GENERATION OF MOUSE REAGENTS FOR STUDYING THE EFFECTS OF ACTIVATED β-CATENIN SIGNALING IN INTESTINAL DEVELOPMENT AND DISEASE

By

Kendra Kay Madrid

A Thesis

Presented to the Department of Cell and Developmental Biology

and the Oregon Health & Science University

School of Medicine

in partial fulfillment of

the requirements for the degree of

Master of Science

August 2004

School of Medicine
Oregon Health & Science University

CERTIFICATE OF APPROVAL

This is to certify that the M.S. thesis of

Kendra Madrid

has been approved

[redacted]
Professor in charge of thesis

[redacted]
Member

[redacted]
Member

[redacted]
Member

Member

TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

CHAPTER 1 INTRODUCTION

CHAPTER 2 METHODS AND MATERIALS

CHAPTER 3 RESULTS

ABREVIATIONS

Apc	Adenomatous polyposis coli
bGH	Bovine growth hormone
CA	Enhanced chicken β-actin promoter
COS-7	Transformed African green monkey kidney fibroblast cells
CMV	Cytomegalovirus
ES cell	Embryonic stem cell
Fabpl	Rat liver fatty acid binding protein transcriptional regulatory elements
Fabpl $^{4x\ at\ -132}$	Nucleotides –596 to +21 of the rat *Fabpl* gene with four additional tandem repeats of its nucleotides –172 to –133 added at nucleotide -132
FAP	Familial Adenomatous Polyposis
Fz	Frizzled
GFP	Green fluorescent protein
Gsk-3β	Glycogen synthase kinase-3β
hGH	Human growth hormone
HMG	High mobility group
IVR	Intervillus region
Lef-1	Lymphoid enhancer factor-1
Lef-1/β-cat	Fusion molecule of Lef-1 and β-catenin
MCS	Multiple cloning site
Min	Multiple intestinal neoplasia
Mmp-7	Matrilysin-7
neo	Neomycin resistance gene
pBigT	Plasmid Big Tamale
pBS	Plasmid Bluescript
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PGK-neo	Phosphoglycerate kinase -neomycin resistance selection cassette
Pparδ	Peroxisome proliferator-activated receptor δ
qRT-PCR	Real-time quantitative RT-PCR
ROSA	Reverse Orientation Splice Acceptor
rtTA	Reverse tetracycline-controlled transactivator
Tcf-1, 3, 4	T cell factor-1, 3, 4
Wnt	Wingless/Int-1

ACKNOWLEDGEMENTS

First and foremost, I give thanks to my Lord, Jesus Christ, and to God, my heavenly Father. I praise you, O Lord, and exalt you, O God, for great is your love and great is your faithfulness; they reach far above the heavens. You are my rock and my fortress; into your hands I commit this work. I trust in you, O LORD; I say, "You are my God." From life's first cry to final breath, Jesus, the events and circumstances of my life are ever in your hands. In you alone my hope is found. You are my light, my strength, my solid ground. O LORD my God, I give you thanks forever.

I also acknowledge and thank my husband Ramon and my son Michael. Their commitment to my success, their unstinting support of my goals, and their continued patience, sacrifice, and encouragement have seen me through these five years at OHSU. You are both prince among men; your unfailing love surrounds me and rests upon me. In you both my heart rejoices. I thank God for you.

I would like to acknowledge Dr. Melissa Wong, my advisor, and the members of the Wong lab: Adria Decker, Dr. Adnan Rizvi and John Swain. I would like to thank Missy for her generosity, her encouragement and support, and her example; I have learned a great deal from her. I would like to thank everyone in the lab for creating a friendly and supportive working environment. I would also like to acknowledge the members of my thesis committee: Dr. Jan Christian, Dr. Michael Liskay, and Dr. Richard Maurer. My thanks to each of them for their much appreciated support and insight.

Further, I would like to acknowledge Dr. Hans Peter Bächinger, Dr. Richard Brennan, Dr. David Hinrichs, Dr. Richard Maurer, and Dr. Karin Rodland, for their investment in me as a scientist. I am deeply grateful to each of them for inspiring me to think in new ways. I would also like to thank Dr. Leslie Patton, Dr. Paul Howard, Dr. Shall Jue, Kara Johnson, Stephanie Willis, Melissa Cunningham, Linda Jauron-Mills, Carolyn Gendron, Pam Wagoner, and Elaine Offield who have shared their time, knowledge, reagents, and protocols. Their generosity has encouraged me throughout my research.

Finally, I would like to acknowledge and thank the Northwest Health Foundation, the Tarter Trust Fellowship, and the Philanthropic Educational Organization for their support of my work.

ABSTRACT

The intestinal epithelium undergoes rapid renewal throughout life. In contrast to tissues that renew by duplication of existing differentiated cells, the continuous replacement of the intestinal lining is sustained by multipotential intestinal stem cells. The balance of proliferation and differentiation in the intestine requires continuous regulation of the intestinal stem cell.

Though some of the factors that coordinate the intestinal stem cell's regulation are just beginning to be studied, the Wnt/β-catenin signaling pathway has been shown to play a role in influencing intestinal stem cell fate. Stimulation of this pathway during development results in apoptosis of the intestinal stem cell, whereas ablation of signaling through this pathway results in premature differentiation of the developing stem cells. In adults, activation of the Wnt/β-catenin signaling pathway leads to hyperproliferation and polyp formation, which in humans can progress to colorectal cancer.

Previous studies have shown that essentially all forms of sporadic and hereditary colorectal tumors contain mutations in the regulatory genes of this pathway. The Wnt/β-catenin pathway mediates its signaling effects through activation of gene transcription by β-catenin. Therefore it is the accumulation of β-catenin, and its subsequent transcriptional activation of target genes, that effect a response critical to normal development or tumorigenesis. However, the early events in the response of the intestinal stem cell and its progenitors to activated β-catenin signaling and, thus, the initial molecular mechanisms underlying the transformation from the normal to disease state are unknown.

In order to define the early molecular events leading to colorectal cancer, and identify possible molecular targets for therapy, as well as gain insight into intestinal stem cell regulation, stimulation of β-catenin signaling in the intestine must be inducible at various developmental and adult time points. The current available mouse models used to study the role of the Wnt/β-catenin pathway in development and disease lack temporal control of β-catenin signaling and instead rely on mutations in the germ line to dysregulate the pathway. Therefore, current mouse models cannot provide information about the immediate molecular events upon β-catenin's transactivation of target genes. To gain spatial and temporal control over activation of β-catenin signaling, reagents for the generation of two transgenic mouse lines have been created using electroporation of ES cells. Both mouse lines will harbor an inducible transgene capable of activating β-catenin signaling in the intestinal epithelium. Studies with these mice will allow characterization of the immediate molecular and cellular responses to activated β-catenin signaling in the intestine, in both development and in disease.

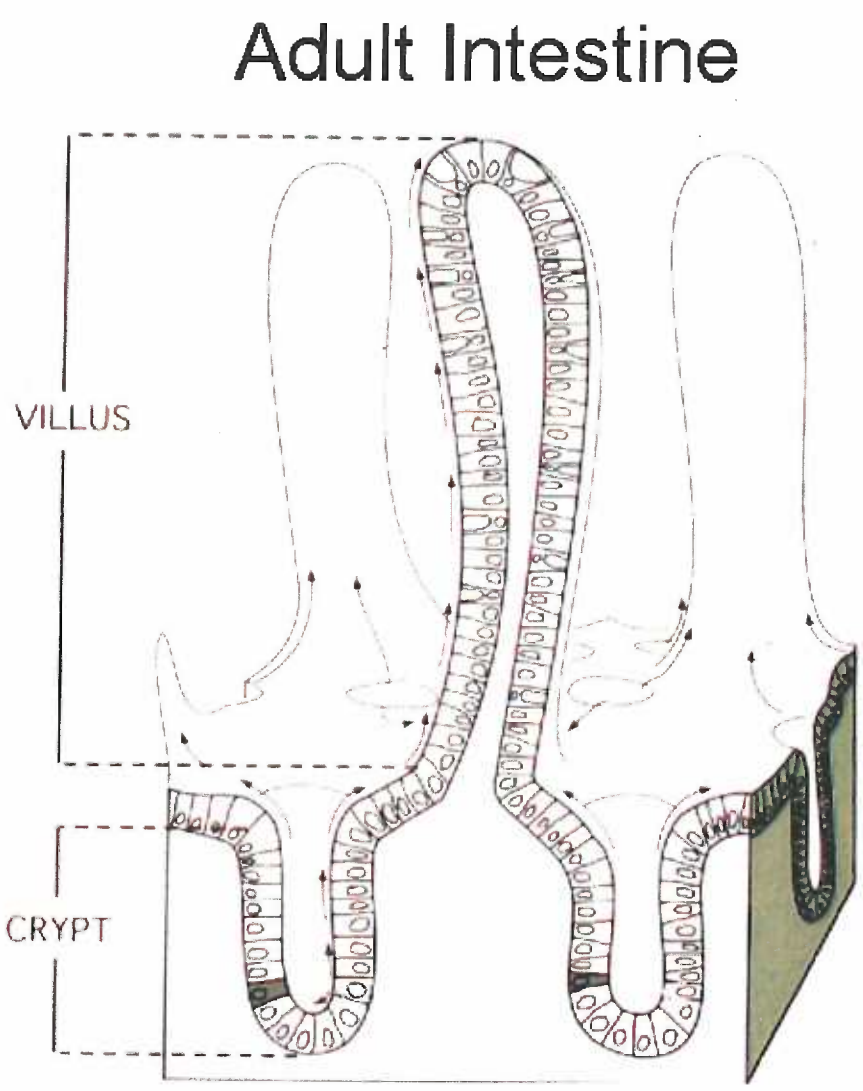
INTRODUCTION

Wnt/β-catenin signaling influences both adult and developmental intestinal epithelium. Dysregulation of the Wnt/β-catenin signaling pathway in the adult intestinal epithelium results in the nuclear accumulation of β-catenin and subsequent transcriptional activation of specific target genes (Behrens et al. 1996; Huber et al. 1996; Molenaar et al. 1996; Batlle et al. 2002; van de Wetering et al. 2002), leading to unregulated epithelial proliferation and polyp formation (Moser et al. 1990; Fodde et al. 1994; Oshima et al. 1995; Shibata et al. 1997). Intestinal polyps in humans can progress to colorectal cancer if left untreated (Powell et al. 1992; Kinzler and Vogelstein 1996; Polakis 2000; Fodde et al. 2001b). The Wnt/β-catenin pathway is also involved in the intestine during development, having been shown to regulate the activity and survival of the developing intestinal epithelial stem cell (Korinek et al. 1998a; Wong et al. 1998; Wong et al. 2002). In contrast to its effects in the adult intestinal epithelium, over stimulation of Wnt/β-catenin signaling in the developing intestinal epithelium results in apoptosis of the stem cell population (Wong et al. 2002). The cascade of early events stimulated by β-catenin signaling and the molecular mechanisms that transform normal epithelium into intestinal polyps in the adult or induce an apoptotic response in developing intestinal stem cells are not well understood. The goal of the work detailed in this thesis is to generate inducible transgenic mouse lines that will facilitate the study of the intestinal epithelial stem cell's response to activated β-catenin signaling, both in development and disease.

The Structure of the Intestine

In vertebrates, digestion takes place in a long tube known as the gastrointestinal tract, which is comprised of the oral cavity, esophagus, stomach, small intestine (duodenum, jejunum, and ileum), large intestine (cecum and colon), and anal canal. The small intestine is characterized by numerous finger-like projections known as villi interposed between invaginations known as crypts of Lieberkühn (see Figure 1). The lumenal surface of the crypts and villi are lined with a single-layered sheet of epithelial cells. The villi are lined with enterocytes responsible for absorption, goblet cells that secrete mucus, and enteroendocrine cells to secrete hormones. The crypts are lined with undifferentiated cells and Paneth cells, which secrete antimicrobrial peptides and growth factors. Several crypts surround the base of each villus; each crypt and adjacent villi form a distinct anatomical domain known as the crypt-villus unit (Cheng and Leblond 1974; Moxey and Trier 1978; Moxey and Trier 1979; Karam 1999).

The mouse intestine begins developing at embryonic day (E)7.5. By E15.5 the tube is lined with pseudostratified epithelium that progressively evolves into an epithelial monolayer. From E16.5, nascent villi arise. The region of epithelium between villi, the intervillus region (IVR), is characterized by high levels of mitotic activity and populated largely with undifferentiated cells. Studies in genetic mosaic mice reveal that the IVR contains multiple stem cells (Schmidt et al. 1988). After birth, the IVRs invaginate to form crypts. During this period, a single stem cell is selected to populate the adult crypt in an undefined process known as crypt purification (Schmidt et al. 1988; Wong et al. 2002). This single stem cell gives rise to 4-6 active stem cells that maintain the adult



modified from *Stappenbeck et al.* (2001)

Figure 1. The Adult Intestinal Stem Cell Niche. This drawing of an intestine section from an adult mouse depicts the invaginated proliferative compartment (crypt) that contains the intestinal stem cell (darkened) and the finger-like projection (villus). The multipotent stem cell gives rise to all four epithelial lineages. Three of the lineages differentiate as they migrate up out of the crypt and onto adjacent villi. In three to five days, these migrating cells reach the villus tip and undergo apoptosis or are exfoliated into the intestinal lumen. The fourth lineage differentiates during downward migration to the base of the crypt. Each adult crypt populates adjacent villi and surrounding crypts populate each villus.

crypt (Potten 1998; Booth and Potten 2000). Completion of intestinal development occurs by postnatal day (P)21 (Trier and Moxey 1979; Calvert and Pothier 1990).

The Adult Intestinal Stem Cell Niche

The adult mouse intestinal lining is organized in a single-layered sheet of epithelial cells that is replaced at a constant and rapid rate under normal conditions throughout life. This continuous renewal of the intestinal epithelium is sustained by multipotential stem cells. The adult intestinal epithelial stem cells reside near the bottom of the crypt (see Figure 1, Potten and Loeffler 1990; Karam 1999; Brittan and Wright 2002) and establish a steady state of proliferation, generating at least one replacement stem cell and one progeny cell each time they divide. The progeny cells divide, creating a transient amplifying population of cells that give rise to the four epithelial lineages (Cheng and Leblond 1974; Kirkland 1988; Karam 1999; Wright 2000). Three of the lineages, the enterocytes, enteroendocrine cells, and goblet cells, differentiate as they migrate up out of the crypt and onto the adjacent villi (Moxey and Trier 1978). In three to five days they reach the villus tip and undergo apoptosis or are exfoliated into the intestinal lumen (Hall et al. 1994). Paneth cells, the fourth lineage, differentiate as they migrate downwards toward the base of the crypt (Cheng 1974). Each adult crypt populates adjacent villi and multiple crypts populate each villus. Thus, within each crypt-villus unit there is a gradient from proliferation to differentiation, with more proliferation near the base of the crypt where the stem cell and the transient amplifying population

reside, to less proliferation near the crypt-villus junction, where cells are undergoing differentiation, to no proliferation on the villus, where cells are terminally differentiated.

The adult intestinal stem cell is influenced by secreted factors and/or cell-cell interactions in its surrounding microenvironment. These influences originate from nearby differentiated and undifferentiated epithelial cells, as well as the underlying mesenchyme and the extracellular matrix. The mesenchyme is composed of the basement membrane, pericryptal myofibroblasts, and the interstitial cells of Cajal, and has been shown to influence epithelial proliferation and differentiation (Powell et al. 1999). The pericryptal fibroblasts secrete Hepatocyte growth factor, Transforming growth factor-β (Tgf-β), and Keratinocyte growth factor (Sonnenberg et al. 1993; Finch et al. 1995; Kaartinen et al. 1995). Because receptors for these growth factors are located on epithelial cells, it is likely that epithelial-mesenchymal interactions play a vital role in regulation of epithelial cell differentiation. Foxl 1, a winged helix/forkhead transcription factor, is expressed in the gastrointestinal mesoderm. Foxl 1 knockout mice have hyperproliferative crypts and elongated villi due to increased epithelial cell proliferation during embryogenesis (Kaestner et al. 1997). These data provide evidence that epithelial cell proliferation is regulated by mesenchymal factors.

Other signaling molecules have roles in epithelial differentiation. For example, Math-1, a component of the Notch signaling pathway, has been shown to regulate the differentiation of goblet, Paneth, and enteroendocrine cells (van Den Brink et al. 2001; Yang et al. 2001). The ability of Notch signaling to direct intestinal epithelial cell fate of progenitor cells into specific cell lineages underscores the critical influential nature of signals from the stem cells' microenvironment.

Thus, these studies support a model that within each stem cell niche a balance between proliferation and differentiation is maintained because signals that affect the intestinal stem cell, from secreted factors, mesenchymal-epithelial cross-talk, and signaling pathways, are tightly controlled to maintain normal crypt homeostasis. Although the direct relationship between these environmental signals and the epithelial stem cell is not well understood, numerous studies have identified that the Wnt/β-catenin signaling pathway plays a critical regulating role in intestinal development, normal proliferation and differentiation in adulthood, and in disease.

The Wnt/β-catenin Signaling Pathway

The Pathway

Wnt genes are a large family of secreted signaling proteins important during embryonic development (Cadigan and Nusse 1997; Moon et al. 1997). During signaling, an extracellular Wnt signal is transduced through the cooperation of two receptors, Frizzled (Fz) and the Low-density lipoprotein receptor-related protein (Bhanot et al. 1996; Tamai et al. 2000; Mao et al. 2001), to activate the intracellular protein Disheveled (see Figure 2). Then, through an undefined mechanism, Dishevelled inhibits Glycogen synthase kinase-3β (Gsk-3β) phosphorylation of Adenomatous polyposis coli (Apc)/Axin/β-catenin complexes (Rubinfeld et al. 1996; Yost et al. 1996; Hart et al. 1998; Itoh et al. 1998; Kishida et al. 1999; Smalley et al. 1999; Yamamoto et al. 1999). Unphosphorylated β-catenin escapes ubiquitin-mediated degradation (Aberle et al. 1997;

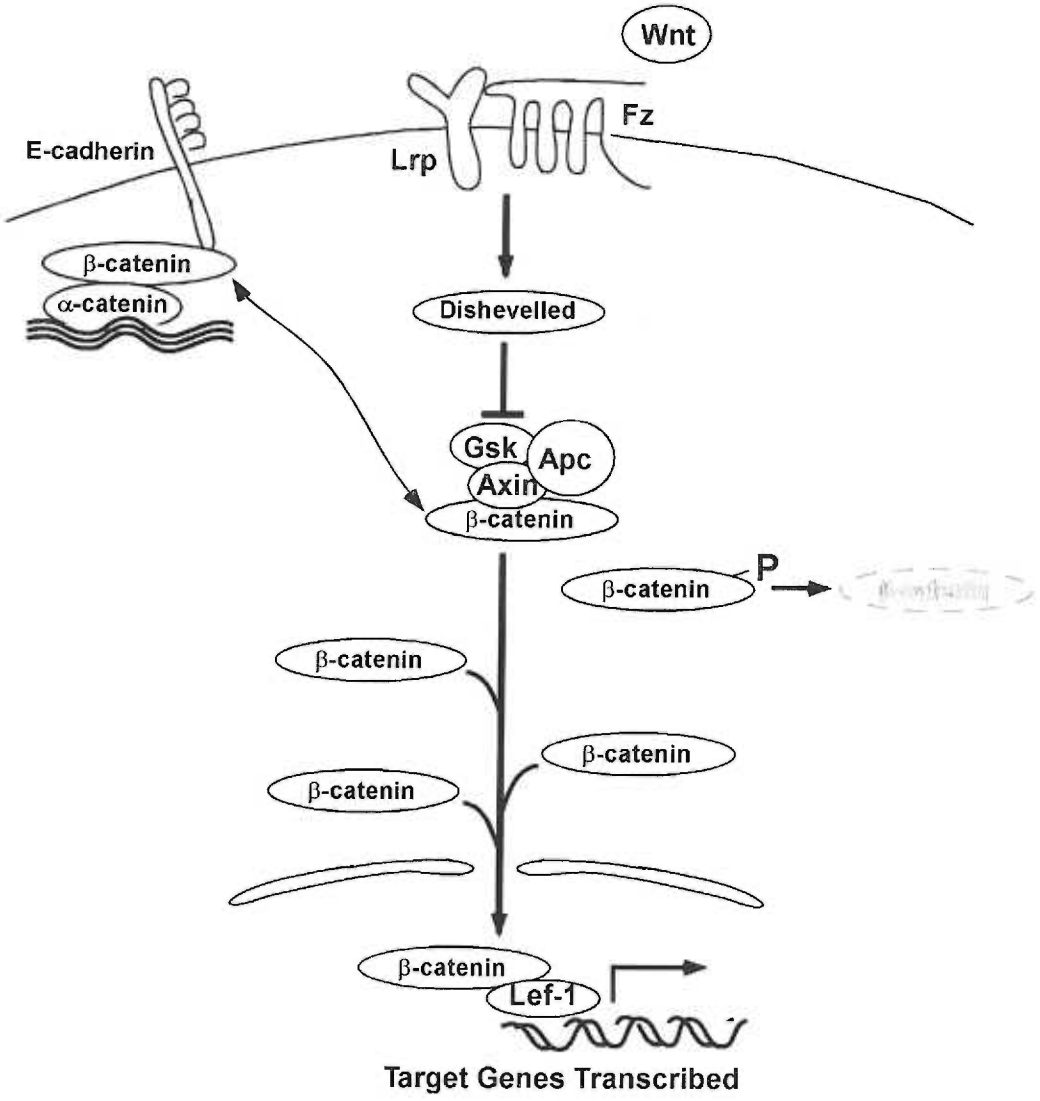


Figure 2. The Wnt/β-catenin Signaling Pathway. Upon binding of Wnt ligand to Frizzled (Fz) and Low-density lipoprotein receptor-related protein (Lrp) receptors, Dishevelled is activated, leading to inhibition of Glycogen synthase kinase-3β (Gsk) by an unknown mechanism. Gsk fails to phosphorylate Adenomatous polyposis coli (Apc)/Axin/β-catenin complexes. Unphosphorylated β-catenin is no longer targeted to the ubiquitin-mediated degradation pathway, but accumulates in the cytoplasm and translocates to the nucleus, where it interacts with transcription factors like Lef-1 to transactivate target gene expression. Lines ending with arrows or bars indicate activating or inhibitory effects, respectively. β-catenin also interacts with α-catenin and the cytoplasmic domain of E-cadherin, linking cell-cell adherens junctions to the cytoskeleton, to mediate cellular adhesion.

Marikawa and Elinson 1998) and translocates to the nucleus, where it can interact with high mobility group (HMG) box transcription factors like Lymphoid enhancer factor-1 (Lef-1) and T-cell factor (Tcf)-1, -3, and -4 to transactivate expression of specific target genes (Behrens et al. 1996; Huber et al. 1996; Molenaar et al. 1996; Hart et al. 1999). Several target genes have been identified, including Peroxisome proliferator-activated receptor δ (Pparδ), Matrilysin (Mmp-7), c-Myc, Tcf-1, CyclinD1, and Cdx-1, (He et al. 1998; Brabletz et al. 1999; Crawford et al. 1999; He et al. 1999; Roose et al. 1999; Shtutman et al. 1999; Lickert et al. 2000).

In Development

Wnt/β-catenin signaling controls numerous processes during embryonic development, including tissue patterning, cell fate specification, and cell proliferation (McCrea et al. 1993; Heasman et al. 1994; Parr and McMahon 1994; Funayama et al. 1995; Cox et al. 1996; Molenaar et al. 1996; Wodarz and Nusse 1998). However, little is known about the role of this pathway during intestinal development. Wnt genes involved in canonical pathway signaling are expressed along the anterior-posterior axis of the digestive tract throughout morphogenesis and differentiation stages of chick embryonic development (Theodosiou and Tabin 2003). The expression of various Wnt/β-catenin signaling molecules during different stages of chick development suggests roles for Wnt/β-catenin signaling throughout patterning and formation of the gastrointestinal tract (Theodosiou and Tabin 2003). Specifically, Fz receptors and the downstream transcription factors Lef-1 and Tcf-4 are expressed during patterning in early

development of the duodenum and large intestine, likely defining morphological boundaries along the anterior-posterior axis of the gastrointestinal tract. Additionally, during morphogenesis, Wnt ligands are expressed in the duodenum endoderm, while their Fz receptors are expressed in adjacent mesoderm, suggesting cross-talk between the endoderm and mesoderm. Wnt/β-catenin signaling is required for the formation of normal crypt-villus units through regulation of proliferation, cell positioning, and differentiation in the small intestinal epithelium, as inferred by analysis of adult transgenic mice expressing the Wnt-inhibitor Dickkopf1 (Dkk1) in the crypt (Pinto et al. 2003), and adult mice deficient for the Wnt/β-catenin target gene EphB3, a receptor tyrosine kinase important in mediating cell mobility (Batlle et al. 2002). Further, the Wnt/β-catenin signaling pathway, through the downstream transcription factor Tcf-4, maintains normal proliferation of the stem cell in the crypts of the small intestine in mice (Korinek et al. 1998a; van de Wetering et al. 2002).

In Disease

Adenomatous polyposis coli

In addition to its role in normal embryonic development, the Wnt/β-catenin pathway plays an important role in colorectal carcinogenesis. Four regulatory genes in this pathway known to be involved in human cancers include Apc, Axin, β-catenin, and Tcf. APC was first identified as a tumor suppressor gene in patients with a form of hereditary colon cancer, Familial Adenomatous Polyposis (FAP, Groden et al. 1991; Kinzler et al. 1991). Patients with FAP have multiple benign adenomatous polyps in the

colon in early adulthood. Invariably, further genetic changes in genes like p53 and K-ras or that result in the loss of responsiveness to Tgf-β (Kinzler and Vogelstein 1996; Takaku et al. 1998) effect the progression from adenoma to invasive and metastatic carcinoma. Both alleles of APC are inactivated in FAP and in most sporadic forms of colorectal cancer (Powell et al. 1992; Nagase and Nakamura 1993; Oshima et al. 1995; Kinzler and Vogelstein 1996). Subsequent studies established that Apc binds β-catenin (Rubinfeld et al. 1993; Su et al. 1993), ultimately linking it to the Wnt signaling pathway (Munemitsu et al. 1995). Mutations in Apc typically result in a protein truncated at its C-terminus, eliminating most of the β-catenin binding sites and all of the Axin binding sites (Rubinfeld et al. 1997; van Es et al. 2001). These mutations prevent Gsk-3β phosphorylation of β-catenin within the multiprotein complex and, consequently, β-catenin is not degraded. The resulting accumulation of β-catenin and its subsequent translocation to the nucleus result in constitutive β-catenin target gene activation, leading to increased intestinal epithelial proliferation, polyp formation, and eventually the development of colorectal cancer (Willert and Nusse 1998; Polakis 1999; Smith et al. 1999; Bienz and Clevers 2000). Interestingly, reintroduction of wildtype APC into colon carcinoma cells with mutant APC can downregulate elevated cytoplasmic pools of β-catenin and suppress cellular growth (Munemitsu et al. 1995; Shih et al. 2000). More recently, Apc has been shown to regulate embryonic stem (ES) cell differentiation by controlling the dosage of β-catenin signaling in the cell (Kielman et al. 2002). Various mutations of Apc resulting in increasing levels of intracellular β-catenin elicit a gradient of ES cell differentiation inhibition. The loss of Apc function to downregulate β-catenin results in a "differentiation defect," suggesting a direct role for β-catenin signaling in

inhibiting differentiation of intestinal stem cells and thus a mechanism for enlargement of the stem cell niche and ultimately polyp formation. Therefore, the primary tumor suppressor function of Apc is to downregulate β-catenin levels.

However, Apc is implicated in cellular functions independent of transcriptional activation by Wnt/β-catenin signaling, including migration, adhesion, and chromosomal instability (Nathke et al. 1996; Yu et al. 1999; Kawasaki et al. 2000; Tirnauer and Bierer 2000; Townsley and Bienz 2000; Fodde et al. 2001a; Kaplan et al. 2001). Apc binds Asef, a Rac-specific guanine nucleotide exchange factor, and is thought to regulate the actin cytoskeleton by inducing migratory activity (Kawasaki et al. 2000). Apc has been shown to associate with adherens junctions of epithelial cells and binds β-catenin, a principle component of adherens junctions. Mutations that cause disassociation of Apc from adherens junctions also cause partial detachment of β-catenin from adherens junctions and disrupt cadherin-mediated cell adhesion (Yu et al. 1999; Townsley and Bienz 2000). Apc also binds the kinetochore proteins Bub1 and EB1 and mediates kinetochore-microtubule attachment, suggesting that mutations in Apc eliminating microtubule binding may contribute to defects in chromosome segregation and spindle formation, and result in aneuploidy (Tirnauer and Bierer 2000; Fodde et al. 2001a). These additional functions of Apc may also contribute to the progression of polyp formation.

One current animal model for FAP is the multiple intestinal neoplasia (Min) mouse (Moser et al. 1990; Su et al. 1992; Levy et al. 1994). Like FAP, the Min mouse harbors an inherited phenotype arising from a germ line mutation in the Apc gene. These heterozygous mice undergo inactivation of the second Apc allele and develop intestinal adenomas at a high frequency throughout their intestinal tract. However, the distribution

of polyps is primarily along the small intestine rather than the colon. Further, these mice do not develop cancer but die from intestinal obstruction. Similar mouse lines carrying Apc mutations have been established to study the effects of Apc loss. Fodde *et al.* introduced a specific chain-termination mutation in the 15th exon of the Apc gene into the mouse germ line, creating the Apc1638 mouse. Mice heterozygous for this Apc gene modification progressively develop intestinal tumors in a manner similar to that observed in Min mice (Fodde et al. 1994). Taketo and colleagues generated $Apc^{\Delta 716}$ knockout mice containing a mutant gene that encodes Apc truncated at codon 716 (Oshima et al. 1995; Oshima et al. 1997). These mice undergo inactivation of the remaining Apc allele by loss of heterozygosity and demonstrate an increase in the size of the proliferating compartment, in contrast to an increase in the rate of proliferation, resulting in the formation of intestinal polyps. Importantly, it is difficult to study the initial events in tumorigenesis with these models because there is no temporal control over inactivation of the second allele in these mice. To circumvent the early embryonic lethality of the homozygous Apc mutation, Noda *et al.* created an inducible Apc^{508S} mutant mouse by conditional targeting of the Apc gene. Using the Cre/loxP recombination system and infection of colorectal epithelial cells of mutant allele homozygotes with an adenovirus encoding the Cre recombinase, they were able to inactivate both alleles of Apc in adults and observe the rapid development of colorectal adenomas (Shibata et al. 1997). Sansom *et al.* showed that in $Cre^{+}Apc^{fl/fl}$ mice which are homozygous for the inducible Apc^{508S} mutation and heterozygous for inducible Cre expressed in the intestine, loss of Apc alters patterns of cellular proliferation, differentiation and migration (Sansom et al. 2004). Moreover, β-catenin localizes to the nuclei three days following induction of Cre

recombinase, coincident with the onset of observed cellular changes. However, the inducible Cre system used results in virtually 100% intestinal recombination and the $Cre^{+}Apc^{fl/fl}$ mice become sick in five days before any polyps form. These mice were likely unable to sustain normal intestinal function due to failure of the villi epithelium to differentiate into functional absorptive cells. Though the inducible Apc mutant mouse model offers temporal control over activation of the Wnt/β-catenin signaling pathway, it is neither commercially nor academically available. Therefore, there is currently no mutant Apc mouse model available to evaluate the early events in tumorigenesis.

Axin

Axin, and its close homolog Conductin, which exhibits all of the binding and regulatory functions of Axin (Behrens et al. 1998), were originally identified as inhibitors of Wnt signaling (Zeng et al. 1997). Both Axin and Conductin interact directly with β-catenin, Apc, and Gsk-3β and function to downregulate β-catenin by promoting its Gsk-3β-phosphorylation (Behrens et al. 1998; Hart et al. 1998; Ikeda et al. 1998; Kishida et al. 1998; Nakamura et al. 1998; Willert et al. 1999). Rare mutations in Axin/Conductin have been found in DNA-mismatch-repair defective colorectal cancer with intact APC by activating β-catenin/Tcf-4 signaling (Liu et al. 2000).

β-catenin

Another key regulator in the Wnt/β-catenin pathway known to be mutated in colorectal cancer is β-catenin. Originally identified as a protein that interacts with the cytoplasmic domain of E-cadherin, a component of epithelial cell-cell adherens junctions,

β-catenin links E-cadherin to the actin cytoskeleton by interacting with α-catenin to mediate cellular adhesion (Nagafuchi and Takeichi 1989; Ozawa et al. 1989; Cox et al. 1996; Orsulic and Peifer 1996; Ben-Ze'ev and Geiger 1998). Subsequent studies revealed that β-catenin is also a critical downstream mediator of the Wnt signaling pathway. The N-terminal region of β-catenin contains phosphorylation sites important for targeted degradation (see Figure 3, Yost et al. 1996; Aberle et al. 1997; Cadigan and Nusse 1997). The central region contains several copies of 42 amino acid long tandemly repeated sequence motifs, known as Armadillo repeats, which mediate binding of β-catenin to other proteins like Apc, Axin, E-cadherin, and Tcf (Hulsken et al. 1994; Behrens et al. 1998; Graham et al. 2000; Huber and Weis 2001). The C-terminal region harbors a potent transactivation domain (van de Wetering et al. 1997; Hsu et al. 1998; Hecht et al. 1999). This domain binds transcriptional coactivators like Creb binding protein (Cbp, Hecht et al. 2000; Takemaru and Moon 2000), as well as the chromatin remodeling complex component, Brg-1 (Barker et al. 2001), promoting target gene activation. Like Apc, the homozygous null mutation of β-catenin results in early embryonic lethality (Haegel et al. 1995; Huelsken et al. 2000). However, several studies explore a role for β-catenin in oncogenic transformation. Among the sporadic forms of human colorectal cancer that retain wildtype Apc, mutations are found in the N-terminal phosphorylation sites of β-catenin, rendering β-catenin refractory to degradation and thus augmenting cytoplasmic and nuclear pools of β-catenin (Ilyas et al. 1997; Morin et al. 1997; Sparks et al. 1998; Miyaki et al. 1999). An intestine-specific β-catenin N-terminal deletion mutation in mice, which removes Gsk-3β phosphorylation sites, was shown to produce adenomatous polyps in the intestine and microadenomas in the colon, resembling those in $Apc^{\Delta 716}$ mice

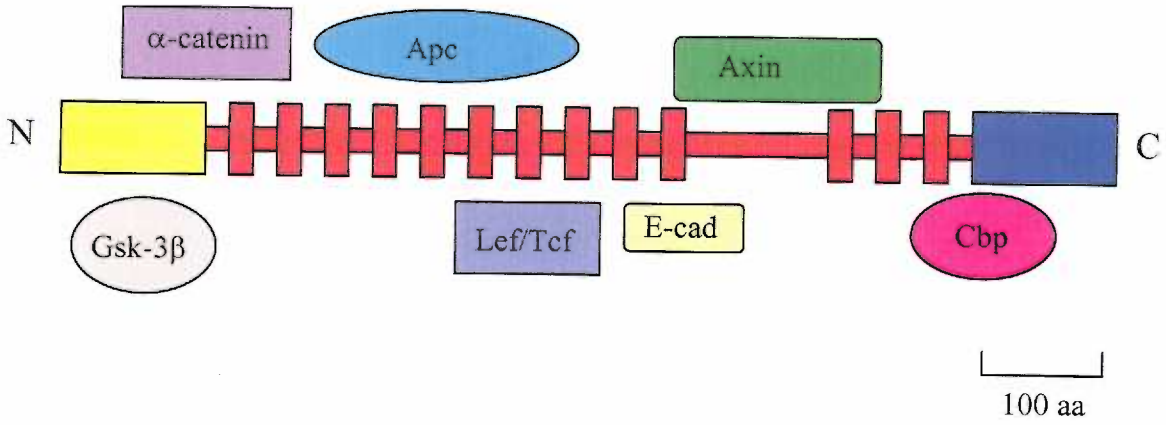


Figure 3. Schematic Representation of the β-catenin Protein and Its Binding Partners. Yellow indicates the N-terminal region of approximately 130 amino acids containing the Glycogen synthase kinase-3β (Gsk-3β) phosphorylation sites important for degradation targeting. This region of β-catenin interacts with Gsk-3β and α-catenin. Red indicates the central region of approximately 550 amino acids containing the Armadillo repeats important for protein binding. This region of β-catenin interacts with Adenomatous polyposis coli (Apc), Axin, E-cadherin and Lymphoid enhancer factor (Lef)/T cell factor (Tcf). Blue indicates the C-terminal region of approximately 110 amino acids containing the potent transactivation domain. This region of β-catenin interacts with Creb binding protein (Cbp). (Adapted from Oving 2002 and the Wnt homepage http;//www.Stanford.edu/rnusee/wntwindow.html)

(Harada et al. 1999). Moreover, an inducible β-catenin knockout mouse, which undergoes deletion of β-catenin by Cre-mediated recombination in response to administration of β-napthoflavone, demonstrates crypt ablation, increased apoptosis, decreased goblet cell differentiation, and deficient enterocyte-matrix attachment upon loss of β-catenin (Ireland et al. 2004).

Target genes of β-catenin transactivation have been implicated as having key roles in oncogenesis. He *et al.* identified c-MYC as a β-catenin target gene, showing it to be repressed by wildtype APC and activated by β-catenin through Tcf-4 binding sites in the c-Myc promoter (He et al. 1998). This study explains previous reports that c-MYC is overexpressed in colorectal cancers, despite the lack of any genetic modifications in c-MYC itself (Sikora et al. 1987; Imaseki et al. 1989; Smith et al. 1993). β-catenin was shown to activate transcription of CyclinD1 (Tetsu and McCormick 1999), also upregulated in colorectal cancers (Bartkova et al. 1994; Arber et al. 1996). PPARδ was identified as an APC-regulated target of the Wnt signaling pathway and its expression is likewise elevated in colorectal cancer cells (He et al. 1999). This study demonstrated that repression by APC was mediated by β-catenin/Tcf-4 responsive elements in the PPARδ promoter. Mmp7 is expressed in colonic adenocarcinomas as well as in intestinal adenomas, indicating a role in the early stages of colorectal cancer (Newell et al. 1994; Takeuchi et al. 1997; Fingleton et al. 1999). The Mmp-7 promoter contains a single optimal Tcf-4 binding site (Korinek et al. 1997). In overexpression studies, Mmp-7 was upregulated by β-catenin, identifying it as a target gene for β-catenin/Tcf-4 transactivation (Crawford et al. 1999). Importantly, genetic ablation of Mmp-7 in Min mice reduces adenoma formation by 60% (Wilson et al. 1997), suggesting that regulation

of Mmp-7 by β-catenin contributes to intestinal tumor establishment and growth. Each of these genes is important in cell cycle progression, transcriptional regulation or tissue remodeling, normal cellular functions that become dysregulated in cancer.

Lymphoid Enhancer Factor/T Cell Factor

The downstream effectors of the Wnt/β-catenin signaling pathway are the Lef/Tcf transcription factors (Behrens et al. 1996; Huber et al. 1996; Molenaar et al. 1996; Brunner et al. 1997; Cadigan and Nusse 1997; Clevers and van de Wetering 1997; van de Wetering et al. 1997). The Lef/Tcf family of HMG box transcription factors includes Lef-1, Tcf-1, Tcf-3, and Tcf-4. Tcf-4 is expressed in the developing and adult intestinal epithelium (Korinek et al. 1998b; Barker et al. 1999; Brittan and Wright 2002). Lef/Tcf transcription factors bind directly to DNA through their HMG domains, but are incapable of activating gene transcription alone (Eastman and Grosschedl 1999; Roose and Clevers 1999). In the absence of signaling, Lef/Tcf transcription factors repress transcription of Wnt/β-catenin target genes by interaction with transcription corepressors like Groucho or with Cbp (Cavallo et al. 1998; Roose et al. 1998; Waltzer and Bienz 1998). However, signaling through this pathway results in the formation of nuclear Lef/Tcf/β-catenin complexes (Korinek et al. 1997; Morin et al. 1997). In these complexes, β-catenin supplies the transactivation domain required for transcriptional activation (Molenaar et al. 1996; van de Wetering et al. 1997).

As previously described, mutations in Apc or β-catenin induce constitutive formation of nuclear β-catenin/Tcf complexes, resulting in activated transcription of target genes. Thus, Tcf-4 can mediate hyperproliferation of intestinal epithelial cells.

Because Tcf-4 is required for maintenance of the proliferative stem cell compartment (Korinek et al. 1998a), it has been proposed that constitutive activation of Tcf-4 by accumulated β-catenin leads to retention of cells in the crypt that would normally undergo differentiation and migration out of the crypt. The result of these cells remaining in the crypt is an abnormal increase in size of the crypt compartment, leading to formation of a polyp (Oshima et al. 1997; Korinek et al. 1998a).

An additional mechanism by which Tcf transcription factors may contribute to cancer is through its activation of Tcf-1, a target gene in epithelial cells. Mice homozygous for mutations in Tcf-1 develop intestinal adenomas (Roose et al. 1999). The most abundantly expressed isoforms of Tcf-1 lack a β-catenin binding site (Castrop et al. 1995; Van de Wetering et al. 1996) but retain their Groucho binding site. Further, the HMG box DNA binding domains of the four Lef/Tcf proteins are essentially identical (Cadigan and Nusse 1997; Clevers and van de Wetering 1997), suggesting that they may regulate the same target genes. Therefore, Tcf-1 may act as a feedback repressor of β-catenin/Tcf target genes (Roose et al. 1999). Crossing Tcf-$1^{-/-}$ mice with Min mice heterozygous for mutant Apc markedly enhanced the number and size of intestinal polyps. This study indicates that Tcf-4 activation of Tcf-1 maintains the tumor suppressor activity of Tcf-1 in intestinal epithelial cells.

Thus, the key regulators in the Wnt signaling pathway, Apc, Axin, and β-catenin, are mutated in colorectal cancer and all of these mutations result in the accumulation of β-catenin by preventing its degradation in the absence of a Wnt signal. Through Tcf-4, these mutations lead to constitutive activation of target genes involved in cellular proliferation and morphology.

Regulation of the Intestinal Stem Cell

While the Wnt/β-catenin signaling pathway and its downstream molecules are strongly implicated in polyp formation, its role in regulating the activity and fate of the intestinal stem cell has been only initially explored. Studies by Korinek *et al.* demonstrated that mice with targeted homozygous deletion of Tcf-4 had no proliferating cells within their small intestinal IVRs and die shortly after birth (Korinek et al. 1998a). The neonatal epithelium of these mice was composed entirely of differentiated, non-dividing cells, indicatating that Tcf-4 is necessary for establishing and maintaining the proliferative stem cell compartment in the IVRs of the small intestine. The authors propose that the mesenchymal cells underlying the crypts secrete a Wnt signal to activate Tcf-4. The loss of the "stem cell phenotype" of the nascent crypts, defined by characteristics like cell cycling and longevity, suggests that the developing intestinal stem cell requires Wnt/β-catenin signaling for maintenance of its niche. Other reports confirm this differentiated phenotype in the IVRs of Tcf-$4^{-/-}$ developing mice (van de Wetering et al. 2002) and in Dkk1 transgenic adult mice (Pinto et al. 2003). Wong and colleagues showed that high levels of β-catenin signaling in the intestine influence stem cell selection during crypt morphogenesis (Wong et al. 2002). In this study, chimeric mice mosaically express a transgenic protein designed to constitutively stimulate β-catenin signaling in the intestinal epithelium. These mice undergo induction of an apoptotic response specifically in the transgenic cells during intestinal development. This apoptotic response resulted in a complete loss of all transgene-expressing stem cells and their progeny by completion of crypt formation, P14. The failure of those stem cells to become

established indicates that overexpression of β-catenin during development is detrimental to intestinal stem cell survival. However, in the adult, activated β-catenin signaling in the intestine results in unregulated proliferation and ultimately polyp formation (Kinzler and Vogelstein 1996; Polakis 2000). More research is needed to understand Wnt/β-catenin's signaling role in intestinal stem cell regulation.

Reagents to Study the Effects of Activated β-catenin Signaling

The Rationale

Colorectal cancer is the second leading cause of all cancer deaths in the United States. Because mutations causing disease of the intestinal epithelium most likely occur in the stem cell for propagation of the disease, it is ultimately the intestinal stem cell that gives rise to intestinal carcinomas (Wright 2000; Brittan and Wright 2002). Although at present there are no known markers for intestinal stem cells (Potten 1998; Booth and Potten 2000; Brittan and Wright 2002), they can be evaluated functionally by their ability to populate crypts and villi (Fuller et al. 1990; Park et al. 1995; Novelli et al. 1996). Numerous studies show clearly that Wnt/β-catenin signaling influences the intestinal stem cell's proliferative potential. However, many other aspects of intestinal stem cell regulation remain to be elucidated. During development, the shape of the intestinal stem cell niche undergoes morphogenesis, a single stem cell is selected, and the gradient of proliferation to differentiation is established. Throughout adulthood, the gradient of proliferation to differentiation is maintained, differentiated cells migrate up and out of the

crypt or to the base of the crypt, and the intestinal stem cells remain anchored to the mid-crypt region. Regulation of the intestinal stem cell and the cells in its niche during development and throughout adulthood is complex and likely involves a coordinated series of cues controlled by Wnt/β-catenin signaling.

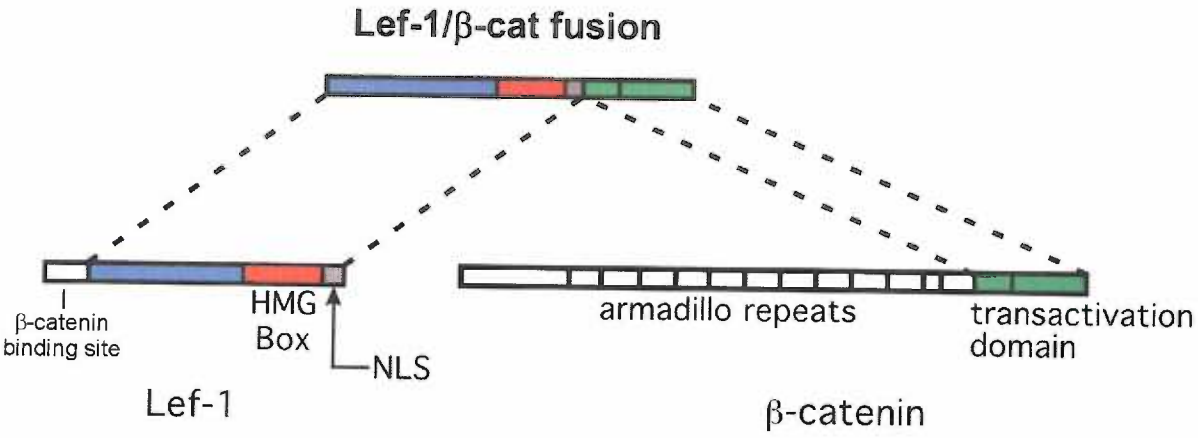
In order to gain insight into how the intestinal stem cell is regulated, future studies will focus on identifying and characterizing the immediate molecular and cellular response of the intestinal stem cell to activated β-catenin signaling in both adult and developing intestinal epithelium. Similarly, to prevent polyp formation in adults, the early changes in gene expression upon activated β-catenin signaling must be identified. Such studies will require animal models that provide temporal control over the activation of β-catenin signaling.

The Approach

In this thesis, I report the generation of multiple ES cell lines that will be used in the generation of two mouse reagents that will provide temporal control of β-catenin signaling; a novel inducible Lef-1/β-cat transgenic mouse, and a novel inducible Lef-1/β-cat knockin ROSA mouse. These mice will harbor a fusion molecule made from the required signaling domains of Lef-1 and β-catenin (Lef-1/β-cat) that can be induced specifically in the intestine to activate Wnt/β-catenin signaling. Activation of β-catenin target gene expression requires both binding of β-catenin to a Lef-1/Tcf transcription factor and binding of Lef-1/Tcf to DNA. The Lef-1/β-cat fusion molecule is composed of the DNA binding domain of Lef-1, residues 33-368 of human Lef-1 encompassing a

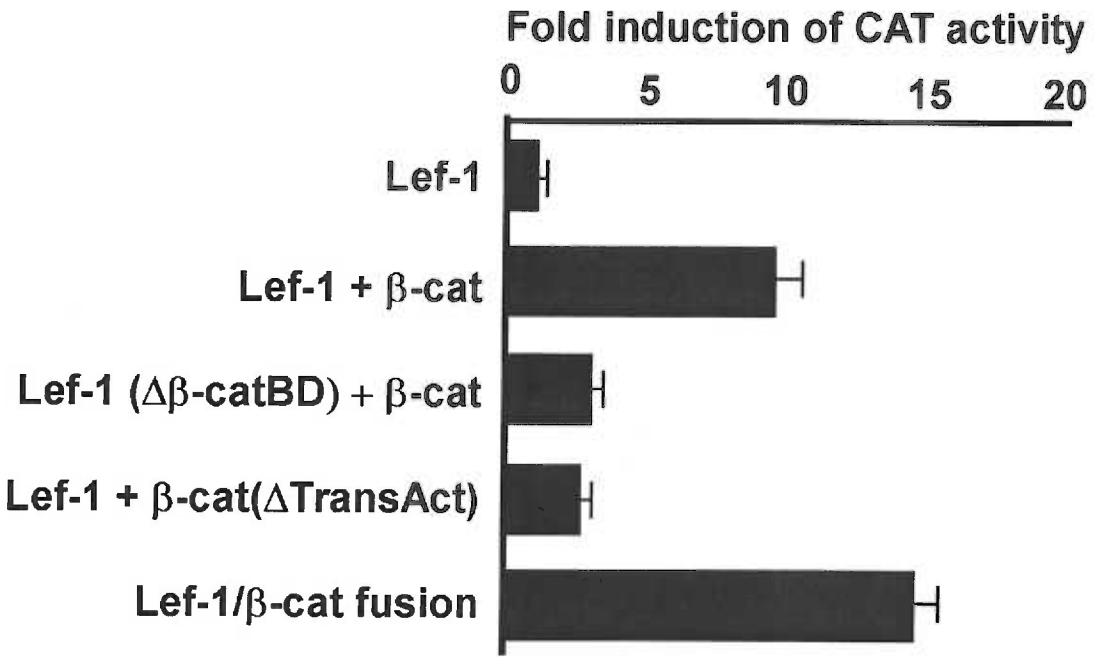
functional HMG box and nuclear localization signal, coupled to the last Armadillo repeat and C-terminal transactivation domain of human β-catenin (see Figure 4). Fusion of these two domains obviates the need for the β-catenin binding site of Lef-1. Also missing from this molecule are the N-terminal region of β-catenin, which contains the phosphorylation sites important for its targeted degradation, and the Armadillo repeats, which mediate the binding of β-catenin to other proteins like Apc, Axin, and E-cadherin. Lacking these two regions allows the fusion protein to escape being targeted for degradation or recruited to adherens junctions, allowing for separation of β-catenin's signaling and adhesive functions. Thus, the Lef-1/β-cat protein translocates directly to the nucleus and activates target gene expression.

In vitro studies have demonstrated the effective signaling capacity of Lef-1/β-cat in cultured mouse IIAI.6 B cells cotransfected with a reporter plasmid for Wnt/β-catenin target gene activation (see Figure 5, Wong et al. 2002). This cell line lacks endogenous Lef-1 mRNA and contains very low levels of β-catenin. IIAI.6 cells were transfected with a reporter plasmid containing seven tandem repeats of the consensus Lef-1/Tcf DNA binding site (CTTTGTT) upstream of a chloramphenicol transferase (CAT) gene along with plasmids encoding wildtype Lef-1, wildtype β-catenin, Lef-1/β-cat, a mutant Lef-1 incapable of binding to β-catenin, or a mutant β-catenin lacking its transactivation domain. Expression of Lef-1/β-cat stimulated induction of CAT activity above that observed in cells transfected with wildtype Lef-1 and β-catenin, confirming that the fusion protein is capable of transactivating transcription of target genes. Lef-1/β-cat is also capable of transactivating target genes *in vivo*, as demonstrated by a 4-fold induction



Wong et al. (2002)

Figure 4. The Lef-1/β-cat Fusion Molecule. The Lef-1/β-cat fusion molecule is composed of the DNA binding domain of Lef-1 with its nuclear localization signal (NLS) coupled to the transactivation domain of β-catenin. Excluded from the fusion molecule are the N-terminal phosphorylation sites and armadillo repeats of β-catenin, and the β-catenin binding site of Lef-1.



Wong et al. (2002)

Figure 5. ***In Vitro*** **Transactivation Potential of Lef-1/β-cat.** Cultured mouse IIAI.6 B cells were cotransfected with a Wnt/β-catenin reporter plasmid containing seven tandem repeats of the Lef-1 DNA binding site (CTTTGTT) linked to a thymidine kinase promoter and a bacterial chloramphenicol transferase (CAT) gene, and one of the following plasmids encoding: (*i*) wildtype Lef-1, (*ii*) wildtype Lef-1 and β-catenin, (*iii*) wildtype β-catenin and mutant Lef-1 lacking a β-catenin binding domain, (*iv*) wildtype Lef-1 and mutant β-catenin lacking a transactivation domain, or (*v*) Lef-1/β-cat. Transactivation potential was measured as CAT activity relative to cells containing the Lef-1 and reporter plasmid.

of the known β-catenin target gene, E-cadherin, in a real time quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) study of E18.5 small intestines from mice expressing Lef-1/β-cat under the control of an intestine specific promoter (Wong et al. 2002). In both of the novel inducible Lef-1/β-cat mice, Lef-1/β-cat fusion molecule sequence will be downstream of marker genes flanked by loxP sites.

Expression of Lef-1/β-cat will be intestine specific and under temporal control of doxycycline-induced Cre recombinase by mating the Lef-1/β-cat transgenic mice and the Lef-1/β-cat knockin ROSA mice into an inducible, intestine-specific Cre mouse system (Saam and Gordon 1999; Wong et al. 2000). This system combines Cre/loxP technology with reverse tetracycline-controlled transactivator (rtTA) responsive regulatable gene expression to induce Cre recombinase expression in the intestinal epithelium upon administration of doxycycline (see Figure 6, Abremski and Hoess 1984; Gossen and Bujard 1992; Gossen et al. 1995). rtTA is a fusion protein consisting of the DNA binding domain of a mutant *E. coli Tn*10 tetracycline resistance operon repressor linked to the acidic activating domain of herpes simplex virus VP16. When the rtTA binds tetracycline analogs, such as doxycycline, it can bind to *tet* operator sequences (*tet*O) from the *E. coli tet* operon and activate transcription of a gene under the control of *tet*O linked to a minimal promoter (Gossen et al. 1995), such as the human cytomegalovirus immediate early gene 1 promoter (P_{hCMV}). In the inducible, intestine-specific Cre system, the rtTA gene is under the control of modified transcriptional regulatory elements derived from a rat fatty acid-binding protein gene (*Fabpl* $^{4X\ at\ -132}$) that drive gene expression in the multipotent intestinal stem cell of epithelium in the small intestine, cecum, and colon of adult mice (*Fabpl* $^{4X\ at\ -132}$-rtTA) (Simon et al. 1997; Saam and Gordon 1999), and

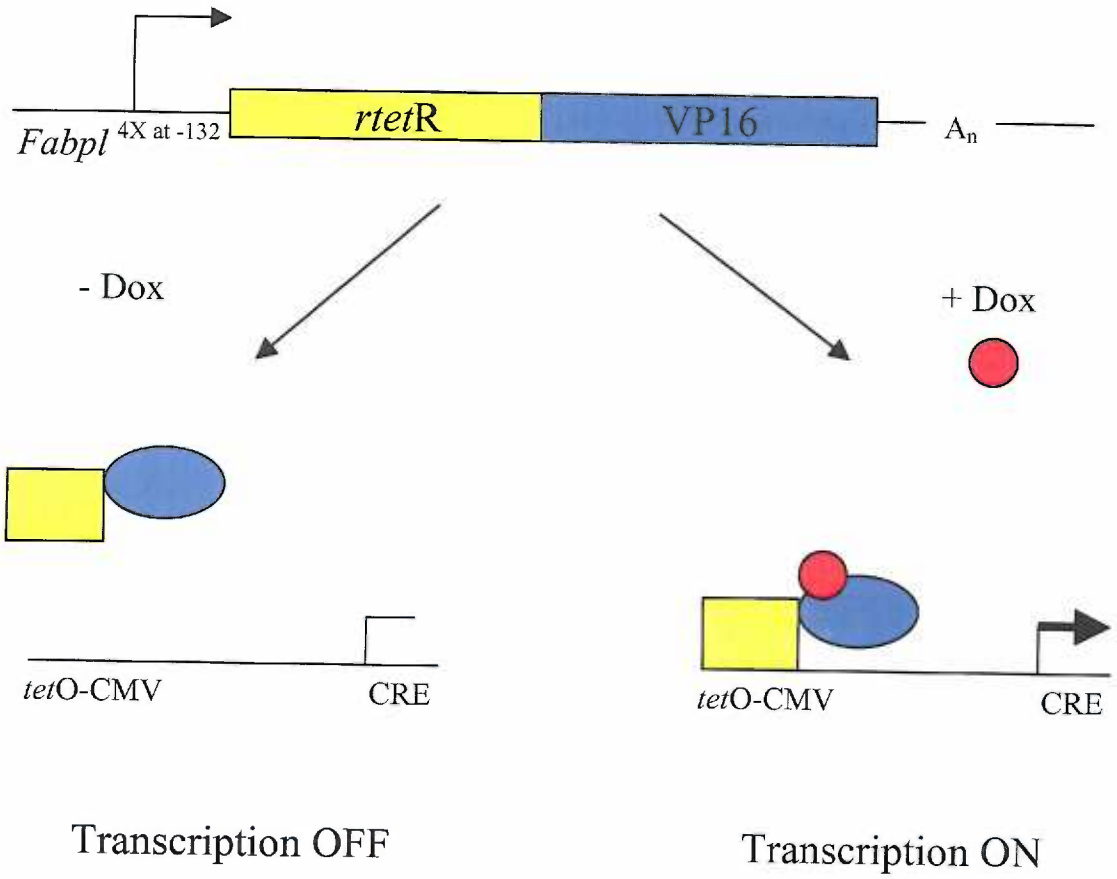


Figure 6. Reverse Tetracycline Transactivation in the Inducible, Intestine-specific Cre Mouse System. Transcription from the modified rat liver fatty acid binding protein promoter (*Fabpl* $^{4X \text{ at } -132}$) results in a transcript encoding the reverse tetracycline transactivator (rtTA) fusion protein, which consists of the reverse *tet* repressor (*rtet*R) coupled to the VP16 transactivator (VP16) and is restricted to the intestinal epithelium. In the absence of doxycycline (Dox), rtTA cannot bind to the *tet* operator sequences (*tet*O) and transcription of Cre recombinase (CRE) is not activated. In the presence of Dox, rtTA binds *tet*O and promotes transcription from the cytomegalovirus (CMV) minimal promoter of a transcript encoding CRE only in those cells where *Fabpl* $^{4X \text{ at } -132}$ is active. Thus, CRE will only be expressed in intestinal epithelial cells.

Cre recombinase is under the control of tetracycline operator sequences and a minimal promoter from human cytomegalovirus (*tet*O-P_{hCMV}-Cre) (Saam and Gordon 1999; Wong et al. 2000). The P1 bacteriophage Cre recombinase catalyzes recombination between two 34-bp consensus sequences called loxP sites, oriented as direct repeats, resulting in the excision of intervening DNA sequence (Sternberg and Hamilton 1981; Abremski and Hoess 1984). Therefore, mice with tetracycline regulatable Cre, intestine-specific rtTA, and a floxed marker allele will undergo Cre-mediated recombination restricted to the intestine upon administration of doxycycline. This system has been thoroughly characterized (Saam and Gordon 1999; Wong et al. 2000). In the absence of doxycycline, no recombination is detectable in the intestine of adult tri-transgenic mice containing *Fabpl* $^{4X \text{ at } -132}$-rtTA, *tet*O-P_{hCMV}-Cre, and a floxed reporter gene. Four days after oral administration of doxycycline, mosaic recombination of the reporter is apparent in the epithelium of the small intestine, the cecum, and the colon. Furthermore, the recombined locus persists for at least 60 days after the withdrawal of doxycycline. Because Paneth cells are phagocytosed in ~ 20 days and stem cell descendents are apoptosed or extruded into the lumen at the villus tip in 3-5 days, this data indicates that recombination has taken place in the intestinal stem cell, the only cell that is retained in the crypt for such an extended period of time.

The Reagents

The Lef-1/β-cat transgenic mouse will harbor an inducible Lef-1/β-cat transgene inserted randomly into the mouse genome by electroporation of ES cells. Thus, upon

crossing to the inducible, intestine-specific Cre mouse system, tri-transgenic mice will mosaically express Lef-1/β-cat in the intestine upon administration of doxycycline, with wildtype control tissue juxtaposed induced tissue.

The Lef-1/β-cat knockin ROSA mouse will harbor an inducible Lef-1/β-cat transgene targeted to the ROSA26 locus by homologous recombination. When mated into the inducible, intestine-specific Cre mouse system, these tri-transgenic mice will mosaically express a robust level of Lef-1/β-cat in the intestine upon administration of doxycycline.

These mice will serve as valuable tools for studies of intestinal stem cell regulation, the early molecular and cellular events involved in polyp formation, and the identification of transcriptional targets for future therapeutic and diagnostic approaches to intestinal disease.

MATERIALS AND METHODS

Lef-1/β-cat Transgene Cloning

Plasmids: **p3021** (Wong et al. 2002) encodes a fusion protein consisting of an N-terminal 15-residue c-Myc epitope tag, amino acids 33-368 of human Lef-1, and the C-terminal 124 residues of human β-catenin (Lef-1/β-cat). **pBS:L1/L2loxP2** is the pBluescript II:KS (pBS:KS) vector (Stratagene) modified to contain two loxP sites (5'-ATAACTTCGTATAGCATACATTATACGAAGTTAT-3') flanking an optimal Kozak consensus sequence (5'-GCCACC-3') (Kozak 1987) immediately upstream of the ATG in the *Nco*I site. The multiple cloning site (MCS) was modified to contain a *Bam*HI-*Pst*I-*Nco*I-*Mfe*I-*Bgl*II-*Xba*I linker. **pEGFP** contains coding sequence for enhanced green fluorescent protein (GFP, Cormack et al. 1996) and the SV40 poly(A) tail sequence in a pBS:KS backbone. **pCAhGH** contains the enhanced chicken β-actin promoter (CA, Miyazaki et al. 1989, from the pCALSL plasmid, a kind gift from Dr. Jie Shen, Harvard) upstream of nucleotides +3 to +2152 of the human growth hormone (hGH) gene, and a phosphoglycerate kinase-neomycin resistance selection cassette (PGK-neo, Hermiston et al. 1996).

Generating an inducible Lef-1/β-cat transgene: To create the CA-loxP-GFP-polyA-loxP-Lef-1/β-cat-hGH-PGK-neo construct, a 1581-bp *Nco*I-*Eco*RI fragment from p3021 containing the Lef-1/β-cat sequence was subcloned into *Nco*I-*Mfe*I sites in pBS:L1/L2loxP2, yielding pBS:L1/L2loxP2/3021. A 1035-bp *Eco*RV-*Bam*HI GFP-

polyA tail fragment was excised from pEGFP and then subcloned into *Eco*RV-*Bam*HI sites of pBS:L1/L2loxP2/3021. A 2.7 Kb *Bgl*II fragment containing loxP-GFP-polyA-loxP-Lef-1/β-cat was excised from this plasmid and subcloned into the *Bam*HI site of pCAhGH, yielding pCA3021hGH, which contains the CA-loxP-GFP-polyA-loxP-Lef-1/β-cat-hGH-PGK-neo transgene.

Lef-1/β-cat Transgene In Vitro *Assays*

2.5 X 10^5 COS-7 cells (ATCC) were transfected with 1 μg (i) pCA3021hGH or (ii) pCA3021hGH plus 1 μg pMC-Cre [a kind gift from Dr. John Adleman, OHSU, (Gu et al. 1994); this plasmid uses the thymidine kinase promoter to direct Cre expression] using Lipofectamine (Invitrogen, Luttrell et al. 1995). Cells were evaluated for GFP expression 48-hours after transfection by fluorescence microscopy. Cells were evaluated for β-catenin or c-Myc expression 72-hours after transfection by immunohistochemical analysis using affinity-purified rabbit antibodies to the C-terminal 14 residues of human/mouse β-catenin (1:500, Sigma) or the c-Myc epitope tag (1:500, Upstate Biotechnology). Antigen-antibody complexes were detected with indocarbocyanine (Cy3)-conjugated donkey anti-rabbit secondary antibodies (1:500, Jackson ImmunoResearch Laboratories). This assay was performed in triplicate in three separate experiments. The number of β-catenin positive staining nuclei was scored for 2,050 total cells counted per experiment.

Electroporation of ES Cells

A 10.3 Kb fragment containing the CA-loxP-GFP-polyA-loxP-Lef-1/β-cat-hGH-PGK-neo transgene was excised from pCA3021hGH with *Xba*I, purified by gel electrophoresis and β-agarase digestion, then electroporated into D3 129/Sv ES cells (Potter et al. 1984; Wurst and Joyner 1993). Approximately 5 X 10^7 ES cells were electroporated with 15 μg of linear DNA at 200 V and 960 μF, in a Bio-Rad Gene Pulser. A second round of electroporation using 2 X 10^7 ES cells and 20 μg linear DNA was also performed. Electroporated ES cells were propagated on monolayers of primary mouse embryonic fibroblast cells mitotically inactivated by 6,000 rads of gamma irradiation. ES cells were expanded in Dulbecco's Modified Eagle's Medium high glucose (Gibco) supplemented with 15% defined fetal bovine serum (DFBS; HyClone), 0.1 mM non-essential amino acids (Gibco), 2 mM nucleoside stock (Sigma), 10^{-6} M β-mercaptoethanol (Sigma), 2 mM L-glutamine (Gibco), 1000 U/ml Leukemia Inhibitory Factor (Chemicon), and 50 μg/ml penicillin and streptomycin (Gibco) at 37°C and 5% CO_2. Two days after electroporation, ES cells were grown in 400μg/ml Geneticin (Calbiochem) for transgene selection. After four to seven days, seventy-five single drug-resistant colonies were identified, picked and expanded in 24- and 6-well plates. Cells from each colony were frozen in cryovials using 25% DFBS and 10% dimethyl sulfoxide (Gibco). Cells from each colony were expanded further into 15 60-mm dishes for genomic DNA, RNA, and protein isolation. ES cells were tested for Mycoplasma contamination using the Mycoplasma Detection Kit (ATCC, Tang et al. 2000).

Screening of ES Clones for Presence of Transgene

Sixty-six stably transfected ES cell clones were identified by visualization of GFP fluorescence in live cells using a Leica DMIRE/2 model inverted microscope. Fifty-six of those clones were confirmed to contain the Lef-1/β-cat transgene by polymerase chain reaction (PCR) using genomic DNA isolated according to standard methods (Wurst and Joyner 1993). Primers to hGH (forward, 5'-AGGTGGCCTTTGACACCTACCAGG-3' and reverse, 5'-TCTGTTGTGTTTCCTCCCTGTTGG-3') amplified a 360-bp product, and primers to actin (forward, 5'-CACCACACCTTCTACAATGAGCTG-3' and reverse, 5'-TCATCAGGTAGTCAGTGAGGTCGC-3') amplified a 450-bp product. Cycle conditions were as follows: denaturation, 94°C for 1 min; annealing, 55°C for 1 min; extension, 72°C for 1.5 min, for a total of 30 cycles.

Screening of ES Clones for Single Copy Transgene Insertion by Southern Blot Analysis

Probes: A GFP-specific fragment was generated using primers designed to amplify a 691-bp amplicon in the 3' region of GFP (forward, 5'-GCTACCCCGACCACATGAAG-3' and reverse, 5'-CCCTGAACCTGAAACATAAAATGAA-3') and subcloned into the pCR2.1 TA Cloning Kit (Invitrogen). The GFP fragment was excised from the pCR2.1 TA cloning vector by sequential restriction digestion with *Eco*RI and *Eag*I. A 484-bp fragment containing the 5' region of the neomycin resistance gene was excised with *Spe*I and *Msc*I from the pGT28 plasmid (New England Biosciences; a kind gift from Dr. Richard Maurer, OHSU). Fifty ng of DNA was used to generate [^{32}P]-dCTP labeled

random primed probes using the Ready-To-Go-DNA Labeling Bead Kit (Amersham Biosciences). The GFP probe hybridizes to the 3' end of the GFP gene in the Lef-1/β-cat transgene and yields a >7 Kb band on genomic ES cell genomic DNA carrying a single transgene copy cut with *Sac*I and a >4.8 Kb band cut with *Bam*HI. The neo probe hybridizes to 5' end of the neomycin resistance gene in the Lef-1/β-cat transgene and yields a >5.7 Kb band on genomic ES cell genomic DNA carrying a single transgene copy cut with *Sac*I and a >7.8 Kb band cut with *Bam*HI.

DNA preparation: Genomic DNA was isolated from the fifty-six candidate ES cell clones. Cells from five overgrown 60-mm dishes were lysed (50 mM Tris at pH 8.0, 100 mM NaCl, 10 mM EDTA, 1% SDS, 1mg/ml proteinase K), pooled, transferred to a 15 ml tube, and incubated at 56°C overnight. DNA was extracted using phenol:chloroform, precipitated with ethanol, spooled out on the tip of a Pasteur pipette, and resuspended in water.

Hybridization: Ten μg of genomic DNA was digested with either *Bam*HI or *Sac*I and then resolved by electrophoresis on a 0.8% agarose gel. Gels were denatured in 0.25 M HCl at 25°C for 10 min, then neutralized in 0.4 M NaOH at 25°C for 30 min. DNA was then transferred to positively charged nylon membrane (GeneScreen Plus; Perkin Elmer) following standard procedures (Southern 1975; Ausubel 1993; Sambrook and Russell 2001). Hybridization with the neo-specific probe was carried out for 18 hours at 68°C in 15 ml hybridization buffer (6X SSC, 5X Denhardt's reagent, 0.5% SDS, 100 μg/ml denatured salmon sperm DNA) containing approximately 4 X 10^7 cpm/ml of labeled probe. The hybridized membranes were washed twice with 30 ml buffer I (2X SSC/0.1% SDS) at 25°C for 5 min each, then twice with 30 ml buffer II (0.2X SSC/0.1% SDS) at

25°C for 5 min each, and finally twice with 30 ml buffer III (0.2X SSC/0.1% SDS pre-warmed) at 42°C for 15 min each. Membranes were exposed to film at -80°C for 16-24 hours. Membranes were stripped at 78-98°C for 30 minutes with buffer S (10 mM Tris-HCl at pH 7.5, 1 mM EDTA, 1% SDS) and re-probed with the GFP-specific probe.

Towards Assessing Transgene Expression Level in ES Clones

For determining mRNA expression level:

cDNA synthesis: RNA was isolated from 30 candidate ES cell clones. Cells from five overgrown 60-mm dishes were lysed [600 µl Buffer RLT (Qiagen)/β-mercaptoethanol per 60-mm dish], pooled, transferred to a 1.5 ml RNase-free tube, and homogenized using a 20-gauge needle. Total RNA was then isolated using the RNeasy RNA isolation Kit (Qiagen) and treated with 0.2 U DNaseI (Roche) at 37°C for 15 min to remove contaminating genomic DNA. cDNA was synthesized using oligo(dT) primers and Superscript II Reverse Transcriptase (Invitrogen). Briefly, 1.5 µg of DNase-treated total RNA was incubated with 250 ng oligo(dT) primers in a 10 µl reaction at 70°C for 5 min and then cooled to 42°C. The reverse transcription reaction was carried out with 200 U reverse transcriptase in the presence of 250 mM Tris-HCl at pH 8.3, 375 mM KCl, 15 mM $MgCl_2$, 100 mM DTT, 40 U RNase inhibitor, and 10 mM dNTPs at 42°C for one hour. PCR using actin primers spanning an intron/exon junction and designed to produce a 300-bp amplicon from β-actin cDNA and 450-bp amplicon from β-actin genomic DNA was used to confirm cDNA synthesis and the absence of genomic DNA contamination.

Primer design: Two sets of primers for use with SYBR Green-based qRT-PCR to Lef-1/β-cat were designed using Primer Express Software (Applied Biosystems). To eliminate amplification of endogenous Lef-1 or β-catenin, one primer pair produces a 111-bp amplicon spanning the Lef-1/β-catenin junction and the other primer pair produces a 116-bp amplicon including the Lef-1/β-catenin junction. Primer pairs were tested to establish a single specific amplicon by PCR using genomic DNA, positive control cDNA, and water. Cycle conditions were as follows: denaturation, 94°C for 2 min; annealing, 55°C for 1 min; extension, 72°C for 2 min, for a total of 35 cycles. Melting curves for each set of primers were determined by qRT-PCR on positive control cDNA and water to identify amplicon melting temperatures. Lef-1/β-cat primer pairs were also tested for linear amplification with primers to the endogenous reference gene Glyceraldehyde 3-phosphate dehydrogenase (Gapdh) by qRT-PCR using serial dilutions of positive control cDNA (undiluted, 1:2, 1:4, 1:8) and water. The threshold cycle, C_T, indicates the fractional cycle number at which the amount of amplified target reaches a fixed threshold. Fluorescence data were collected at 0.5°C lower than the identified amplicon melting temperature for each primer pair and plotted as the ratio of target gene C_T to Gapdh C_T against the log of sample cDNA concentration. A slope equal to or less than 0.1 indicated acceptable linear amplification efficiencies. qRT-PCR was carried out in triplicate using SYBR Green and a 7900HT Sequence Detector (Applied Biosystems). Each 25 µl reaction contained 2X SYBR Green master mix (Applied Biosystems), 0.25 units UDP-*N*-glycosidase (Life Technologies), 900 nM forward and reverse primers, and 2-4 µl of sample cDNA (see Table 1).

Primer Name	Primer Sequence	Length	T_M	m
111-bp amplicon spanning Lef-1/β-catenin junction Forward Primer	5'-CGACTTCAGGTACAGGTCCCA-3'	21	81.4°C	0.08
111-bp amplicon spanning Lef-1/β-catenin junction Reverse Primer	5'-TCAGCTCAACTGAAAGCCGTT-3'	21		
116-bp amplicon including Lef-1/β-catenin junction Forward Primer	5'-CAGCTGCCTACATCGGAGATC-3'	21	81.8°C	0.10
116-bp amplicon including Lef-1/β-catenin junction Reverse Primer	5'-AGCCATTGGCTCTGTTCTGAA-3'	21		

Table 1. Primer Pairs for Use with SYBR Green-based qRT-PCR to Assess Lef-1/β-cat Transgene Expression Level in ES Clones. Both primer pairs were designed to Lef-1/β-cat fusion molecule sequence to yield amplicons of ~150 base pairs spanning or including the Lef-1/β-catenin junction. Amplicon melting temperatures (T_M) were determined by melting curve analysis. Linearity slopes (m) equal to or less that 0.1 indicated acceptable linear amplification efficiencies between primers to Lef-1/β-cat and Gapdh.

For determining protein expression level: Protein was isolated from 30 candidate ES cell clones. Cells from five overgrown 6-mm dishes plates were lysed (100 mM $NaPO_4$ at pH7.8, 0.1% NP40), then frozen and thawed five times. Lysates were centrifuged at 13,200 rpm for 2 min and supernatants pooled.

Detection of Wnt/β-catenin Target Gene Expression

Paraffin embedded tissue preparation: The small intestine and colon were removed *en bloc* from adult mice, linearized, and gently flushed with ice-cold phosphate buffered saline (PBS). Intestines were divided into proximal, mid, and distal regions, flushed with fixative [paraformaldehyde-lysine-periodate (McLean and Nakane 1974), 4% paraformaldehyde (PFA), 2% PFA or 10% basic normal formalin], opened longitudinally, and pinned onto wax. Intestines were then fixed for 2 hours at 25°C, washed with PBS, and incubated overnight in 70% ethanol at 25°C. Intestinal regions were embedded in 2% agar, subdivided into 3 cm strips, and cut down the midline. Half-strips were aligned side by side to preserve the cephalo-caudal orientation and the crypt-villus orientation, and the entire stack was embedded in agar. Excess agar was trimmed and the stack transferred to a cassette for paraffin embedding and sectioning by the OHSU Cancer Institute Histology Core Facility under the supervision of Dr. Christopher Corless.

Immunohistochemistry: Fixed, paraffin-embedded sections were deparaffinized (xylene for 5 min X 3; 100% isopropanol for 3 min X 3; ddH_2O for 5 min), washed (PBS for 3 min X 3), and incubated in blocking buffer (1%BSA, 0.3% Triton X-100 in 1X PBS) for 30 min at 25°C. Sections were then incubated with affinity purified rabbit polyclonal

antibodies to Pparδ (1:250, Alexis Biochemicals) for 1 hour at 25°C. Antigen-antibody complexes were detected with Cy3-conjugated donkey anti-rabbit secondary antibodies (1:500, Jackson ImmunoResearch Laboratories). Stained sections were viewed with a Leica DC500 model microscope.

Lef-1/β-cat Knockin ROSA Cloning

Plasmids: **pBS:L1/L2** is the pBS:KS vector modified to contain a *Bam*HI-*Pst*I-*Nco*I-*Mfe*I-*Bgl*II-*Xba*I linker in its MCS with an optimal Kozak consensus sequence (5'-GCCACC-3') immediately upstream of the ATG in the *Nco*I site. **pBigT** (a kind gift from Dr. Shankar Srinivas, Columbia, Srinivas et al. 2001) is the pBS:KS vector modified to contain a PGK-neo cassette flanked by loxP sites upstream of a MCS and the bovine growth hormone (bGH) gene, and downstream of an Adenovirus splice acceptor (SA) site. The loxP-PGK-neo-loxP-*gene-of-interest*-bGH transgene is flanked by *Pac*I and *Asc*I restriction digestion sites. **pROSA26PA** (a kind gift from Dr. Shankar Srinivas, Columbia, Zambrowicz et al. 1997; Srinivas et al. 2001) contains the genomic sequences with which to target the ROSA26 locus. Immediately adjacent *Pac*I and *Asc*I restriction digestion sites are situated between the ROSA26 5' fragment and the ROSA26 3' fragment.

Subcloning an inducible Lef-1/β-cat gene into the ROSA targeting vector: To create the ROSA26 genomic sequence-ROSA promoter-loxP-PGK-neo-polyA-loxP-Lef-1/β-cat-bGH-ROSA26 genomic sequence construct, a 1581-bp *Nco*I-*Eco*RI fragment from p3021

containing the Lef-1/β-cat sequence was subcloned into *Nco*I-*Mfe*I sites in pBS:L1/L2, yielding pBS:L1/L2/3021, with Lef-1/β-cat directly following an optimal Kozak consensus sequence (5'-GCCACC-3') immediately upstream of the ATG in the *Nco*I site. This plasmid was then digested with *Hind*III and *Xba*I to yield a 1645-bp fragment of pBS:L1/L2/3021containing Lef-1/β-cat. The DNA overhangs of this fragment were filled in with Klenow to create blunt ends and subcloned into the cut and filled-in *Xho*I site of pBigT, yielding pBigT/3021. A 4.8 Kb *Pac*I-*Asc*I fragment from pBigT/3021 containing loxP-PGK-neo-polyA-loxP-Lef-1/β-cat-bGH was then subcloned into the *Pac*I-*Asc*I site of pROSA26PA, yielding pRT3 which contains the ROSA26 genomic sequence-loxP-PGK-neo-polyA-loxP-Lef-1/β-cat-bGH-ROSA26 genomic sequence transgene. Thirty μg of pRT3 was linearized with *Xho*I and prepared for ES cell electroporation by the OHSU Transgenics/Gene Targeting Facility under the supervision of Dr. Manfred Baetscher.

Screening of Lef-1/β-cat Knockin ROSA ES Clones for Homologous Recombination

Stably transfected ES cells were identified by PCR using Immomix Red (Bioline). Genomic DNA was isolated from ES cell clones. One hundred and ninety two unique ES cell clones grown in triplicate 96-well plates were lysed (10 mM Tris at pH 7.5, 10 mM EDTA at pH 8.0, 10 mM NaCl, 0.5% Sarcosyl, and 1 mg/ml proteinase K) overnight at 60°C in a humidified chamber. DNA was precipitated with 75 mM NaCl in ethanol for 30 min at 25°C, washed with 70% ethanol using a multichannel pipettor, resuspended in 30 μl water/well, and transferred to a 1.5 ml tube. Primers to the endogenous ROSA promoter (forward, 5'-CCTAAAGAAGAGGCTGTGCTTTGG-3') and to the SA of

BigT (reverse, 5'-CATCAAGGAAACCCTGGACTACTG-3', a kind gift from Dr. Stefan Lanker, OHSU) amplified a 1.2 Kb product. Cycle conditions were as follows: denaturation, 95°C for 30 sec; annealing, 53°C for 30 sec; extension, 72°C for 1 min, for a total of 30 cycles.

RESULTS

Lef-1/β-cat Transgene Cloning

The inducible Lef-1/β-cat transgene contains the Lef-1/β-cat gene downstream of a floxed GFP gene under the control of the chicken β-actin promoter (see Figure 7). The GFP gene is followed by a stop codon and a poly A tail to prevent expression of the downstream Lef-1/β-cat sequence. GFP is constitutively expressed and serves as a negative marker for the recombination event in that GFP identifies cells that have not undergone Cre-mediated recombination. The Lef-1/β-cat sequence contains a c-Myc epitope tag for tracking expression of the fusion protein. The hGH gene contains intron-exon structure and a poly A tail, and thus provides message stability for the entire transgene. A neomycin resistance cassette is included for the selection of ES cells that express the transgene. Antibodies to the c-Myc epitope tag or to the junction of the Lef-1/β-cat fusion protein can be used as positive markers for recombination. Both GFP and Lef-1/β-cat sequences contain optimal Kozak consensus sequences. Because the two loxP sites flanking the GFP gene lie in the same orientation, the presence of Cre recombinase will catalyze recombination between the loxP sites, excising the intervening GFP sequence, and result in the expression of Lef-1/β-cat.

Figure 7. Schematic Representation of the Inducible Lef-1/β-cat Transgene. The inducible Lef-1/β-cat transgene contains the enhanced chicken β-actin promoter (CA), the enhanced green fluorescent protein (GFP), the Lef-1/β-cat fusion protein (Lef-1/β-cat), the human growth hormone (hGH) and a phosphoglycerate kinase-neomycin resistance selection cassette (PGK-neo). Triangles represent loxP sites.

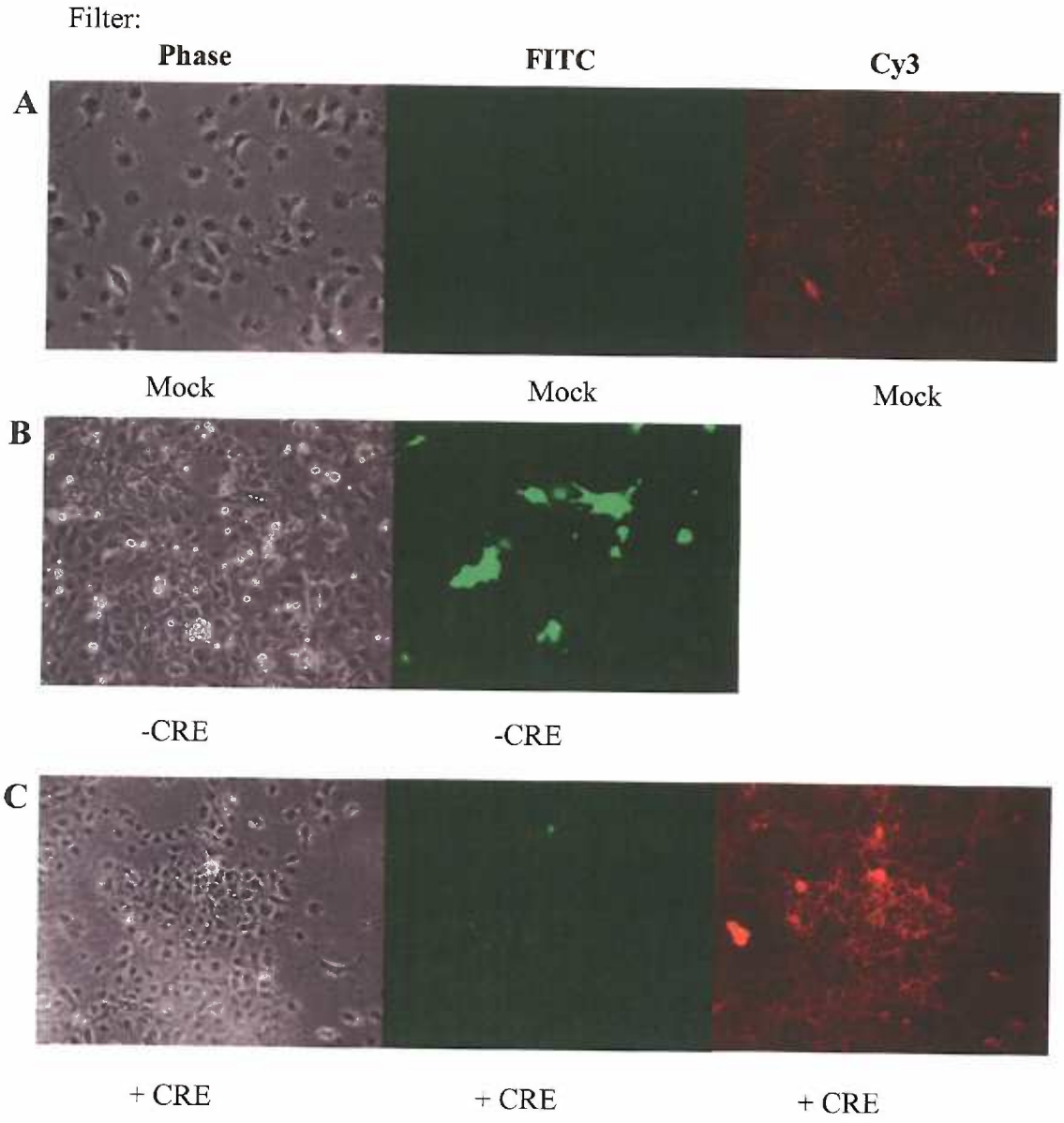


Figure 8. *In Vitro* Activity of the Inducible Lef-1/β-cat Transgene. (A) Mock transfected COS-7 cells expressed no GFP and only endogenous membrane-specific-β-catenin. (B) COS-7 cells transfected with the Lef-1/β-cat construct expressed GFP in the absence of Cre recombinase. (C) COS-7 cells cotransfected with Lef-1/β-cat and a CMV-Cre plasmid express robust nuclear-specific β-catenin in the presence of Cre recombinase. Upon expression of Cre recombinase, floxed GFP sequences were excised, allowing expression of the Lef-1/β-cat fusion protein. Live cells were evaluated for GFP expression 48 hours after transfection by fluorescence microscopy. Permeabilized cells were evaluated for β-catenin expression 72 hours after transfection by immunohistochemical analysis using rabbit anti-β-catenin primary antibodies and indocarbocyanine (Cy3)-conjugated donkey anti-rabbit secondary antibodies. Bars = 25 μm.

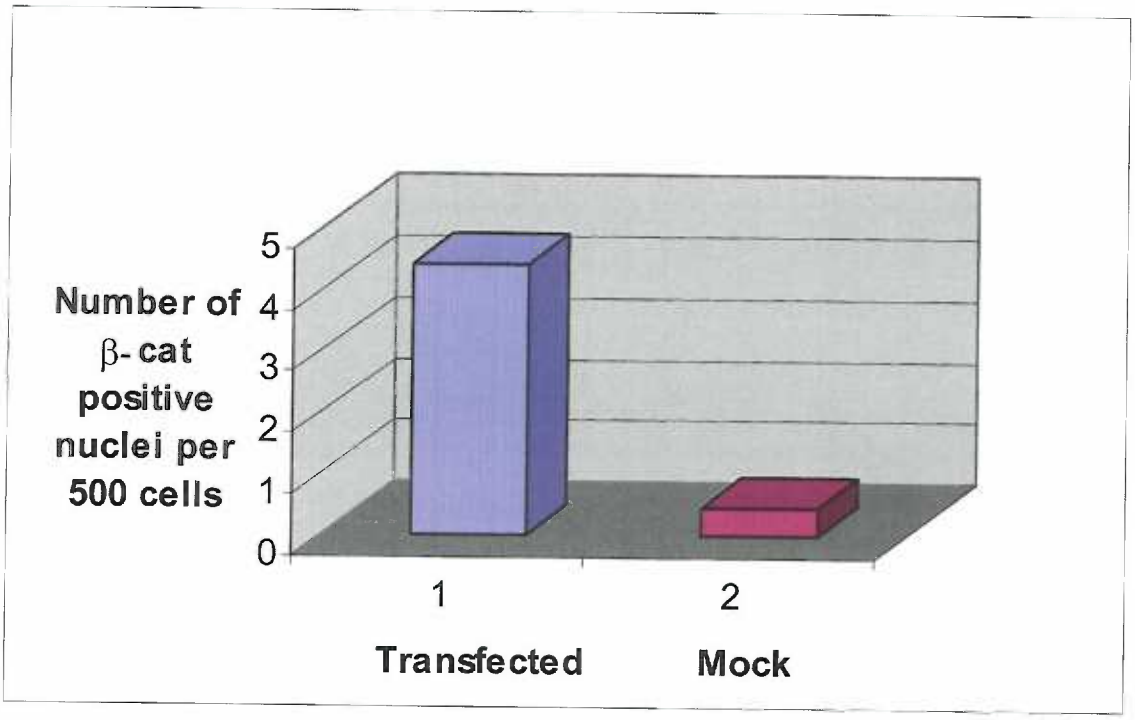


Figure 9. Quantitative Analysis of the *In Vitro* Assay Results for Inducible Lef-1/β-cat Trangene Activity. COS-7 cells were evaluated for β-catenin expression by immunohistochemical analysis 72-hours after transfection with or without plasmids encoding Lef-1/β-cat and Cre recombinase. A total of 2,050 cells were counted. The number of β-catenin positive staining nuclei was reported as per 500 cells counted. The number of β-catenin positive nuclei showed a >10 fold difference between transfected and mock transfected cells.

Lef-1/β-cat Transgene In Vitro *Assays*

The Lef-1/β-cat transgene is designed to express GFP prior to Cre-mediated recombination and the Lef-1/β-cat fusion protein after Cre-mediated recombination. *In vitro* testing of this transgene in COS-7 cells revealed that cells transfected with the Lef-1/β-cat construct express GFP in the absence of Cre recombinase (see Figure 8B). Cells cotransfected with Lef-1/β-cat and a CMV-Cre plasmid express robust and nuclear-specific β-catenin, dramatically higher than levels seen for endogenous β-catenin expression, indicating Lef-1/β-cat fusion protein expression (see Figure 8A and C). A total 2,050 cells were counted per experiment. The number of β-catenin positive nuclei was >10-fold higher in transfected as compared to mock transfected cells (see Figure 9). Staining for c-Myc yielded similar results. This data confirmed that Lef-1/β-cat expression is inducible with Cre recombinase and that the transgene functions as designed.

Electroporation of ES Cells

Because expression of the Lef-1/β-cat transgene is induced by Cre-mediated recombination, it is critical that only a single copy of the transgene be incorporated into the mouse genome. Insertion of multiple transgene copies can yield multiple recombination derivatives, potentially eliminating Lef-1/β-cat expression. Electroporation into ES cells provides a greater chance of single copy transgene integration into the mouse genome than the more traditional approach of pro-nuclear

injection (Andreason and Evans 1988; Lewandoski and Martin 1997; Lobe et al. 1999). Therefore, to generate the Lef-1/β-cat transgenic mouse with only a single copy inserted into its genome, the transgene was electroporated into ES cells. A total of seventy-five colonies were identified in two rounds of electroporation using ratios of DNA concentration per number of cells adjusted to enhance single copy insertion.

Screening of ES Clones for Presence of Transgene

ES cell clones were screened for presence of the transgene by GFP visualization (see Figure 10) and by PCR using primers to hGH and actin (see Figure 11). These data identified fifty-six clones that express GFP and contain the Lef-1/β-cat transgene.

Screening of ES Clones for Single Copy Transgene Insertion by Southern Blot Analysis

Probes to the GFP and neo genes were generated for use in Southern blot analysis. The neo probe recognizes a single >5.7 Kb fragment from *Sac*I digested ES cell genomic DNA carrying a single copy of the transgene. A double transgene insertion is expected to yield multiple bands or a single 11.3 Kb band, depending upon the orientation and configuration of the multiple transgene copies. To distinguish between a single insertion displaying an 11.3 Kb band and a double insertion, membranes were re-evaluated with the GFP probe. The GFP probe recognizes a single 14 Kb fragment if two transgene copies are present. Analysis of banding patterns identified two clones as having single copy insertion (see Figure 12).

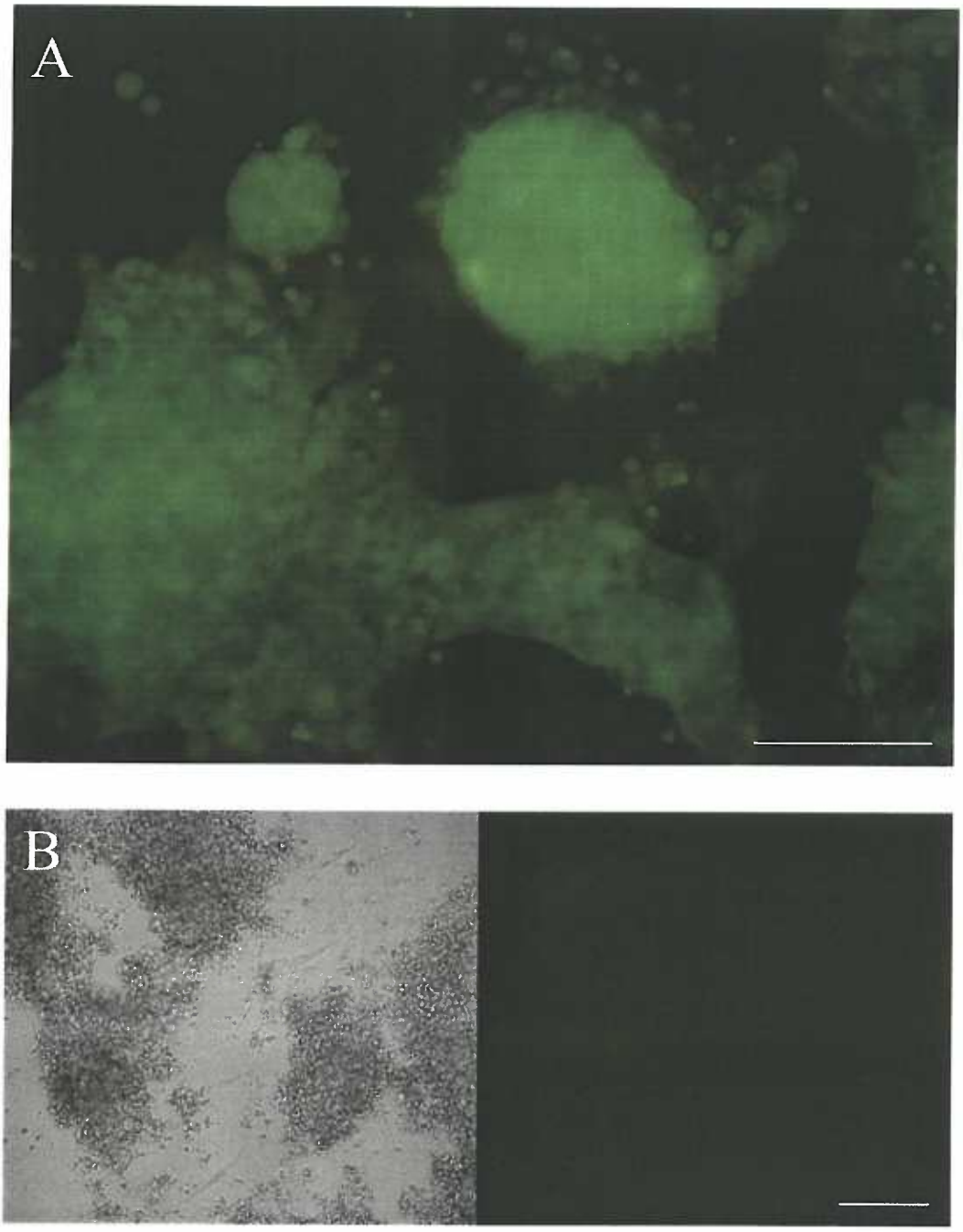


Figure 10. Identification of ES Clones Containing the Lef-1/β-cat Transgene by GFP Visualization. Individual neomycin resistant ES cell colonies were picked and expanded for eight days. Clones containing the inducible Lef-1/β-cat transgene were then identified by visualization of GFP fluorescence. (A) GFP expression in Lef-1/β-cat transfected ES cell clone indicating presence of the transgene (FITC channel). (B) Lack of GFP expression in Lef-1/β-cat transfected ES cell clone indicating absence of the transgene (Phase and FITC channels). Bars = 3 mm.

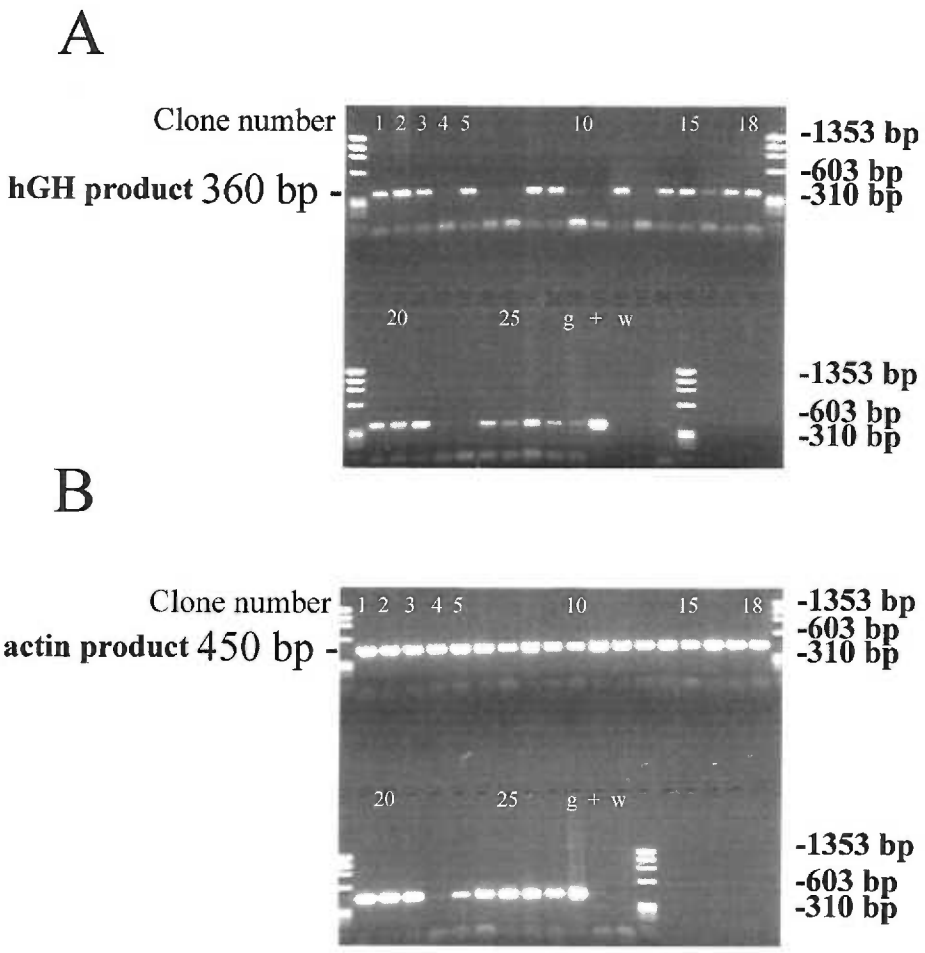


Figure 11. Identification of ES Clones Containing the Lef-1/β-cat Transgene by PCR with Primers to hGH and Actin. Templates of genomic DNA from 27 ES cell clones (1-27), genomic tail DNA (g), Lef-1/β-cat containing plasmid DNA (+) and water (w) were used in PCR reactions. (A) Primers to hGH amplified a 360-bp product and identified clones containing the inducible Lef-1/β-cat trangene. (B) True negative clones were verified using primers to actin, which amplified a 450-bp product, for the presence of DNA.

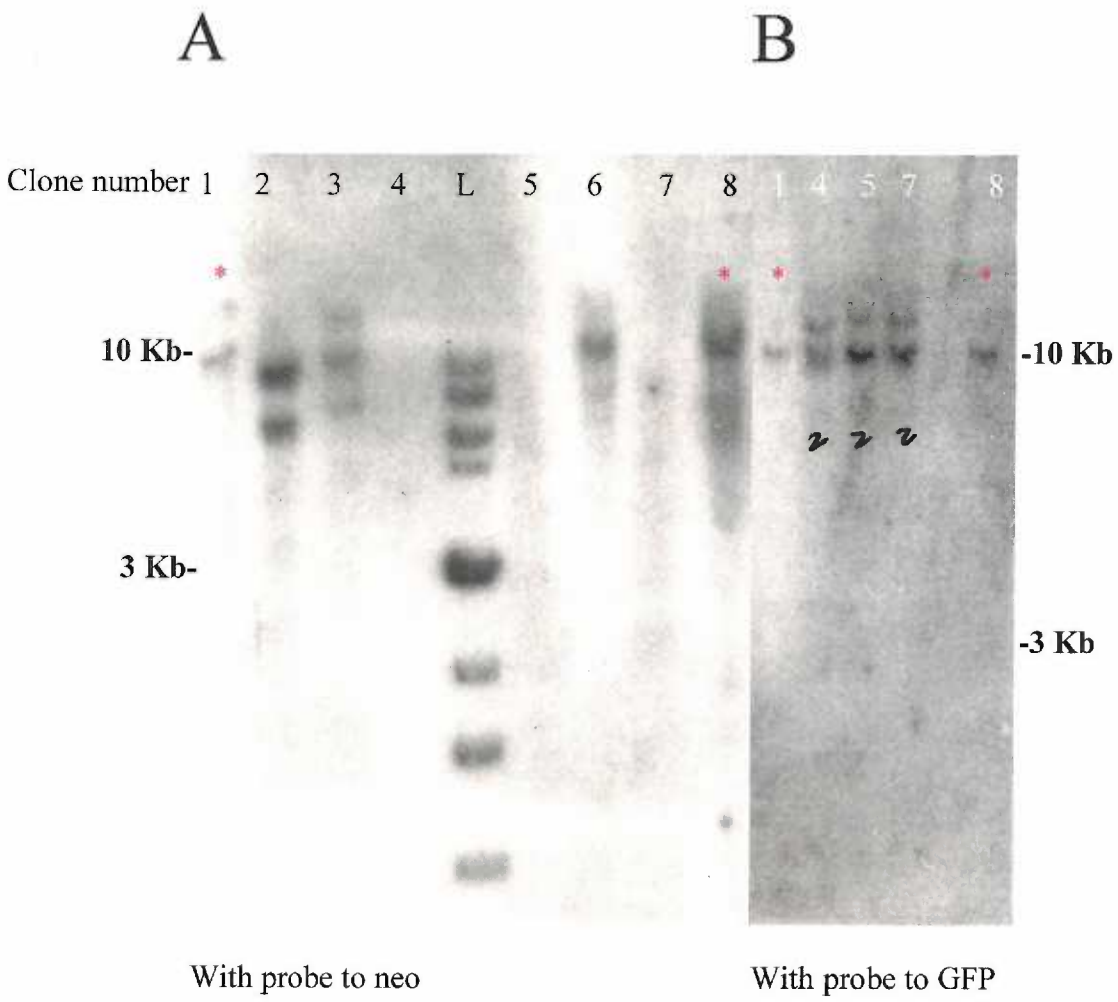


Figure 12. Identification of ES Clones with Single Copy Transgene Insertion by Southern Blot Analysis. Ten μg of genomic DNA from 8 candidate ES cell clones (1-8) was digested with *Sac*I and resolved by electrophoresis prior to transfer to positively charged nylon membrane. (A) Membrane was probed with a radiolabeled fragment from the neomycin resistance gene, which yields a single >5.7 Kb band for a single transgene insertion and a single 11.3 Kb band for a double transgene insertion. (B) Membrane was probed with a fragment from the GFP gene, which yields a >7 Kb band for a single transgene insertion and a single 14 Kb band for a double transgene insertion. Red asterisks (*) indicate candidate clones that have a single copy of the transgene. L = 1 Kb ladder.

Baseline Peroxisome Proliferator-activated Receptor δ Protein Expression Pattern Determined by Immunohistochemistry

In preparation for using the Lef-1/β-cat transgenic mouse to evaluate protein expression responses of various β-catenin target genes to activated β-catenin signaling, a baseline expression pattern in wildtype mice was established for the selected β-catenin target gene, Pparδ. Pparδ protein localizes to the base of the intestinal crypt (see Figure 13), suggesting that it may be specifically expressed in Paneth cells.

Lef-1/β-cat Knockin ROSA Cloning and Screening for Homologous Recombination

The transgenic approach for obtaining temporal control over β-catenin signaling involved electroporating ES cells with a randomly integrating inducible Lef-1/β-cat transgene. With this approach, expression of the Lef-1/β-cat protein, and resulting activation of β-catenin signaling, will vary between mouse lines, depending upon the site of transgene integration for each line. To complement the variable expression levels associated with this approach, a knockin approach was taken to ensure robust expression of Lef-1/β-cat in the intestinal epithelium. With the knockin approach, a transgene with Lef-1/β-cat downstream of a floxed PGK-neo cassette is embedded into the ROSA genomic sequences of the ROSA targeting vector (see Figure 14). The ROSA targeting vector containing this inducible Lef-1/β-cat transgene was then electroporated into ES cells. Genomic DNA from 192 unique ES cell clones was isolated. Initial screening for homologous recombination by PCR revealed a 27% recombination rate (n = 22) and

narrowed the number of ES clones to be screened by a functional *in vitro* assay using Cre recombinase. Two of these six clones were selected for injection.

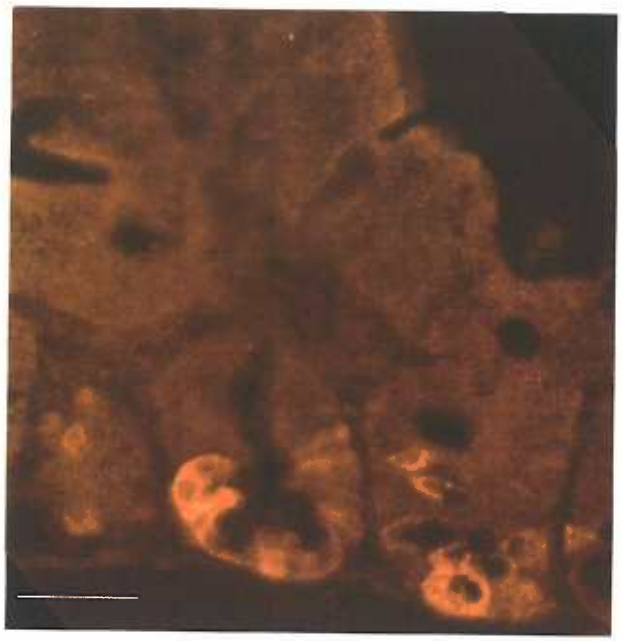
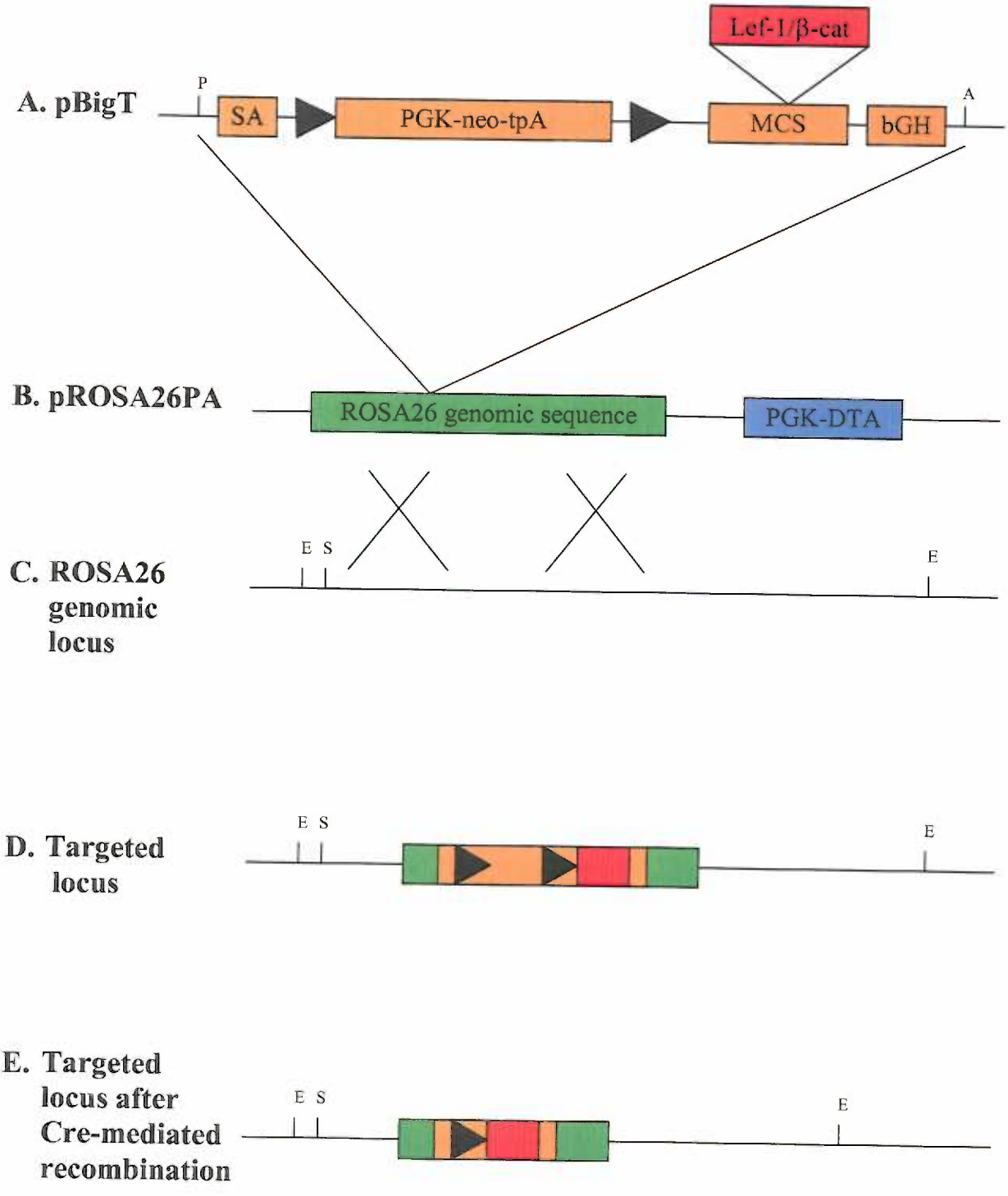


Figure 13. Protein Expression of Peroxisome proliferator-activated receptor δ (Pparδ) in Wildtype Adult Small Intestine. Fixed, paraffin-embedded tissue sections were evaluated for Pparδ protein expression by immunohistochemical analysis using antibodies to Pparδ. Antigen-antibody complexes were detected with indocarbocyanin (Cy3)-conjugated secondary antibodies. Pparδ protein locates to the base of the intestinal crypt. Bar = 25 μm.


A. pBigT
P
SA
PGK-neo-tpA
Lef-1/β-cat
MCS
bGH
A
B. pROSA26PA
ROSA26 genomic sequence
PGK-DTA
C. ROSA26 genomic locus
E S
E
D. Targeted locus
E S
E
E. Targeted locus after Cre-mediated recombination
E S
E

DISCUSSION

Lef-1/β-cat Transgenic and Knockin Reagents

This thesis reports the generation of reagents for the production of two novel inducible mouse lines capable of β-catenin signaling activation in the intestinal stem cell and its progeny. The Lef-1/β-cat transgenic mouse will harbor Lef-1/β-cat downstream of a floxed GFP gene. A functional *in vitro* assay for the inducible expression of Lef-1/β-cat demonstrates that the transgene functions as designed. Transfected COS-7 cells expressed GFP prior to Cre-mediated recombination and Lef-1/β-cat after Cre-mediated recombination. Stably transfected ES cells were identified by visualization of GFP fluorescence and by PCR analysis confirming presence of the Lef-1/β-cat transgene. Southern blot analysis identified clones with a single copy of the transgene. Choosing ES cell clones with a single Lef-1/β-cat transgene insertion and a high, medium or low level of expression of the transgene will permit generation of transgenic mice that express different levels of Lef-1/β-cat upon Cre-mediated induction. Upon crossing into the inducible, intestine-specific Cre mouse system, these mice will mosaically express Lef-1/β-cat in the intestine upon administration of doxycycline and provide a means to evaluate the effects of different levels of activated β-catenin signaling in the intestinal stem cell.

To ensure insertion of a single copy of the transgene and a robust level of Lef-1/β-cat expression, a knockin approach was taken to complement the transgenic approach. The Lef-1/β-cat knockin ROSA mouse will harbor Lef-1/β-cat downstream of

a floxed PGK-neo cassette at the ROSA26 genomic locus. ES cell clones electroporated with the ROSA targeting vector containing this inducible Lef-1/β-cat transgene were screened for homologous recombination and two clones were identified for injection. Upon crossing into the inducible, intestine-specific Cre mouse system, these mice will mosaically express a robust level of Lef-1/β-cat in the intestine upon administration of doxycycline and provide a means to evaluate the effects of a high level of activated β-catenin signaling in the intestinal stem cell.

Strategy for Inducible, Intestine-specific Activation of β-catenin Signaling

There are several approaches to study gene function in mice. Gene targeting in mouse ES cells can be used to knock out a specific gene *in vivo*, creating mice that completely lack expression of that gene, or to knock in a specific gene into a particular locus, creating mice that express that gene in a tissue-specific or stage-specific manner. Mouse ES cells can also be used to generate transgenic mice with extra copies, altered copies or a copy of a foreign gene randomly integrated into their genome (Melton 1994; Mueller and Sarvetnick 1995; St-Jacques and McMahon 1996; Shastry 1998; Champtiaux and Changeux 2004). These approaches to manipulating the mouse genome result in a gene alteration in the germ line and thus the gene alteration is present in all cells at all times of the animal's life, from development throughout adulthood. Alteration of a gene essential for development or survival of the animal often results in early lethality and precludes assessment of gene function at later stages of development or adulthood. Similarly, manipulation of a gene that is expressed in multiple tissues or contributes to a

complex process can produce a multi-component effect and complicate analysis of its function.

β-catenin participates in both cell adhesion and cell signaling (Nusse 1997). Further, it is absolutely required for embryogenesis, as the homozygous null mutation results in early embryonic lethality (Haegel et al. 1995). To gain spatial and temporal control over activation of β-catenin signaling for the study of the molecular and cellular responses to activated Wnt/β-catenin-mediated signaling in the intestine, a strategy for conditional expression of an exogenous transgene was developed using the Cre/loxP recombination system and transgenic technologies. Cre recombinase will catalyze the excision of DNA located between flanking loxP sites (Sternberg and Hamilton 1981; Abremski and Hoess 1984). This strategy for conditional gene targeting involves the generation of two mouse lines. Both mouse lines will harbor inducible Lef-1/β-cat sequences and will therefore be capable of Wnt/β-catenin signaling activation. One line, the Lef-1/β-cat transgenic mouse, will harbor floxed GFP sequence upstream of Lef-1/β-cat sequence. Thus, upon Cre-mediated recombination, GFP will be excised and Lef-1/β-cat will be expressed. The other line, the Lef-1/β-cat knockin ROSA mouse, will harbor a floxed PGK-neo cassette upstream of Lef-1/β-cat targeted to the ROSA locus. To restrict recombination to the intestinal stem cell and its progeny, and to any given stage in development or adulthood, the two inducible Lef-1/β-cat mouse lines can be crossed into the inducible, intestine-specific *Fabpl* $^{4X\ at\ -132}$-rtTA, *tet*O-P_{hCMV}-Cre mouse system (the inducible, intestine-specific Cre mouse system, Saam and Gordon 1999; Wong et al. 2000). Cre activity in these mice is inducible by administration of doxycycline because the Cre recombinase coding sequence is expressed under the regulation of *tet*O

sequences. Cre activity is intestine-specific because the rtTA sequence is under the control of the *Fabpl* $^{4X \text{ at } -132}$ promoter. These *Fabpl* $^{4X \text{ at } -132}$ regulatory elements are known to reliably direct expression of gene products to the multipotent intestinal stem cell and its progeny, from E14.5 through adulthood, in small intestinal, cecal, and colonic epithelium (Wong et al. 2000).

Cre-mediated recombination, and thus activation of β-catenin signaling, will occur only upon administration of doxycycline and only in intestinal epithelial cells in offspring derived from crosses between these novel lines and the inducible, intestine-specific Cre mouse system. The GFP or neo marker genes will remain expressed in cells of all other tissues where Cre recombinase is not expressed and β-catenin signaling will remain unaffected. This strategy allows for temporal and spatial regulation of Lef-1/β-cat expression. Therefore, at any given time in development or adulthood, β-catenin signaling can be activated in the intestinal stem cell and the immediate molecular and cellular *in vivo* response evaluated.

Advantages of Analysis with Inducible Lef-1/β-cat

The Strategy

Use of the Lef-1/β-cat transgenic mouse and the Lef-1/β-cat knockin ROSA mouse with the inducible, intestine-specific Cre mouse system provides an excellent system for studying the effects of activated β-catenin signaling in the intestine. Cre-mediated recombination in the inducible, intestine-specific Cre mouse system is mosaic

and therefore allows for direct comparisons between juxtaposed Lef-1/β-cat expressing cells and non-Lef-1/β-cat expressing cells. Because both the wildtype and Lef-1/β-cat expressing cells are exposed to the identical temporal and spatial environmental cues, the wildtype cells are the ideal control cell population. Significantly, mosaic expression of Cre results in mosaic activation of β-catenin signaling, permitting survival of the mouse and the ability to study the progression of polyp formation. Unlike mice that do not survive long enough to develop polyps due to strategies that result in uniform Cre-mediated recombination, the inducible Lef-1/β-cat mice will more accurately mimic the onset and progression of human disease. Further, the intestinal stem cell's response to activated β-catenin signaling requires the complex and dynamic interaction between the stem cell and its surrounding mesenchyme. While cell culture is useful for dissecting the molecular aspects of signaling pathways, an *in vivo* analysis within the intact intestinal epithelium is essential for a complete and comprehensive understanding of the epithelial response. The Lef-1/β-cat transgenic mouse and the Lef-1/β-cat knockin ROSA mouse provide for an *in vivo* analysis and ensure maintenance of the required interactions between the mesenchyme and the epithelium. Moreover, in contrast to comparisons between normal tissue and carcinoma biopsies, these mice give access to the immediate molecular response to activated β-catenin signaling rather than simply an end-point result of gene expression changes.

Most importantly, these mice allow for two independent and different types of analysis. Characterization of the intestinal stem cells' early molecular and cellular responses to activated β-catenin signaling with these mice will provide insight into the

role of the Wnt/β-catenin pathway in regulating the intestinal stem cell during development, adulthood, and disease. Additionally, defining the immediate changes in gene expression resulting from activated β-catenin signaling in adults will facilitate the identification of early molecular events leading to polyp formation and colorectal cancer.

The Transgene Design

The design of the inducible Lef-1/β-cat transgene, which either utilizes the enhanced chicken β-actin promoter to drive expression of the floxed GFP-Lef-1/β-cat sequences or the ROSA26 promoter to drive expression of the floxed PGK-neo-Lef-1/β-cat sequences, provides for several useful and valuable features. First, the enhanced chicken β-actin promoter is ubiquitously expressed and has strong promoter activity in mice (Miyazaki et al. 1989; Sands et al. 1993). Using this promoter to drive expression of the floxed GFP-Lef-1/β-cat sequences lends versatility to this reagent, as mice harboring this transgene can be mated into other inducible Cre mouse systems for evaluating the role of activated β-catenin signaling in other organ systems. Second, GFP serves as a marker for recombination in that GFP expression identifies epithelial cells that have not yet undergone recombination. Using the coding sequence for GFP within the floxed allele of the transgene simplifies the overall mating scheme for the Lef-1/β-cat transgenic mice because it serves as a monitor of Cre recombinase activity. An additional mouse harboring a marker for recombination, like the Cre recombination marker R26R mouse (Soriano 1999), is therefore unnecessary. In addition, GFP is routinely expressed in

transgenic mice. The protein has been genetically modified for increased expression and enhanced levels of fluorescence for optimal detection (Kozak 1987; Cormack et al. 1996; Haas et al. 1996). GFP expression can be detected directly by fluorescence microscopy or by immunodetection with anti-GFP antibodies. Should these approaches prove problematic, antibodies to the c-Myc epitope tag in the Lef-1/β-cat protein or to the Lef-1-β-catenin junction of the Lef-1/β-cat fusion protein can be used to identify cells that have undergone recombination. Third, targeting the inducible Lef-1/β-cat transgene to the ROSA26 locus ensures integration of a single copy and a robust expression level of Lef-1/β-cat in the intestinal epithelium.

Fourth, for both the Lef-1/β-cat transgenic and knockin mice, the Lef-1/β-cat fusion protein lacks the ability to be regulated in the cytoplasm through interactions with Apc/Axin or to participate in adherens junctions through interactions with E-cadherin. Stimulating β-catenin signaling by means of a fusion protein that contains only the signaling domains of β-catenin allows for the separation of β-catenin's signaling and adhesive functions. For example, in an analysis of the early epithelial events preceding polyp formation resulting from activated β-catenin signaling, only the signaling activities of Lef-1/β-cat will contribute to the immediate changes in gene expression. Although Apc mutants allowing accumulation of β-catenin result in polyp formation, the Lef-1/β-cat transgenic mouse and the Lef-1/β-cat knockin ROSA mouse may not elicit adenomatous polyps. The dynamic regulation of β-catenin levels between the various functional pools (plasma membrane, cytoplasmic, and nuclear) is not yet clearly defined, however, several studies indicate that control is exerted through competitive interactions between binding partners. These studies predict a mechanism whereby disruption of β-

catenin binding to E-cadherin or Apc/Axin complexes releases β-catenin to translocate to the nucleus and bind Lef-1/Tcf transcription factors, resulting in increased activation of target genes. Because the Lef-1/β-cat fusion protein lacks the Armadillo repeat domain, which mediates binding to other proteins like E-cadherin, Apc, and Axin, it escapes this regulation and translocates directly to the nuclear pool. Therefore, a lack of polyps in mice that undergo activation of β-catenin signaling from a Lef-1/β-cat fusion protein incapable of participating in adherens junctions would suggest that β-catenin's cell adhesion role is critical to colorectal carcinogenesis.

The Model

There are a number of experimental mouse models currently available that have been useful in studying the molecular mechanism of polyp formation. The Min mouse harbors a germ line mutation in the Apc gene and recapitulates the human disease FAP (Moser et al. 1990; Su et al. 1992). Similarly, the Apc1638 mouse and the $Apc^{\Delta 716}$ mouse express a truncated Apc and develop multiple polyps throughout the intestinal tract (Fodde et al. 1994; Oshima et al. 1995; Oshima et al. 1997). However, inactivation of the second Apc allele in these mice occurs by loss of heterozygosity and thereby prevents analysis of the early events in polyp formation. In the homozygous Apc^{580S} inducible Apc knockout mouse, inactivation of Apc directed specifically to the colorectal epithelium is achieved by Cre-mediated recombination (Shibata et al. 1997). Upon introduction of Cre recombinase, Apc function is lost and adenomas develop within four weeks. In the $Cre^{+}Apc^{fl/fl}$ mouse, loss of Apc upon induction of Cre recombinase in the intestine causes

increased proliferation, failed differentiation, and aberrant migration in intestinal crypts, resulting in morbidity in five days (Sansom et al. 2004). Although loss of Apc in this mouse produces many of the phenotypes associated with early colorectal lesions, Cre-mediated recombination is uniform and this mouse does not develop polyps before it suffers severe intestinal compromise from the reduction in villus differentiation. Unfortunately, the inducible Apc mutant mouse is neither academically nor commercially available. The N-terminal truncated β-catenin $Catnb^{\Delta ex3}$ mouse undergoes deletion of all the Gsk-3β phosphorylation target serine and threonine residues when crossed with a mouse that expresses Cre recombinase in the intestinal epithelium (Harada et al. 1999). The compound heterozygous offspring express a dominant and stable form of β-catenin and develop numerous intestinal adenomatous polyps two weeks after birth resembling those in the $Apc^{\Delta 716}$ knockout mice (Oshima et al. 1995; Oshima et al. 1997). Because Cre recombinase in this mouse is controlled by the regulatory region of either the cytokeratin 19 (Ck19, Quaroni et al. 1991) or rat liver fatty acid binding protein (*Fabpl)* gene (Simon et al. 1993), deletion of exon 3 of one of the alleles of β-catenin by Cre-mediated recombination is initiated upon activation of the endogenous Ck19 or *Fabpl* transgene during development. Moreover, this mouse is similarly not available. Therefore, though each of these available mouse models provide evidence for the involvement of Wnt signaling in intestinal tumorigenesis, they preclude the study of the early events in tumorigenesis by a lack of temporal control over activation of β-catenin signaling. The Lef-1/β-cat transgenic mouse and the Lef-1/β-cat knockin ROSA mouse models overcome this limitation in that they can be induced to simulate β-catenin signaling at a given time in adulthood, and thereby allow a precise analysis of the

immediate impact of stimulated β-catenin signaling on the intestinal epithelium in polyp formation. Furthermore, Cre-mediated recombination is mosaic in the inducible Lef-1/β-cat mouse models, permitting survival of the mice and thereby the ability to evaluate progression to polyp formation. Additionally, because the Lef-1/β-cat fusion protein can only mediate signaling, the Lef-1/β-cat transgenic mouse and the Lef-1/β-cat knockin ROSA mouse models will compare the differences between stimulating signaling alone, and stimulating signaling as well as adhesion, and thus may determine the role each plays in polyp formation.

Importantly, the inducible Lef-1/β-cat mouse models also allow a precise analysis of the immediate impact of stimulated β-catenin signaling on the intestinal stem cell.

Significance and Potential Uses of the Inducible Lef-1/β-cat Mice

In humans, the transformation of normal epithelium into intestinal polyps involves a series of molecular events over a considerable period of time, up to five to ten years. Identifying the early events will not only provide therapeutic and diagnostic targets for the prevention of polyp formation and subsequent progression to colorectal cancer, but also elucidate the normal molecular regulation of intestinal stem cell proliferation and differentiation. Such insight into intestinal stem cell regulation is critical for the development of any future stem cell applications.

Stem cells can transform into a myriad of differentiated cell types, providing a potential therapeutic approach for treating disease. Characterizing the regulation of stem cells is the key to using them therapeutically. The ability to manipulate stem cells will

allow for the control of tissue regeneration, the complete replacement of diseased tissue, and even the delivery of therapeutic agents. For example, adult hematopoietic stem cells are currently used to replace defective blood cells in treating Hodgkin's lymphoma and other leukemia (Dreger and Montserrat 2002). The rapid renewal of the intestinal epithelium continues throughout life, suggesting that the intestinal epithelium has a prominent capacity for regeneration, especially in response to tissue damage. However, despite what is known about the maintenance and regeneration of the intestinal epithelia, a molecular basis for regenerative therapy is currently lacking. Once identified, exogenous intestinal stem cells may be used as a source for transplantation in diseased patients. Endogenous intestinal stem cells could be manipulated to correct dysregulated epithelial regeneration in patients with intestinal inflammation or disease. Alternatively, bone marrow-derived cells have been shown to contribute to the regeneration of the intestinal epithelia (Okamoto et al. 2002). In bone marrow transplant recipients, donor-derived epithelial cells repopulated the gastrointestinal tract during epithelial regeneration after graft-versus-host disease or ulcer formation. Utilization of these different types of stem cells may someday lead to the therapeutic regeneration of the damaged intestinal epithelia of colorectal cancer patients. Studies that address how Wnt/β-catenin signaling effects stem cell regulation will shed light onto how stem cells can be manipulated and ultimately utilized for treating intestinal disease.

Studies using the Lef-1/β-cat transgenic mouse and the Lef-1/β-cat knockin ROSA mouse will impact our understanding of Wnt/β-catenin's signaling role in intestinal stem cell regulation. Establishment and maintenance of the intestinal stem cell niche are dynamic processes that require temporal and spatial expression of signals that

control adhesion, migration, proliferation, and differentiation. Inducing Lef-1/β-cat in stem cells at critical time points during development will faciliate determining if the temporal onset of Wnt/β-catenin signaling is involved in shaping the physical intestinal stem cell niche, proliferation within the stem cell niche, selection of a single stem cell to populate the adult crypt, and the onset of epithelial differentiation. Inducing Lef-1/β-cat in adult stem cells will aid in determining if the spatial organization of Wnt/β-catenin signaling is involved in maintaining the gradient of proliferation to differentiation from the base of the adult crypt to the crypt-villus junction.

Further, elucidating the early events in response to activating Wnt/β-catenin signaling in the intestinal stem will ultimately allow for therapeutic and diagnostic approaches to treating intestinal diseases like colorectal cancer. For example, studies that detect early changes in gene expression in response to activated β-catenin signaling will identify genes that can be used as targets in diagnostic screening for early detection of colorectal cancer.

The Lef-1/β-cat transgenic mouse and the Lef-1/β-cat knockin ROSA mouse are valuable tools for these types of studies because they provide spatial and temporal control over the activation of β-catenin signaling. Hence, they offer several potential uses. These mice could be used to identify developmental β-catenin target gene expression in the mouse intestine. Lef-1/β-cat expression could be induced in the developing intestines of embryos by feeding the pregnant females with doxycycline 2g/kg chow. Studies using doxycycline to prevent expression of a transgene under control of tetracycline regulatable response elements in the embryo have demonstrated successful transmission of the drug through the placenta of pregnant females to inhibit gene expression in the embryos

(Tumbar et al. 2004). Alterations in the formation of the stem cell niche upon induction of Lef-1/β-cat expression at critical time points in intestinal crypt morphogenesis would suggest a temporal dependence on β-catenin signaling. The immediate cellular response to activated β-catenin signaling could be defined in the inducible Lef-1/β-cat mice by detecting changes in mitotic activity, apoptotic activity, and lineage allocation upon induction of Lef-1/β-cat. Such studies would assess the significance of β-catenin signaling in establishing the developing stem cell niche or maintaining the gradient of cell proliferation to cell differentiation in the adult crypt. These mice could be used to determine β-catenin target gene expression in developing, adult, and diseased intestines. Comparison among gene expression profiles will identify differential responses between normal and β-catenin-activated intestines. In the event that some intestinal neonatal genes normally silenced in the adult become stimulated in disease, as is the case with myocardial hypertrophy (Ghatpande et al. 1999), intestinal development could represent a powerful model for characterizing the molecular mechanism that drives polyp formation. Further, global gene expression comparisons using DNA microarray analysis could identify novel genes that are immediately up- or downregulated upon β-catenin signaling in the intestinal epithelium and augment the list of already known target genes. It is anticipated that comparisons will identify changes in genes that are implicated in the regulation of the intestinal stem cell niche, including growth potential, differentiation status, cell signaling capacity, structural components, and apoptosis. It is possible that these studies may lead to the identification of intestinal stem cells. Novel genes identified by global gene expression comparisons between activated and wildtype adults that correlate with high levels of proliferation or between cells from the IVRs and the adult

crypts that correlate with the developing intestine could potentially represent intestinal stem cell markers. Importantly, these comparisons could provide the basis for establishing the molecular mechanism of polyp formation. Genes identified in these studies could be used as targets for therapeutic agents to prevent disease or diagnostic indicators of the onset of disease. Further, these mice could be used to elucidate the role of β-catenin signaling in establishing the intestinal stem cell niche.

Both the Lef-1/β-cat transgenic mouse and the Lef-1/β-cat knockin ROSA mouse will be valuable tools to investigate the early events in the intestinal stem cell's response to β-catenin signaling *in vivo*. These mice should prove especially useful in studies of β-catenin's signaling role in intestinal carcinoma, normal regulation of the intestinal stem cell, and establishment and maintenance of the intestinal stem cell niche.

SUMMARY AND CONCLUSIONS

Reagents for the generation of two transgenic mouse lines in which the activation of β-catenin signaling can be temporally and spatially regulated have been created by electroporation of ES cells with an inducible Lef-1/β-cat transgene. The inducible Lef-1/β-cat transgene contains sequence for the Lef-1/β-cat fusion molecule, capable of stimulating β-catenin signaling, downstream of a floxed reporter or selection gene. Inducible expression of Lef-1/β-cat was demonstrated in a functional *in vitro* assay using transfected COS-7 cells. ES cells were stably transfected with the inducible Lef-1/β-cat transgene either randomly integrated into the genome, or targeted to the ROSA26 genomic locus by homologous recombination. ES cell clones demonstrating the presence of a single copy of the transgene have been identified and selected for injection. Studies with these mice to determine protein expression patterns of β-catenin target genes in response to activated β-catenin signaling will require baseline expression in wildtype mice for comparisons. The protein expression pattern for the β-catenin target gene Pparδ was determined by immunohistochemical analysis and identified localization in cells at the base of the crypt.

Future studies with the inducible Lef-1/β-cat mice will elucidate the role of Wnt/β-catenin signaling in intestinal stem cell regulation, ultimately allowing for the utilization of stem cells in regenerative therapy. Further, studies with adult mice to characterize the immediate molecular and cellular response to β-catenin signaling will allow a precise analysis of the early epithelial events preceding polyp formation, which is critical to identifying therapeutic and diagnostic targets for preventing formation of

polyps and subsequent progression to colorectal cancer. The ability to control activation of Wnt/β-catenin signaling in the intestine or in other organs will permit *in vivo* studies to enhance basic understanding of Wnt/β-catenin signaling in development and disease.

REFERENCES

Aberle, H., Bauer, A., Stappert, J., Kispert, A., and Kemler, R. (1997). beta-catenin is a target for the ubiquitin-proteasome pathway. *Embo J* **16,** 3797-3804.

Abremski, K., and Hoess, R. (1984). Bacteriophage P1 site-specific recombination. Purification and properties of the Cre recombinase protein. *J Biol Chem* **259,** 1509-1514.

Andreason, G. L., and Evans, G. A. (1988). Introduction and expression of DNA molecules in eukaryotic cells by electroporation. *Biotechniques* **6,** 650-660.

Arber, N., Hibshoosh, H., Moss, S. F., Sutter, T., Zhang, Y., Begg, M., Wang, S., Weinstein, I. B., and Holt, P. R. (1996). Increased expression of cyclin D1 is an early event in multistage colorectal carcinogenesis. *Gastroenterology* **110,** 669-674.

Ausubel, F. (1993). "Current Protocols in Molecular Biology." John Wiley and Sons, Inc., USA.

Barker, N., Huls, G., Korinek, V., and Clevers, H. (1999). Restricted high level expression of Tcf-4 protein in intestinal and mammary gland epithelium. *Am J Pathol* **154,** 29-35.

Barker, N., Hurlstone, A., Musisi, H., Miles, A., Bienz, M., and Clevers, H. (2001). The chromatin remodelling factor Brg-1 interacts with beta-catenin to promote target gene activation. *Embo J* **20,** 4935-4943.

Bartkova, J., Lukas, J., Strauss, M., and Bartek, J. (1994). The PRAD-1/cyclin D1 oncogene product accumulates aberrantly in a subset of colorectal carcinomas. *Int J Cancer* **58,** 568-573.

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

Behrens, J., Jerchow, B. A., Wurtele, M., Grimm, J., Asbrand, C., Wirtz, R., Kuhl, M., Wedlich, D., and Birchmeier, W. (1998). Functional interaction of an axin homolog, conductin, with beta-catenin, APC, and GSK3beta. *Science* **280,** 596-599.

Behrens, J., von Kries, J. P., Kuhl, M., Bruhn, L., Wedlich, D., Grosschedl, R., and Birchmeier, W. (1996). Functional interaction of beta-catenin with the transcription factor LEF-1. *Nature* **382,** 638-642.

Ben-Ze'ev, A., and Geiger, B. (1998). Differential molecular interactions of beta-catenin and plakoglobin in adhesion, signaling and cancer. *Curr Opin Cell Biol* **10,** 629-639.

Bhanot, P., Brink, M., Samos, C. H., Hsieh, J. C., Wang, Y., Macke, J. P., Andrew, D., Nathans, J., and Nusse, R. (1996). A new member of the frizzled family from Drosophila functions as a Wingless receptor. *Nature* **382,** 225-230.

Bienz, M., and Clevers, H. (2000). Linking colorectal cancer to Wnt signaling. *Cell* **103,** 311-320.

Booth, C., and Potten, C. S. (2000). Gut instincts: thoughts on intestinal epithelial stem cells. *J Clin Invest* **105,** 1493-1499.

Brabletz, T., Jung, A., Dag, S., Hlubek, F., and Kirchner, T. (1999). beta-catenin regulates the expression of the matrix metalloproteinase-7 in human colorectal cancer. *Am J Pathol* **155,** 1033-1038.

Brittan, M., and Wright, N. A. (2002). Gastrointestinal stem cells. *J Pathol* **197,** 492-509.

Brunner, E., Peter, O., Schweizer, L., and Basler, K. (1997). pangolin encodes a Lef-1 homologue that acts downstream of Armadillo to transduce the Wingless signal in Drosophila. *Nature* **385,** 829-833.

Cadigan, K. M., and Nusse, R. (1997). Wnt signaling: a common theme in animal development. *Genes Dev* **11,** 3286-3305.

Calvert, R., and Pothier, P. (1990). Migration of fetal intestinal intervillous cells in neonatal mice. *Anat Rec* **227,** 199-206.

Castrop, J., van Wichen, D., Koomans-Bitter, M., van de Wetering, M., de Weger, R., van Dongen, J., and Clevers, H. (1995). The human TCF-1 gene encodes a nuclear DNA-binding protein uniquely expressed in normal and neoplastic T-lineage lymphocytes. *Blood* **86,** 3050-3059.

Cavallo, R. A., Cox, R. T., Moline, M. M., Roose, J., Polevoy, G. A., Clevers, H., Peifer, M., and Bejsovec, A. (1998). Drosophila Tcf and Groucho interact to repress Wingless signalling activity. *Nature* **395,** 604-608.

Champtiaux, N., and Changeux, J. P. (2004). Knockout and knockin mice to investigate the role of nicotinic receptors in the central nervous system. *Prog Brain Res* **145,** 235-251.

Cheng, H. (1974). Origin, differentiation and renewal of the four main epithelial cell types in the mouse small intestine. IV. Paneth cells. *Am J Anat* **141,** 521-535.

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

Clevers, H., and van de Wetering, M. (1997). TCF/LEF factor earn their wings. *Trends Genet* **13,** 485-489.

Cormack, B. P., Valdivia, R. H., and Falkow, S. (1996). FACS-optimized mutants of the green fluorescent protein (GFP). *Gene* **173,** 33-38.

Cox, R. T., Kirkpatrick, C., and Peifer, M. (1996). Armadillo is required for adherens junction assembly, cell polarity, and morphogenesis during Drosophila embryogenesis. *J Cell Biol* **134,** 133-148.

Crawford, H. C., Fingleton, B. M., Rudolph-Owen, L. A., Goss, K. J., Rubinfeld, B., Polakis, P., and Matrisian, L. M. (1999). The metalloproteinase matrilysin is a target of beta-catenin transactivation in intestinal tumors. *Oncogene* **18,** 2883-2891.

Dreger, P., and Montserrat, E. (2002). Autologous and allogeneic stem cell transplantation for chronic lymphocytic leukemia. *Leukemia* **16,** 985-992.

Eastman, Q., and Grosschedl, R. (1999). Regulation of LEF-1/TCF transcription factors by Wnt and other signals. *Curr Opin Cell Biol* **11,** 233-240.

Finch, P. W., Cunha, G. R., Rubin, J. S., Wong, J., and Ron, D. (1995). Pattern of keratinocyte growth factor and keratinocyte growth factor receptor expression during mouse fetal development suggests a role in mediating morphogenetic mesenchymal-epithelial interactions. *Dev Dyn* **203,** 223-240.

Fingleton, B. M., Heppner Goss, K. J., Crawford, H. C., and Matrisian, L. M. (1999). Matrilysin in early stage intestinal tumorigenesis. *Apmis* **107,** 102-110.

Fodde, R., Edelmann, W., Yang, K., van Leeuwen, C., Carlson, C., Renault, B., Breukel, C., Alt, E., Lipkin, M., Khan, P. M., and et al. (1994). A targeted chain-termination mutation in the mouse Apc gene results in multiple intestinal tumors. *Proc Natl Acad Sci U S A* **91,** 8969-8973.

Fodde, R., Kuipers, J., Rosenberg, C., Smits, R., Kielman, M., Gaspar, C., van Es, J. H., Breukel, C., Wiegant, J., Giles, R. H., and Clevers, H. (2001a). Mutations in the APC tumour suppressor gene cause chromosomal instability. *Nat Cell Biol* **3,** 433-438.

Fodde, R., Smits, R., and Clevers, H. (2001b). APC, signal transduction and genetic instability in colorectal cancer. *Nat Rev Cancer* **1,** 55-67.

Fuller, C. E., Davies, R. P., Williams, G. T., and Williams, E. D. (1990). Crypt restricted heterogeneity of goblet cell mucus glycoprotein in histologically normal human colonic mucosa: a potential marker of somatic mutation. *Br J Cancer* **61,** 382-384.

Funayama, N., Fagotto, F., McCrea, P., and Gumbiner, B. M. (1995). Embryonic axis induction by the armadillo repeat domain of beta-catenin: evidence for intracellular signaling. *J Cell Biol* **128,** 959-968.

Ghatpande, S., Goswami, S., Mascareno, E., and Siddiqui, M. A. (1999). Signal transduction and transcriptional adaptation in embryonic heart development and during myocardial hypertrophy. *Mol Cell Biochem* **196,** 93-97.

Gossen, M., and Bujard, H. (1992). Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proc Natl Acad Sci U S A* **89,** 5547-5551.

Gossen, M., Freundlieb, S., Bender, G., Muller, G., Hillen, W., and Bujard, H. (1995). Transcriptional activation by tetracyclines in mammalian cells. *Science* **268,** 1766-1769.

Graham, T. A., Weaver, C., Mao, F., Kimelman, D., and Xu, W. (2000). Crystal structure of a beta-catenin/Tcf complex. *Cell* **103,** 885-896.

Groden, J., Thliveris, A., Samowitz, W., Carlson, M., Gelbert, L., Albertsen, H., Joslyn, G., Stevens, J., Spirio, L., Robertson, M., and et al. (1991). Identification and characterization of the familial adenomatous polyposis coli gene. *Cell* **66,** 589-600.

Gu, H., Marth, J. D., Orban, P. C., Mossmann, H., and Rajewsky, K. (1994). Deletion of a DNA polymerase beta gene segment in T cells using cell type-specific gene targeting. *Science* **265,** 103-106.

Haas, J., Park, E. C., and Seed, B. (1996). Codon usage limitation in the expression of HIV-1 envelope glycoprotein. *Curr Biol* **6,** 315-324.

Haegel, H., Larue, L., Ohsugi, M., Fedorov, L., Herrenknecht, K., and Kemler, R. (1995). Lack of beta-catenin affects mouse development at gastrulation. *Development* **121,** 3529-3537.

Hall, P. A., Coates, P. J., Ansari, B., and Hopwood, D. (1994). Regulation of cell number in the mammalian gastrointestinal tract: the importance of apoptosis. *J Cell Sci* **107 (Pt 12),** 3569-3577.

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

Hart, M., Concordet, J. P., Lassot, I., Albert, I., del los Santos, R., Durand, H., Perret, C., Rubinfeld, B., Margottin, F., Benarous, R., and Polakis, P. (1999). The F-box protein beta-TrCP associates with phosphorylated beta-catenin and regulates its activity in the cell. *Curr Biol* **9,** 207-210.

Hart, M. J., de los Santos, R., Albert, I. N., Rubinfeld, B., and Polakis, P. (1998). Downregulation of beta-catenin by human Axin and its association with the APC tumor suppressor, beta-catenin and GSK3 beta. *Curr Biol* **8,** 573-581.

He, T. C., Chan, T. A., Vogelstein, B., and Kinzler, K. W. (1999). PPARdelta is an APC-regulated target of nonsteroidal anti-inflammatory drugs. *Cell* **99,** 335-345.

He, T. C., Sparks, A. B., Rago, C., Hermeking, H., Zawel, L., da Costa, L. T., Morin, P. J., Vogelstein, B., and Kinzler, K. W. (1998). Identification of c-MYC as a target of the APC pathway. *Science* **281,** 1509-1512.

Heasman, J., Crawford, A., Goldstone, K., Garner-Hamrick, P., Gumbiner, B., McCrea, P., Kintner, C., Noro, C. Y., and Wylie, C. (1994). Overexpression of cadherins and underexpression of beta-catenin inhibit dorsal mesoderm induction in early Xenopus embryos. *Cell* **79,** 791-803.

Hecht, A., Litterst, C. M., Huber, O., and Kemler, R. (1999). Functional characterization of multiple transactivating elements in beta-catenin, some of which interact with the TATA-binding protein in vitro. *J Biol Chem* **274,** 18017-18025.

Hecht, A., Vleminckx, K., Stemmler, M. P., van Roy, F., and Kemler, R. (2000). The p300/CBP acetyltransferases function as transcriptional coactivators of beta-catenin in vertebrates. *Embo J* **19,** 1839-1850.

Hermiston, M. L., Wong, M. H., and Gordon, J. I. (1996). Forced expression of E-cadherin in the mouse intestinal epithelium slows cell migration and provides evidence for nonautonomous regulation of cell fate in a self-renewing system. *Genes Dev* **10,** 985-996.

Hsu, S. C., Galceran, J., and Grosschedl, R. (1998). Modulation of transcriptional regulation by LEF-1 in response to Wnt-1 signaling and association with beta-catenin. *Mol Cell Biol* **18,** 4807-4818.

Huber, A. H., and Weis, W. I. (2001). The structure of the beta-catenin/E-cadherin complex and the molecular basis of diverse ligand recognition by beta-catenin. *Cell* **105,** 391-402.

Huber, O., Korn, R., McLaughlin, J., Ohsugi, M., Herrmann, B. G., and Kemler, R. (1996). Nuclear localization of beta-catenin by interaction with transcription factor LEF-1. *Mech Dev* **59,** 3-10.

Huelsken, J., Birchmeier, W., and Behrens, J. (1994). E-cadherin and APC compete for the interaction with beta-catenin and the cytoskeleton. *J Cell Biol* **127,** 2061-2069.

Huelsken, J., Vogel, R., Brinkmann, V., Erdmann, B., Birchmeier, C., Birchmeier, W. (2000). Requirement for beta-catenin in anterior-posterior axis formation in mice. *J Cell Biol* **148**, 567-578.

Ikeda, S., Kishida, S., Yamamoto, H., Murai, H., Koyama, S., and Kikuchi, A. (1998). Axin, a negative regulator of the Wnt signaling pathway, forms a complex with GSK-3beta and beta-catenin and promotes GSK-3beta-dependent phosphorylation of beta-catenin. *Embo J* **17,** 1371-1384.

Ilyas, M., Tomlinson, I. P., Rowan, A., Pignatelli, M., and Bodmer, W. F. (1997). Beta-catenin mutations in cell lines established from human colorectal cancers. *Proc Natl Acad Sci U S A* **94,** 10330-10334.

Imaseki, H., Hayashi, H., Taira, M., Ito, Y., Tabata, Y., Onoda, S., Isono, K., and Tatibana, M. (1989). Expression of c-myc oncogene in colorectal polyps as a biological marker for monitoring malignant potential. *Cancer* **64,** 704-709.

Ireland, H., Kemp, R., Houghton, C., Howard, L., Clarke, A. R., Sansom, O. J., and Winton, D. J. (2004). Inducible Cre-mediated control of gene expression in the murine gastrointestinal tract: effect of loss of beta-catenin. *Gastroenterology* **126,** 1236-1246.

Itoh, K., Krupnik, V. E., and Sokol, S. Y. (1998). Axis determination in Xenopus involves biochemical interactions of axin, glycogen synthase kinase 3 and beta-catenin. *Curr Biol* **8,** 591-594.

Kaartinen, V., Voncken, J. W., Shuler, C., Warburton, D., Bu, D., Heisterkamp, N., and Groffen, J. (1995). Abnormal lung development and cleft palate in mice lacking TGF-beta 3 indicates defects of epithelial-mesenchymal interaction. *Nat Genet* **11,** 415-421.

Kaestner, K. H., Silberg, D. G., Traber, P. G., and Schutz, G. (1997). The mesenchymal winged helix transcription factor Fkh6 is required for the control of gastrointestinal proliferation and differentiation. *Genes Dev* **11,** 1583-1595.

Kaplan, K. B., Burds, A. A., Swedlow, J. R., Bekir, S. S., Sorger, P. K., and Nathke, I. S. (2001). A role for the Adenomatous Polyposis Coli protein in chromosome segregation. *Nat Cell Biol* **3,** 429-432.

Karam, S. M. (1999). Lineage commitment and maturation of epithelial cells in the gut. *Front Biosci* **4,** D286-298.

Kawasaki, Y., Senda, T., Ishidate, T., Koyama, R., Morishita, T., Iwayama, Y., Higuchi, O., and Akiyama, T. (2000). Asef, a link between the tumor suppressor APC and G-protein signaling. *Science* **289,** 1194-1197.

Kielman, M. F., Rindapaa, M., Gaspar, C., van Poppel, N., Breukel, C., van Leeuwen, S., Taketo, M. M., Roberts, S., Smits, R., and Fodde, R. (2002). Apc modulates embryonic stem-cell differentiation by controlling the dosage of beta-catenin signaling. *Nat Genet* **32,** 594-605.

Kinzler, K. W., Nilbert, M. C., Su, L. K., Vogelstein, B., Bryan, T. M., Levy, D. B., Smith, K. J., Preisinger, A. C., Hedge, P., McKechnie, D., and et al. (1991). Identification of FAP locus genes from chromosome 5q21. *Science* **253,** 661-665.

Kinzler, K. W., and Vogelstein, B. (1996). Lessons from hereditary colorectal cancer. *Cell* **87,** 159-170.

Kirkland, S. C. (1988). Polarity and differentiation of human rectal adenocarcinoma cells in suspension and collagen gel cultures. *J Cell Sci* **91 (Pt 4),** 615-621.

Kishida, S., Yamamoto, H., Hino, S., Ikeda, S., Kishida, M., and Kikuchi, A. (1999). DIX domains of Dvl and axin are necessary for protein interactions and their ability to regulate beta-catenin stability. *Mol Cell Biol* **19,** 4414-4422.

Kishida, S., Yamamoto, H., Ikeda, S., Kishida, M., Sakamoto, I., Koyama, S., and Kikuchi, A. (1998). Axin, a negative regulator of the wnt signaling pathway, directly interacts with adenomatous polyposis coli and regulates the stabilization of beta-catenin. *J Biol Chem* **273,** 10823-10826.

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

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

Korinek, V., Barker, N., Willert, K., Molenaar, M., Roose, J., Wagenaar, G., Markman, M., Lamers, W., Destree, O., and Clevers, H. (1998b). Two members of the Tcf family implicated in Wnt/beta-catenin signaling during embryogenesis in the mouse. *Mol Cell Biol* **18,** 1248-1256.

Kozak, M. (1987). An analysis of 5'-noncoding sequences from 699 vertebrate messenger RNAs. *Nucleic Acids Res* **15,** 8125-8148.

Levy, D. B., Smith, K. J., Beazer-Barclay, Y., Hamilton, S. R., Vogelstein, B., and Kinzler, K. W. (1994). Inactivation of both APC alleles in human and mouse tumors. *Cancer Res* **54,** 5953-5958.

Lewandoski, M., and Martin, G. R. (1997). Cre-mediated chromosome loss in mice. *Nat Genet* **17,** 223-225.

Lickert, H., Domon, C., Huls, G., Wehrle, C., Duluc, I., Clevers, H., Meyer, B. I., Freund, J. N., and Kemler, R. (2000). Wnt/(beta)-catenin signaling regulates the expression of the homeobox gene Cdx1 in embryonic intestine. *Development* **127,** 3805-3813.

Liu, W., Dong, X., Mai, M., Seelan, R. S., Taniguchi, K., Krishnadath, K. K., Halling, K. C., Cunningham, J. M., Boardman, L. A., Qian, C., Christensen, E., Schmidt, S. S., Roche, P. C., Smith, D. I., and Thibodeau, S. N. (2000). Mutations in AXIN2 cause colorectal cancer with defective mismatch repair by activating beta-catenin/TCF signalling. *Nat Genet* **26,** 146-147.

Lobe, C. G., Koop, K. E., Kreppner, W., Lomeli, H., Gertsenstein, M., and Nagy, A. (1999). Z/AP, a double reporter for cre-mediated recombination. *Dev Biol* **208,** 281-292.

Luttrell, L. M., Hawes, B. E., Touhara, K., van Biesen, T., Koch, W. J., and Lefkowitz, R. J. (1995). Effect of cellular expression of pleckstrin homology domains on Gi-coupled receptor signaling. *J Biol Chem* **270,** 12984-12989.

Mao, J., Wang, J., Liu, B., Pan, W., Farr, G. H., 3rd, Flynn, C., Yuan, H., Takada, S., Kimelman, D., Li, L., and Wu, D. (2001). Low-density lipoprotein receptor-related protein-5 binds to Axin and regulates the canonical Wnt signaling pathway. *Mol Cell* **7,** 801-809.

Marikawa, Y., and Elinson, R. P. (1998). beta-TrCP is a negative regulator of Wnt/beta-catenin signaling pathway and dorsal axis formation in Xenopus embryos. *Mech Dev* **77,** 75-80.

McCrea, P. D., Brieher, W. M., and Gumbiner, B. M. (1993). Induction of a secondary body axis in Xenopus by antibodies to beta-catenin. *J Cell Biol* **123,** 477-484.

McLean, I. W., and Nakane, P. K. (1974). Periodate-lysine-paraformaldehyde fixative. A new fixation for immunoelectron microscopy. *J Histochem Cytochem* **22,** 1077-1083.

Melton, D. W. (1994). Gene targeting in the mouse. *Bioessays* **16,** 633-638.

Miyaki, M., Iijima, T., Kimura, J., Yasuno, M., Mori, T., Hayashi, Y., Koike, M., Shitara, N., Iwama, T., and Kuroki, T. (1999). Frequent mutation of beta-catenin and APC genes in primary colorectal tumors from patients with hereditary nonpolyposis colorectal cancer. *Cancer Res* **59,** 4506-4509.

Miyazaki, J., Takaki, S., Araki, K., Tashiro, F., Tominaga, A., Takatsu, K., and Yamamura, K. (1989). Expression vector system based on the chicken beta-actin promoter directs efficient production of interleukin-5. *Gene* **79,** 269-277.

Molenaar, M., van de Wetering, M., Oosterwegel, M., Peterson-Maduro, J., Godsave, S., Korinek, V., Roose, J., Destree, O., and Clevers, H. (1996). XTcf-3 transcription factor mediates beta-catenin-induced axis formation in Xenopus embryos. *Cell* **86,** 391-399.

Moon, R. T., Brown, J. D., and Torres, M. (1997). WNTs modulate cell fate and behavior during vertebrate development. *Trends Genet* **13,** 157-162.

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

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

Moxey, P. C., and Trier, J. S. (1978). Specialized cell types in the human fetal small intestine. *Anat Rec* **191,** 269-285.

Moxey, P. C., and Trier, J. S. (1979). Development of villus absorptive cells in the human fetal small intestine: a morphological and morphometric study. *Anat Rec* **195,** 463-482.

Mueller, R., and Sarvetnick, N. (1995). Transgenic/knockout mice--tools to study autoimmunity. *Curr Opin Immunol* **7,** 799-803.

Munemitsu, S., Albert, I., Souza, B., Rubinfeld, B., and Polakis, P. (1995). Regulation of intracellular beta-catenin levels by the adenomatous polyposis coli (APC) tumor-suppressor protein. *Proc Natl Acad Sci U S A* **92,** 3046-3050.

Nagafuchi, A., and Takeichi, M. (1989). Transmembrane control of cadherin-mediated cell adhesion: a 94 kDa protein functionally associated with a specific region of the cytoplasmic domain of E-cadherin. *Cell Regul* **1,** 37-44.

Nagase, H., and Nakamura, Y. (1993). Mutations of the APC (adenomatous polyposis coli) gene. *Hum Mutat* **2,** 425-434.

Nakamura, T., Hamada, F., Ishidate, T., Anai, K., Kawahara, K., Toyoshima, K., and Akiyama, T. (1998). Axin, an inhibitor of the Wnt signalling pathway, interacts with beta-catenin, GSK-3beta and APC and reduces the beta-catenin level. *Genes Cells* **3,** 395-403.

Nathke, I. S., Adams, C. L., Polakis, P., Sellin, J. H., and Nelson, W. J. (1996). The adenomatous polyposis coli tumor suppressor protein localizes to plasma membrane sites involved in active cell migration. *J Cell Biol* **134,** 165-179.

Newell, K. J., Witty, J. P., Rodgers, W. H., and Matrisian, L. M. (1994). Expression and localization of matrix-degrading metalloproteinases during colorectal tumorigenesis. *Mol Carcinog* **10,** 199-206.

Novelli, M. R., Williamson, J. A., Tomlinson, I. P., Elia, G., Hodgson, S. V., Talbot, I. C., Bodmer, W. F., and Wright, N. A. (1996). Polyclonal origin of colonic adenomas in an XO/XY patient with FAP. *Science* **272,** 1187-1190.

Nusse, R. (1997). A versatile transcriptional effector of Wingless signaling. *Cell* **89,** 321-323.

Okamoto, R., Yajima, T., Yamazaki, M., Kanai, T., Mukai, M., Okamoto, S., Ikeda, Y., Hibi, T., Inazawa, J., and Watanabe, M. (2002). Damaged epithelia regenerated by bone marrow-derived cells in the human gastrointestinal tract. *Nat Med* **8,** 1011-1017.

Orsulic, S., and Peifer, M. (1996). An in vivo structure-function study of armadillo, the beta-catenin homologue, reveals both separate and overlapping regions of the protein required for cell adhesion and for wingless signaling. *J Cell Biol* **134,** 1283-1300.

Oshima, H., Oshima, M., Kobayashi, M., Tsutsumi, M., and Taketo, M. M. (1997). Morphological and molecular processes of polyp formation in Apc(delta716) knockout mice. *Cancer Res* **57,** 1644-1649.

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

Ozawa, M., Baribault, H., and Kemler, R. (1989). The cytoplasmic domain of the cell adhesion molecule uvomorulin associates with three independent proteins structurally related in different species. *Embo J* **8,** 1711-1717.

Park, H. S., Goodlad, R. A., and Wright, N. A. (1995). Crypt fission in the small intestine and colon. A mechanism for the emergence of G6PD locus-mutated crypts after treatment with mutagens. *Am J Pathol* **147,** 1416-1427.

Parr, B. A., and McMahon, A. P. (1994). Wnt genes and vertebrate development. *Curr Opin Genet Dev* **4,** 523-528.

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

Polakis, P. (1999). The oncogenic activation of beta-catenin. *Curr Opin Genet Dev* **9,** 15-21.

Polakis, P. (2000). Wnt signaling and cancer. *Genes Dev* **14,** 1837-1851.

Potten, C. S. (1998). Stem cells in gastrointestinal epithelium: numbers, characteristics and death. *Philos Trans R Soc Lond B Biol Sci* **353,** 821-830.

Potten, C. S., and Loeffler, M. (1990). Stem cells: attributes, cycles, spirals, pitfalls and uncertainties. Lessons for and from the crypt. *Development* **110,** 1001-1020.

Potter, H., Weir, L., and Leder, P. (1984). Enhancer-dependent expression of human kappa immunoglobulin genes introduced into mouse pre-B lymphocytes by electroporation. *Proc Natl Acad Sci U S A* **81,** 7161-7165.

Powell, D. W., Mifflin, R. C., Valentich, J. D., Crowe, S. E., Saada, J. I., and West, A. B. (1999). Myofibroblasts. II. Intestinal subepithelial myofibroblasts. *Am J Physiol* **277,** C183-201.

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

Quaroni, A., Calnek, D., Quaroni, E., and Chandler, J. S. (1991). Keratin expression in rat intestinal crypt and villus cells. Analysis with a panel of monoclonal antibodies. *J Biol Chem* **266,** 11923-11931.

Roose, J., and Clevers, H. (1999). TCF transcription factors: molecular switches in carcinogenesis. *Biochim Biophys Acta* **1424,** M23-37.

Roose, J., Huls, G., van Beest, M., Moerer, P., van der Horn, K., Goldschmeding, R., Logtenberg, T., and Clevers, H. (1999). Synergy between tumor suppressor APC and the beta-catenin-Tcf4 target Tcf1. *Science* **285,** 1923-1926.

Roose, J., Molenaar, M., Peterson, J., Hurenkamp, J., Brantjes, H., Moerer, P., van de Wetering, M., Destree, O., and Clevers, H. (1998). The Xenopus Wnt effector XTcf-3 interacts with Groucho-related transcriptional repressors. *Nature* **395,** 608-612.

Rubinfeld, B., Albert, I., Porfiri, E., Fiol, C., Munemitsu, S., and Polakis, P. (1996). Binding of GSK3beta to the APC-beta-catenin complex and regulation of complex assembly. *Science* **272,** 1023-1026.

Rubinfeld, B., Albert, I., Porfiri, E., Munemitsu, S., and Polakis, P. (1997). Loss of beta-catenin regulation by the APC tumor suppressor protein correlates with loss of structure due to common somatic mutations of the gene. *Cancer Res* **57,** 4624-4630.

Rubinfeld, B., Souza, B., Albert, I., Muller, O., Chamberlain, S. H., Masiarz, F. R., Munemitsu, S., and Polakis, P. (1993). Association of the APC gene product with beta-catenin. *Science* **262,** 1731-1734.

Saam, J. R., and Gordon, J. I. (1999). Inducible gene knockouts in the small intestinal and colonic epithelium. *J Biol Chem* **274,** 38071-38082.

Sambrook, J., and Russell, D. (2001). Molecular Cloning: A Laboratory Manual, pp. 6.33-36.55. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sands, A. T., Hansen, T. N., Demayo, F. J., Stanley, L. A., Xin, L., and Schwartz, R. J. (1993). Cytoplasmic beta-actin promoter produces germ cell and preimplantation embryonic transgene expression. *Mol Reprod Dev* **34,** 117-126.

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

Schmidt, G. H., Winton, D. J., and Ponder, B. A. (1988). Development of the pattern of cell renewal in the crypt-villus unit of chimaeric mouse small intestine. *Development* **103,** 785-790.

Shastry, B. S. (1998). Gene disruption in mice: models of development and disease. *Mol Cell Biochem* **181,** 163-179.

Shibata, H., Toyama, K., Shioya, H., Ito, M., Hirota, M., Hasegawa, S., Matsumoto, H., Takano, H., Akiyama, T., Toyoshima, K., Kanamaru, R., Kanegae, Y., Saito, I., Nakamura, Y., Shiba, K., and Noda, T. (1997). Rapid colorectal adenoma formation initiated by conditional targeting of the Apc gene. *Science* **278,** 120-123.

Shih, I. M., Yu, J., He, T. C., Vogelstein, B., and Kinzler, K. W. (2000). The beta-catenin binding domain of adenomatous polyposis coli is sufficient for tumor suppression. *Cancer Res* **60,** 1671-1676.

Shtutman, M., Zhurinsky, J., Simcha, I., Albanese, C., D'Amico, M., Pestell, R., and Ben-Ze'ev, A. (1999). The cyclin D1 gene is a target of the beta-catenin/LEF-1 pathway. *Proc Natl Acad Sci U S A* **96,** 5522-5527.

Sikora, K., Chan, S., Evan, G., Gabra, H., Markham, N., Stewart, J., and Watson, J. (1987). c-myc oncogene expression in colorectal cancer. *Cancer* **59,** 1289-1295.

Simon, T. C., Cho, A., Tso, P., and Gordon, J. I. (1997). Suppressor and activator functions mediated by a repeated heptad sequence in the liver fatty acid-binding protein gene (Fabpl). Effects on renal, small intestinal, and colonic epithelial cell gene expression in transgenic mice. *J Biol Chem* **272,** 10652-10663.

Simon, T. C., Roth, K. A., and Gordon, J. I. (1993). Use of transgenic mice to map cis-acting elements in the liver fatty acid-binding protein gene (Fabpl) that regulate its cell lineage-specific, differentiation-dependent, and spatial patterns of expression in the gut epithelium and in the liver acinus. *J Biol Chem* **268,** 18345-18358.

Smalley, M. J., Sara, E., Paterson, H., Naylor, S., Cook, D., Jayatilake, H., Fryer, L. G., Hutchinson, L., Fry, M. J., and Dale, T. C. (1999). Interaction of axin and Dvl-2 proteins regulates Dvl-2-stimulated TCF-dependent transcription. *Embo J* **18,** 2823-2835.

Smith, D. R., Myint, T., and Goh, H. S. (1993). Over-expression of the c-myc proto-oncogene in colorectal carcinoma. *Br J Cancer* **68,** 407-413.

Smith, K., Bui, T. D., Poulsom, R., Kaklamanis, L., Williams, G., and Harris, A. L. (1999). Up-regulation of macrophage wnt gene expression in adenoma-carcinoma progression of human colorectal cancer. *Br J Cancer* **81,** 496-502.

Sonnenberg, E., Meyer, D., Weidner, K. M., and Birchmeier, C. (1993). Scatter factor/hepatocyte growth factor and its receptor, the c-met tyrosine kinase, can mediate a signal exchange between mesenchyme and epithelia during mouse development. *J Cell Biol* **123,** 223-235.

Soriano, P. (1999). Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nat Genet* **21,** 70-71.

Southern, E. M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J Mol Biol* **98,** 503-517.

Sparks, A. B., Morin, P. J., Vogelstein, B., and Kinzler, K. W. (1998). Mutational analysis of the APC/beta-catenin/Tcf pathway in colorectal cancer. *Cancer Res* **58,** 1130-1134.

Srinivas, S., Watanabe, T., Lin, C. S., William, C. M., Tanabe, Y., Jessell, T. M., and Costantini, F. (2001). Cre reporter strains produced by targeted insertion of EYFP and ECFP into the ROSA26 locus. *BMC Dev Biol* **1,** 4.

Stappenbeck, T. S., and Gordon, J. I. (2001). Extranuclear sequestration of phospho-Jun N-terminal kinase and distorted villi produced by activated Rac1 in the intestinal epithelium of chimeric mice. *Development* **128,** 2603-2614.

Sternberg, N., and Hamilton, D. (1981). Bacteriophage P1 site-specific recombination. I. Recombination between loxP sites. *J Mol Biol* **150,** 467-486.

St-Jacques, B., and McMahon, A. P. (1996). Early mouse development: lessons from gene targeting. *Curr Opin Genet Dev* **6,** 439-444.

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

Su, L. K., Vogelstein, B., and Kinzler, K. W. (1993). Association of the APC tumor suppressor protein with catenins. *Science* **262,** 1734-1737.

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

Takemaru, K. I., and Moon, R. T. (2000). The transcriptional coactivator CBP interacts with beta-catenin to activate gene expression. *J Cell Biol* **149,** 249-254.

Takeuchi, N., Ichikawa, Y., Ishikawa, T., Momiyama, N., Hasegawa, S., Nagashima, Y., Miyazaki, K., Koshikawa, N., Mitsuhashi, M., and Shimada, H. (1997). Matrilysin gene expression in sporadic and familial colorectal adenomas. *Mol Carcinog* **19,** 225-229.

Tamai, K., Semenov, M., Kato, Y., Spokony, R., Liu, C., Katsuyama, Y., Hess, F., Saint-Jeannet, J. P., and He, X. (2000). LDL-receptor-related proteins in Wnt signal transduction. *Nature* **407,** 530-535.

Tang, J., Hu, M., Lee, S., and Roblin, R. (2000). A polymerase chain reaction based method for detecting Mycoplasma/Acholeplasma contaminants in cell culture. *J Microbiol Methods* **39,** 121-126.

Tetsu, O., and McCormick, F. (1999). Beta-catenin regulates expression of cyclin D1 in colon carcinoma cells. *Nature* **398,** 422-426.

Theodosiou, N. A., and Tabin, C. J. (2003). Wnt signaling during development of the gastrointestinal tract. *Dev Biol* **259,** 258-271.

Tirnauer, J. S., and Bierer, B. E. (2000). EB1 proteins regulate microtubule dynamics, cell polarity, and chromosome stability. *J Cell Biol* **149,** 761-766.

Townsley, F. M., and Bienz, M. (2000). Actin-dependent membrane association of a Drosophila epithelial APC protein and its effect on junctional Armadillo. *Curr Biol* **10,** 1339-1348.

Trier, J. S., and Moxey, P. C. (1979). Morphogenesis of the small intestine during fetal development. *Ciba Found Symp,* 3-29.

Tumbar, T., Guasch, G., Greco, V., Blanpain, C., Lowry, W. E., Rendl, M., and Fuchs, E. (2004). Defining the epithelial stem cell niche in skin. *Science* **303,** 359-363.

Van de Wetering, M., Castrop, J., Korinek, V., and Clevers, H. (1996). Extensive alternative splicing and dual promoter usage generate Tcf-1 protein isoforms with differential transcription control properties. *Mol Cell Biol* **16,** 745-752.

van de Wetering, M., Cavallo, R., Dooijes, D., van Beest, M., van Es, J., Loureiro, J., Ypma, A., Hursh, D., Jones, T., Bejsovec, A., Peifer, M., Mortin, M., and Clevers, H. (1997). Armadillo coactivates transcription driven by the product of the Drosophila segment polarity gene dTCF. *Cell* **88,** 789-799.

van de Wetering, M., Sancho, E., Verweij, C., de Lau, W., Oving, I., Hurlstone, A., van der Horn, K., Batlle, E., Coudreuse, D., Haramis, A. P., Tjon-Pon-Fong, M., Moerer, P., van den Born, M., Soete, G., Pals, S., Eilers, M., Medema, R., and Clevers, H. (2002). The beta-catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells. *Cell* **111,** 241-250.

van Den Brink, G. R., de Santa Barbara, P., and Roberts, D. J. (2001). Development. Epithelial cell differentiation--a Mather of choice. *Science* **294,** 2115-2116.

van Es, J. H., Giles, R. H., and Clevers, H. C. (2001). The many faces of the tumor suppressor gene APC. *Exp Cell Res* **264,** 126-134.

Waltzer, L., and Bienz, M. (1998). Drosophila CBP represses the transcription factor TCF to antagonize Wingless signalling. *Nature* **395,** 521-525.

Willert, K., and Nusse, R. (1998). Beta-catenin: a key mediator of Wnt signaling. *Curr Opin Genet Dev* **8,** 95-102.

Willert, K., Shibamoto, S., and Nusse, R. (1999). Wnt-induced dephosphorylation of axin releases beta-catenin from the axin complex. *Genes Dev* **13,** 1768-1773.

Wilson, C. L., Heppner, K. J., Labosky, P. A., Hogan, B. L., and Matrisian, L. M. (1997). Intestinal tumorigenesis is suppressed in mice lacking the metalloproteinase matrilysin. *Proc Natl Acad Sci U S A* **94,** 1402-1407.

Wodarz, A., and Nusse, R. (1998). Mechanisms of Wnt signaling in development. *Annu Rev Cell Dev Biol* **14,** 59-88.

Wong, M. H., Huelsken, J., Birchmeier, W., and Gordon, J. I. (2002). Selection of multipotent stem cells during morphogenesis of small intestinal crypts of Lieberkuhn is perturbed by stimulation of Lef-1/beta-catenin signaling. *J Biol Chem* **277,** 15843-15850.

Wong, M. H., Rubinfeld, B., and Gordon, J. I. (1998). Effects of forced expression of an NH2-terminal truncated beta-Catenin on mouse intestinal epithelial homeostasis. *J Cell Biol* **141,** 765-777.

Wong, M. H., Saam, J. R., Stappenbeck, T. S., Rexer, C. H., and Gordon, J. I. (2000). Genetic mosaic analysis based on Cre recombinase and navigated laser capture microdissection. *Proc Natl Acad Sci U S A* **97,** 12601-12606.

Wright, N. A. (2000). Epithelial stem cell repertoire in the gut: clues to the origin of cell lineages, proliferative units and cancer. *Int J Exp Pathol* **81,** 117-143.

Wurst, W., and Joyner, A. (1993). Production of targeted embryonic stem cell clones. In: Joyner, A., editor. Gene Targeting: A Practical Approach. *In* "The Practical Approach Series" (H. Rickwood D., B.D., Ed.), pp. 33-62. Oxford: IRL Press.

Yamamoto, H., Kishida, S., Kishida, M., Ikeda, S., Takada, S., and Kikuchi, A. (1999). Phosphorylation of axin, a Wnt signal negative regulator, by glycogen synthase kinase-3beta regulates its stability. *J Biol Chem* **274,** 10681-10684.

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

Yost, C., Torres, M., Miller, J. R., Huang, E., Kimelman, D., and Moon, R. T. (1996). The axis-inducing activity, stability, and subcellular distribution of beta-catenin is regulated in Xenopus embryos by glycogen synthase kinase 3. *Genes Dev* **10,** 1443-1454.

Yu, X., Waltzer, L., and Bienz, M. (1999). A new Drosophila APC homologue associated with adhesive zones of epithelial cells. *Nat Cell Biol* **1,** 144-151.

Zambrowicz, B. P., Imamoto, A., Fiering, S., Herzenberg, L. A., Kerr, W. G., and Soriano, P. (1997). Disruption of overlapping transcripts in the ROSA beta geo 26 gene trap strain leads to widespread expression of beta-galactosidase in mouse embryos and hematopoietic cells. *Proc Natl Acad Sci U S A* **94,** 3789-3794.

Zeng, L., Fagotto, F., Zhang, T., Hsu, W., Vasicek, T. J., Perry, W. L., 3rd, Lee, J. J., Tilghman, S. M., Gumbiner, B. M., and Costantini, F. (1997). The mouse Fused locus encodes Axin, an inhibitor of the Wnt signaling pathway that regulates embryonic axis formation. *Cell* **90,** 181-192.