleus in the *Dlx1*/*Dlx*2

mutant intestinal tissue at this particular time point. So far, we can only theorize the outcome in the GIT postnatally in the absence of *Dlx* genes, which could potentially be one of cellular proliferation defects and ultimately leading to malignancy.

Western immunoblotting was performed to semi-quantitatively assess β-catenin protein levels. Our immunoblotting results confirmed our qualitative analysis in that β-catenin protein levels were higher in the *Dlx1*/*Dlx*2 mutant GIT tissue (Figure 15A). So far, the β-catenin levels assessed in our Western immunoblotting do not discriminate a particular cellular compartment. Further analysis will be required to investigate the precise location and amounts of β-catenin in the *Dlx1*/*Dlx*2 mutant GIT tissue by cell fractionation analysis.

Dlx2* silencing leads to higher β-catenin levels *in-vitro

Colo320 colonic carcinoma cells were used to analyze the downstream effects of knocking down *Dlx*2 transcript levels. We had previously determined that Colo320 colonic carcinoma cells were DLX2 positive (data not shown). Silencing the *Dlx2* transcript in these cell lines will mimic tissue where the *Dlx2* gene had been knocked out (*Dlx1*/*Dlx2* mutant GIT tissue). Upon the transient knockdown of the *Dlx2* message, we were able to show that the β-catenin levels in the silenced cell lines increased in comparison to the cell lines that were not transfected with the *Dlx2* shRNA or were transfected with the empty construct (no sh*Dlx2* RNA) (Figure 15B). This result concurs with our *in-vivo* analyses where DLX2 was absent in our *Dlx1*/*Dlx2* mutant GIT tissue (Figure 15A). We observed an increase in β-catenin levels despite the less than expected knockdown efficiency.

CONCLUSIONS AND FUTURE DIRECTIONS

The study presented here is the first report to characterize *Dlx2* expression pattern in the developing GIT. We have also investigated the transcriptional regulation of *Apc* by the DLX2 homeodomain transcription factor. Our results consolidate the previous preliminary findings that *Dlx* genes are expressed in the developing murine GIT. However, our studies have specifically explored *Dlx2* expression in a more systematic and detailed approach.

The robust *Dlx2* expression in all four differentiated cell types in the adult mouse GIT epithelium, maybe indicative of a potential role that is necessary postnatally. Other studies have reported the importance of *Dlx* genes in the developing forebrain and retina (Anderson, D D Eisenstat et al. 1997b; Anderson et al. 1997b; D D Eisenstat et al. 1999; de Melo et al. 2003; de Melo et al. 2005b). Given the important function of *Dlx* genes during development and that GIT development concludes postnatally, it will be of great importance to elucidate the role *Dlx* genes may be playing in the developing GIT. However, it is important to note that *Dlx2* expression was also present in the proliferative crypt region. Moreover, we were able to show that the gastrointestinal stem cell is actively expressing DLX2. This raises the issue about *Dlx2* expression in the differentiated and proliferative compartments. However, this phenomenon has been observed in the developing mouse forebrain. For instance, the E16.5 mouse brain contains DLX2 positive cells in the subventricular zone (proliferative) as well as in the cortex (differentiating) (Le et al. 2007). *Dlx* genes have been implicated in various aberrant phenotypes as a consequence of their absence such as impaired migration and differentiation of interneurons (Anderson et al. 1997a; Anderson, M. Qiu et al. 1997a; Le et al. 2007), and increased apoptosis in the mouse retina (de Melo et al. 2005a). Therefore, further studies will need to be performed to assess cell cycle and cell death parameters in the *Dlx1*/*Dlx2* mutant GIT to determine whether

one or both of these assessments will generate similar results as reported in the brain and retina. A *Dlx1* and /or *Dlx2* conditional knockout mouse would yield critical information on the function of *Dlx* genes postnatally in the GIT. Possible outcomes of removing *Dlx2* gene postnatally could lead to a number of abnormal cellular behaviors such as impaired differentiation, atypical cell death or uncontrolled cell proliferation. Embryonically, it will be worth investigating the status of each cell type precursor in the *Dlx1*/*Dlx2* mutant GIT. By performing gene expression analysis using specific antibodies to cell type precursors, we will be able to assess whether any of the precursors or the mature cell types will be affected due to the absence of *Dlx2*.

We have also concluded that DLX2 binds to the *Apc* promoter *in-vivo*. Of the 23 putative binding motifs (divided into 10 regions) present in the proximal *Apc* promoter, DLX2 binds to six regions (regions 1, 2, 6, 7, 8 and 10 in SI and 1, 2, 4, 6, 8 and 10 in the LI) in the adult GIT. However in the E18.5 GIT, DLX2 only binds to one region (region 1 and with possible binding at region 10). Two *in-vitro* analyses also have indicated that DLX2 is binding to the *Apc* promoter and activating gene expression (demonstrated by EMSA and luciferase, respectively). Performing EMSA using GIT nuclear extracts will be informative as this is representative of what is occurring *in-vivo* and will include any other proteins that DLX2 may be in complex with at the *Apc* promoter. Furthermore, to confirm binding specificity on individual regions, site directed mutagenesis would need to be performed to compromise DLX2 binding specificity on the *Apc* promoter and thus confirm the direct activity of DLX2 on *Apc* expression. Although we clearly observed higher levels of β-catenin in the E18.5 *Dlx1*/*Dlx2* mutant GIT tissue by IF and Western blotting, we could not conclude in which cellular compartment β-catenin was

accumulating. Even though our IF results indicate that β-catenin appeared to have accumulated in the cytoplasm and cellular membrane, GIT epithelial cellular fractionation followed by Western immunoblotting will need to be performed to answer and verify this particular question. Ultimately, in order to test our hypothesis that DLX2 is activating the *Apc* gene and the Wnt signalling cascade, it would be critical to assess the interaction of DLX2 and *Apc* by using both *in-vivo* and *in-vitro* models. The Wnt signalling pathway can be tested *in-vivo* by using mouse models. A number of transgenic mouse models (Wnt/β-catenin/TCF reporters) have been made in order to study Wnt or β-catenin-mediated signalling *in-vivo* (reviewed in Barolo 2006). Crossing our *Dlx1*/*Dlx2* mutant mice with a Wnt reporter mouse would provide us with critical information about the role *Dlx2* gene may play during the canonical Wnt signalling cascade. Crossing the *Dlx2* single knockout mouse with a Wnt reporter mouse may yield similar results given that *Dlx1* is not expressed at E18.5 and postnatally (data not shown). However, the scenario may be different at earlier stages given that *Dlx1* has been reported to be expressed in the developing murine GIT (Vohra et al. 2006; Heanue & Pachnis 2007). Another approach that could provide evidence in support of our hypothesis is the *in-vitro* analysis known as the TOP-flash assay. The TOP-flash assay is a robust and well-established quantitative technique to determine β-catenin-Tcf/Lef-mediated gene transcription. The TOP-flash approach could be combined with *Dlx2* shRNA silencing followed by quantifying Tcf/Lef-mediated gene transcription (M van de Wetering et al. 1997).

Further characterization on the role of *Dlx2* in both the embryonic and postnatal stages in the mouse GIT will illuminate our understanding with respect to the function of the murine intestinal epithelium. Furthermore, this knowledge will help us understand the role *Dlx2* genes may play

in the human intestinal epithelium since *Dlx2* is also expressed in the human colon (M Fonseca and D Eisenstat, unpublished observations). It is imperative to understand the molecular players involved in regulating *Apc* transcription. This may not only shed additional information on modulators of the Wnt signalling pathway, but it will enhance our knowledge about the homeostasis of the intestinal epithelium. It is precisely this feature, which when perturbed it leads to malignancy in the form of tumour formation not just in the GIT but also in other organ systems.

Therefore, the findings presented in this thesis have shown another molecular player in the complex network of transcription factors found in the mouse GIT. Further investigation about the role of *Dlx* genes and their involvement in the Wnt signalling pathway will be crucial to better understand intestinal homeostasis in the developing and postnatal vertebrate GIT. This information will be crucial, since it can advance our knowledge regarding tumour biology in the colon.

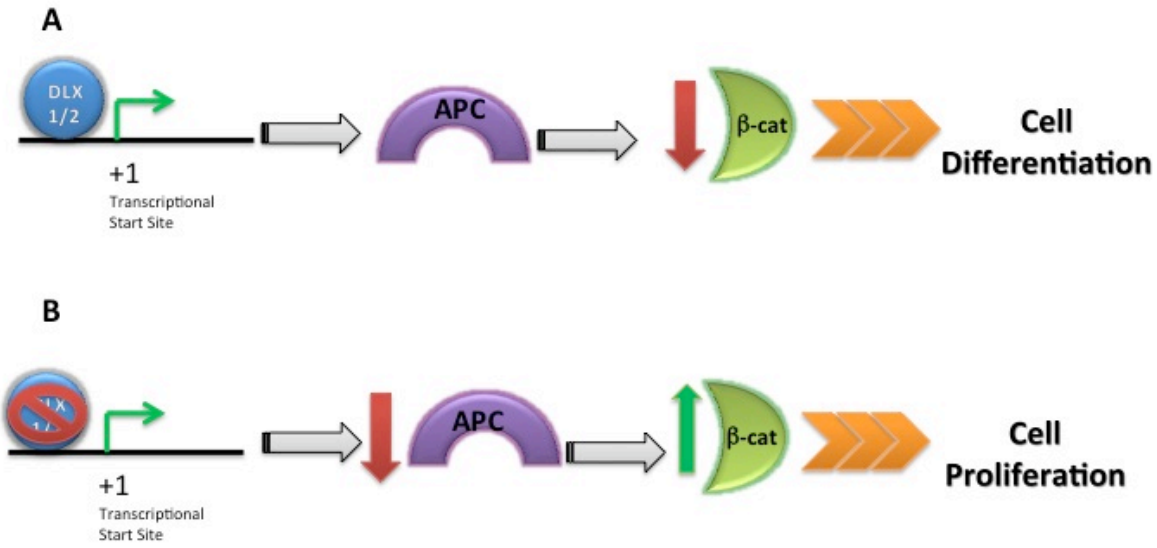

Figure 18: Proposed Model for the Regulation of *Apc* by DLX1/2 transcription factors

This model proposes that in the GIT, DLX2 directly activates the *Apc* promoter. (A) Upon *Apc* activation by DLX2, β-catenin levels are maintained at controlled levels as needed by the cell. This would lead the cell to a differentiation fate. (B) In the absence of the DLX2 protein, (*Dlx1/2* DKO GIT) Apc levels would decrease leading to higher levels of β-catenin in the cell. This would lead the cell to a proliferative fate (M. Fonseca and V.Pinto)

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