SUPPRESSOR OF CYTOKINE SIGNALING-2 (SOCS2) AND EPIDERMAL GROWTH FACTOR (EGF) MODULATE INSULIN-LIKE GROWTH FACTOR-I RECEPTOR (IGF-IR) SIGNALING IN INTESTINAL CANCER

VICTORIA A. NEWTON

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Approved by

Advisor: Dr. Pauline Kay Lund

Reader: Dr. James Anderson

Reader: Dr. Adrienne Cox

Reader: Dr. Carol Otey

Reader: Dr. David Threadgill

ABSTRACT

VICTORIA A. NEWTON: SOCS2 and EGF modulate insulin-like growth factor signaling in intestinal cancer.
(Under the direction of Dr. Pauline Kay Lund)

The insulin-like growth factor (IGF-I) pathway is associated with increased risk and progression of colorectal cancer. Understanding mechanisms that inhibit or promote this key pathway would be useful in developing better therapies and strategies for the treatment of colorectal cancer. Suppressor of cytokine signaling-2 (SOCS2) limits the trophic effects of IGF-I in the intestine and therefore, may negatively regulate intestinal tumorigenesis. We crossed SOCS2$^{-/-}$ mice with *Apc*$^{Min/+}$ mice, a widely used model of spontaneous intestinal tumorigenesis, to test whether loss of SOCS2 promotes increased intestinal tumorigenesis and to identify possible mechanisms. Epidermal growth factor (EGF) is also associated with establishment and progression of colorectal cancer. Studies suggest that EGF impacts on IGF-IR signaling to synergistically increase growth in non-transformed intestinal epithelial cells and that combined inhibition of EGF and IGF-I would be more effective in reducing growth and survival of intestinal tumors than inhibition of either pathway alone. Whether EGF and IGF-I synergistically promote activation of tumor-promoting pathways in normal intestinal epithelial cells is unknown. In addition, combined inhibition of IGF-I and EGF pathways in a model of sporadic intestinal tumorigenesis has not been tested. To test synergistic effects of EGF and IGF-I, we used IEC-6 cells, a non-transformed intestinal epithelial cell line, to assess

mechanisms of additive or synergistic activation of the IGF-IR and a key tumor promoting pathway in colorectal cancer, the β-catenin pathway. Combined inhibition of IGF-IR and EGFR signaling on intestinal tumor development was assessed using *Apc*$^{Min/+}$ mice lacking one allele of IRS-1, a downstream signaling molecule that mediates trophic effects of IGF signaling, and given a specific EGFR inhibitor. Loss of SOCS2 promoted significant increases in tumor number, size, and load in the small intestine and colon of *Apc*$^{Min/+}$ mice. This was associated with increases local IGF-I, serine-phosphorylation of STAT3, a downstream mediator of IGF-I action and a target of SOCS2, and enhanced AP-1 DNA binding. In IEC-6 cells, EGF treatment increases IGF-IR, activation and combined treatment of EGF and IGF-I additively increases nuclear β-catenin and its transcriptional activation. Combined heterozygous deletion of IRS-1 and inhibition of the EGFR dramatically reduced tumor number, size, and load, as well as tumor incidence, in the colon of *Apc*$^{Min/+}$ mice. Surprisingly, this effect was confined to female mice and was not observed in male mice. Together, these studies identified novel mechanisms that regulate IGF-IR signaling. Our studies indicate that SOCS2 may be a useful biomarker of colorectal cancer. The interactions between IGF-I and EGF suggest that combined inhibition of these pathways will be more effective in treating colorectal cancer, than use of either treatment alone. Future studies using microarrays will identify molecular pathways that are activated tumors and normal tissue in response to complete loss of SOCS2 in *Apc*$^{Min/+}$ mice or in response to combined activation or inhibition of EGF and IGF-I signaling.

DEDICATION

I would like to dedicate my thesis to my father, Terry, my mother, Sharon, and my sister Stephanie—Love you guys…and to my soon-to be husband, Monu Bali, for his support, love, and patience despite my obvious nerdiness.

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LIST OF ABBREVIATIONS

Ad	Adenovirus
Amph	Amphiregullin
AMV	Avian Myeloblastosis Virus
ANOVA	Analysis of Variance
ANS	Autonomic Nervous System
AOM	azoxymethane
AP-1	Activator Protein-1
APC	Adenomatous Polyposis Coli
CACC	Colitis-Associated Colorectal Cancer
CIN	Chromosomal instability
CIS	Cytokine-inducible SH2-containing protein
CML	Chronic Myeloid Leukemia
CNS	Central Nervous System
COX-2	Cyclooxygenase-2
CpG	Cytosine-phosphate-Guanine, refers to a nucleotide sequence
CPM	Counts per Minute
CRC	Colorectal Cancer
CSC	Cancer Stem Cell
CYP	Cytochrome P450
DAB	3,3'-Diaminobenzidine
DSS	Dextran Sodium Sulfate
EB	Enteroblast
EC	Enterocyte Columnar (Absorptive Enterocyte)
EE	Enteroendocrine Enterocyte

EGFR	Epidermal growth factor receptor
ELISA	Enzyme-Linked Immunosorbant Assay
EPO	Erythropoietin
ERK	Extracellular Signal-Regulated Kinase
FAP	Familial Adenomatous Polyposis
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
GH	Growth Hormone
GHRH	Growth Hormone Releasing Hormone
GI	Gastrointestinal
GMCSF	Granulocyte Macrophage Colony-Stimulating Factor
GNMT	Glycine N-Methyltransferase
GSK3β	Glycogen Synthase Kinase 3 Beta
HCC	Hepatocellular Carcinoma
HDAC	Histone Deacetylase
HG	High Growth
HMBS	Hydroxymethylbilane synthase
Hop	Hopscotch (Drosophila JAK homologue)
HPNCC	Hereditary non-polyposis colorectal cancer
HRT	Hormone Replacement Therapy
IEC	Intestinal epithelial Cell
IGFBP	Insulin-like growth factor Binding Protein
IGF-I	Insulin-like growth factor I
IGF-IR	Type I IGF receptor
IL	Interleukin

INF	Interferon
IR	Insulin Receptor
IRS-1	Insulin Receptor Substrate
JAK	Janus Kinase
JNK	c-Jun N-terminal Kinase
KIR	Kinase Inhibitor Region
LEF	Lymphoid Enhancer Factor
LIF	Leukemia Inhibitory Factor
LOH	Loss of Heterozygosity
LOI	Loss of Imprinting
MAPK	Mitogen-Activate Protein Kinase
MMR	Mismatch Repair
MSI	Microsatellite Instability
MT	Metallothionein
MWM	Molecular Weight Marker
NE	Nuclear Extract
NRG	Neuregulin
NSCLC	Non-Small Cell Lung Carcinoma
OSM	Oncostatin M
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate-Buffered Saline
PI-3K	Phosphoinositide 3-Kinase
PMSF	Phenylmethanesulfonylfluoride
pS	Phosphoserine
PTB	Phosphotyrosine Binding Domain

PTEN	Phosphatase and Tensin Homolog
pY	Phosphotyrosine
RE	Response Element
SDS	Sodium Dodecyl Sulfate
SEM	Standard Error of the Mean
SFM	Serum-Free Medium
SH2	Src Homology-2
SHC	Src Homology-2 Containing Protein
SM	Smooth muscle
SOCS	Suppressor of Cytokine Signaling
STAT	Signal Transducers and Activators of Transcription
TA	Transit Amplifying
TCF	T-cell Factor
Tg	Transgenic
TGF	Transforming Growth Factor
TISC	Tumor-initiating Stem Cell
TK	Thymidine Kinase
TPN	Total Parenteral Nutrition
Upd	Unpaired
V	Vehicle
WCE	Whole Cell Extract
WT	Wild-type

CHAPTER I

INTRODUCTION AND BACKGROUND

A. The structure and physiology of the small and large intestine

The primary function of the small intestine is to digest and absorb nutrients, water, and electrolytes.[1] The large intestine concentrates waste and reabsorbs water and electrolytes.[1] The intestine is a barrier to luminal toxins and microorganisms.[1] The musocal layer consists of a lining of epithelial cells in contact with the lumen, the underlying lamina propria, and the muscularis mucosa (Figure 1.1A). The lamina propria is a supporting network of loose connective tissue containing fibroblasts, myofibroblasts, smooth muscle cells, capillaries, enteric neurons, and immune cells. The muscularis mucosa is a thin layer of smooth muscle found below the lamina propria. Under the mucosal layer lies the submucosa, a loose connective tissue that houses the submucosal neuronal plexus, large blood vessels, and fibroblasts. In the duodenum, the submucosa contains Brunner's glands (Figure 1.1A). The muscularis externa contains two layers of smooth muscle, a circular inner layer and a longitudinal outer layer and is responsible for the peristalic waves of contraction that propel and churn luminal contents.[1] Peristalic contractions are produced by the intrinsic rhythmic contractility of the smooth muscle cells, where gap junctions allow the propagation of electrical signals throughout the intestine.[1] This process is regulated by the enteric nervous system, which functions as part of the autonomic nervous system (ANS). The submucosal (Meisner's) plexus (located in the submucosa) and the myenteric (Auerbach plexus, located between the circular and longitudinal muscle layers) regulate intestinal motility and also epithelial function.[1] While the enteric nervous system can operate independently of the central nervous system (CNS), the CNS can alter intestinal programs in response to external stimuli such as the sight or smell of food.

Digestion of food begins during mastication. Chewed material is propelled through the esophagus and into the stomach, where it is further broken down by mechanical churning and enzymatic and acidic digestion.[1] When contents are almost completely digested into a mix called chyme, it enters the duodenum, the proximal portion of the small intestine. At that time, the gallbladder releases bile acids and the pancreas releases enzymes to chemically break down nutrients.[1] Digestion continues as chyme moves from the duodenum to jejunum. Brush border enzymes in the intestinal epithelium finalize digestion and transport monosaccharides and amino acids across the epithelial layer into the interstitial space and into the bloodstream.[1] Nutrients are taken through the portal circulation in the liver where they are distributed throughout the body.[1] Lipids are re-esterified into triglycerides and packaged into chylomicrons. Nutrients enter lymphatic vessels and then re-enter the venous circulation. Bile acids are reabsorbed in the ileum, the distal portion of the small intestine. Fecal contents are moved into the large intestine, where water and electrolytes are reclaimed and waste is concentrated and excreted.[1] Immune cells and immune aggregates called Peyer's patches aid in destroying pathogenic bacteria that are constantly exposed to the luminal environment, while maintaining normal intestinal microflora that aid in the breakdown of non-digestible components, promote fat deposition and prevent the overgrowth of pathogenic bacteria.[1]

B. The intestinal epithelium and intestinal adaption.

The intestinal epithelium is organized in a crypt-villus axis (Figure 1.1B). Villi are finger-like projections that start at the top of the crypt and are found only in the small

intestine. Crypts are deep invaginations that are throughout the entire intestine. This organization allows for a vast surface area, which aids in efficient nutrient digestion and the transport of nutrients, water, and electrolytes.[1] Intestinal stem cells (ISCs) reside in the crypt base and undergo asymmetric cell division to maintain the resident stem cell and produce an undifferentiated transit amplifying (TA) progenitor cell, which continues to divide for several cycles and migrate up the crypt towards the lumen. TA cells differentiate, migrate onto the villus, towards the villus tip where they are sloughed off in a process called anoikis (Figure 1.1B). Thus, the crypt-villus axis functions to separate the proliferative zone in the crypt from the differentiation zone in the villus. This process completely replenishes of the intestinal epithelium every 3-5 days, though this varies with species.[2]

Small intestine stem cells differentiate into four main cell types: 1) entero-endocrine, 2) absorptive, 3) goblet, and 4) Paneth cells. Entero-endocrine cells store and secrete hormones that regulate digestive processes in response to neural or mechanical stimuli.[3] Absorptive cells act to transport small peptides, amino acids, lipids, and basic sugars from the lumen to the bloodstream.[4] Goblet cells secrete a lubricating layer of mucus to protect the epithelium and aid the movement of digestive contents.[5] Paneth cells are differentiated cells that do not move up the crypt, but instead migrate downwards and reside at the very base of the crypt.[6] These cells are highly granulated and are thought to play a role in microbial defense.[7]

The intestinal epithelium must balance the rate of proliferation with the rate of apoptosis to maintain intestinal integrity, digestion, and nutrient uptake. In response to changes in these processes, the intestine alters its rate of proliferation and apoptosis to

produce morphological and functional changes in the mucosa, in a process called intestinal adaption.[8] Fasted rats or those fed by total parenteral nutrition (TPN), which are fed intravenously, demonstrate a compensatory atrophy in the intestinal mucosa and show decreases in both villus height and crypt depth.[8] Mice that have undergone surgical resection, where a large portion of the small intestine is removed, demonstrate increases in mucosal mass due to increases in villus height, crypt fission and overall increase in circumference of the small intestine to compensate for the loss of surface area.[9] Growth factors influence the rates of these adaptive processes.[10] GH or IGF-I promote increases in mucosal mass following TPN-feeding and transgenic models overexpressing GH or IGF-I show increases in the length and mass of the small intestine, as well as hyperplasia.[11,12,13] EGF is also required for intestinal adaptation, as pharmacological or genetic inhibition of EGFR prevented the adaptive response in mice that had undergone proximal small bowel resection.[14,15]

In contrast to the small intestine, the colon is organized into a crypt-surface axis. However, processes of stem and progenitor proliferation in the crypt and migration and differentiation of cells are similar to small intestine. The colon does not show as great a degree of adaptive growth as the small intestine, with less atrophy during fasting or TPN and less adaptive increases in mass after removal of the small intestine.[16] The colon does exhibit dramatic regenerative changes and hyperplasia during mucosal healing in response to damage by acute or chronic inflammation or other insults such as irradiation.[17, 18] The colon is also more susceptible to dysplastic changes leading to colon cancer.

C. Genetics of Colon Cancer

Despite advances in screening and detection, colorectal cancer is the 3rd most common cancer among men and women and is the second-leading cause of cancer-related deaths in the United States.[19] Several risk factors have been identified that promote increased risk of colorectal cancer, including family history, lifestyle (i.e. diet, physical activity, etc), and bowel inflammation.[20,21,22] These risk factors promote two major colorectal cancer pathways, sporadic colorectal cancer and inflammation-associated colorectal cancer (Figure 1.2). Identification of the underlying mechanisms that promote colorectal cancer has lead to the development of targeted and combined therapies.[23,24]

a. Sporadic colon cancer

Sporadic colon cancer arises in the absence of overt family history and occurs as a result of activation of oncogenes and inactivation of tumor suppressor genes.[25] Genomic instability leads to mutational accumulation and occurs primarily through two genetic pathways in colorectal cancer.[26] One pathway is chromosomal instability (CIN), where allelic loss of tumor suppressor genes drives the formation of adenomas to large adenomas and adenocarcinomas.[26] In CIN, the APC (adenomatous polyposis coli) gene is frequently mutated and promotes the development of adenomas (Figure 1.2).[27] Adenomas develop subsequent *K-ras* mutations and *p53* deletions and further allelic loss occurs during progression to adenocarcinoma (Figure 1.2).[26] This progression occurs in patients who have familial adenomatous polyposis (FAP). These patients have germ-line

mutations in the *APC* gene and develop hundreds to thousands of adenomas in their colon.[28]

Another pathway to colorectal cancer is microsatellite instability (MSI). MSI is characterized by alterations in the number of tandem repeats of simple DNA sequences or microsatellites and is caused by mutations or epigenetic silencing of DNA mismatch repair genes (MMR).[26] These repeats cause inactivating frameshift mutations in tumor suppressor genes.[29] MSI is associated with another form of hereditary colon cancer called hereditary non-polyposis colorectal cancer (HPNCC) and is characterized by defects in DNA mismatch repair enzymes.[26] CIN or MSI are found in sporadic colorectal cancer and a subset of MSI-high tumors have mutations in *APC* and *p53* genes, characteristic of CIN.[26]

b. Inflammation-associated colon cancer.

Colon cancer can also develop in a setting of chronic inflammation and this is called inflammation- or colitis-associated colorectal cancer (CACC). Patients with ulcerative colitis and Crohn's disease have increased life-time risk of developing colon cancer.[30] In this setting, excessive immune responses drive chronic inflammation and mucosal damage leading to repeated cycles of would healing, involving the proliferation of damaged cells. As opposed to sporadic colon cancer, in CACC, cancerous lesions arise from areas of flat non-polypoid dysplasia that progress from low to high grade (Figure 1.2).[31] The development of cancerous lesions from regions of dysplasia is promoted by the accumulation of mutations in various regulatory pathways. Both sporadic colon

cancer and CACC develop mutations resulting from CIN or MSI at relatively the same frequency; however, the timing of the accumulated mutations differs between sporadic colon cancer and CACC (Figure 1.2).[31] In CACC, APC mutations occur late in tumor development and are less frequent compared to sporadic CRC.[30] Mutations in p53, while a late event in sporadic colon cancer, are detected relatively early in CACC and often in areas of non- or indefinite dysplasia.[31] In addition, epigenetic silencing plays a large role in the early stages of CACC, where CpG methylation is found to precede the development of dysplasia and is frequently found in CAC tumors.[31]

D. The APC gene in Colon Cancer

APC is mutated in up to 85% of sporadic colon cancers and is mutated in most familial colon cancers such as FAP and Gardner syndrome.[28,32] APC normally exists as a complex with axin, glycogen-synthase 3β (GSK-3β), and β-catenin (Figure 1.3).[33] In the absence of Wnt ligands, β-catenin is serine/threonine phosphorylated by GSK-3β and targeted for proteasomal degradation.[33] In the presence of Wnt ligands, GSK3-β is inactivated and β-catenin accumulates in the cytosol and translocates into the nucleus where it induces the activation of t-cell factor (TCF)/lymphoid enhancer factor (LEF) transcription factors (Figure 1.3).[34] TCF/β-catenin complexes activate the expression of target genes such as c-myc and cyclin D1.[35] β-catenin is also complexed at the lateral membranes and sites of cellular adhesion in normal intestinal epithelial cells (IEC).[25] Mutations in APC prevent docking with GSK-3β and β-catenin is not degraded.[27]

Mouse models with inactivation of the APC/β-catenin pathway have established the role of aberrant β-catenin activation in the initiation of colorectal cancer. *Apc*$^{Min/+}$ mice are heterozygous for a mutation that results in a truncated form of APC, which lacks the GSK-3β binding site.[36] Depending on the genetic background, these mice develop tens to hundreds of spontaneous adenomas in the small intestine that typically arise due to focal loss of the remaining wild-type (WT) APC allele through somatic recombination resulting in cells homozygous for the APC mutation.[37,34] This is a process called loss-of-heterozygosity (LOH).[34] The WT APC allele also may be epigenetically silenced due to failure of MMR pathways.[38] *Apc*$^{Min/+}$ mice develop tumors in colon as well as small intestine, but this is heavily dependent on genetic background.[39] The *Apc*$^{Min/+}$ model is widely used to study mediators and mechanisms that govern the initiation, establishment and progression of intestinal tumors.

E. GH/IGF-I axis

a. GH and physiological actions

Growth hormone is a polypeptide hormone that regulates growth and metabolism. It is secreted by the anterior pituitary in a pulsatile manner into the bloodstream in response to growth hormone releasing hormone (GHRH) and ghrelin from the hypothalamus.[1] GH acts on a number of organs, namely liver, muscle and bone. GH exerts its effects by binding to the growth hormone receptor (GHR). The GHR is a single transmembrane glycoprotein that is a member of the cytokine superfamily of receptors.[40] Because the GHR lacks an intrinsic kinase domain, it exerts signaling effects through

Janus Kinase 2 (JAK2), a cytoplasmic tyrosine kinase that is physically and functionally associated with the GHR.[40] Upon receptor dimerization, JAK2 trans- and auto-phosphorylates itself and the GHR. JAK2 also phosphorylates signal-transducers and activators of transcription (STATs) at SH2-domains, resulting in homo- or hetero-dimerization. STAT dimers translocate into the nucleus and mediate gene-transcription by directly binding to the 5' regulatory region of certain DNA sequences via their DNA-binding domain. This induces the expression of genes associated with growth and metabolism, such as insulin, IGF-I, CYP genes and SOCS.[40]

GH is most widely known for its role in promoting growth and also plays an important role in metabolism. GH is responsible for increases in post-natal growth and muscle mass.[41] Excess secretion of GH, a condition called acromegaly, causes dramatic enlargement and thickening of the bones, coarse facial features and visceromegaly.[42] In contrast, GH deficiencies are associated with proportionally short-stature and delayed bone and muscle growth.[43] These effects are mimicked in mouse studies, where transgenic (Tg) overexpression of the bovine GH gene promotes gigantism and aberrant activation of GH/IGF-I signaling.[12] In addition, 'little mice", which have a deficiency in GH due to a recessive mutation in the growth hormone releasing hormone (GHRH) exhibit dwarfism and severely reduced levels of serum IGF-I.[44] GH is also exerts a number of metabolic effects, such as promoting lipolysis, protein synthesis, and hyperglycemia.[45]

b. IGF-I family

The IGF system is composed of the ligands, IGF-I, IGF-II, and insulin, whose effects are mediated by the type I and type 2 IGF receptors (IGF-IR and IGF-IIR) and the insulin receptor (IR)[46] (Figure 1.4). In addition, a family of 7 binding proteins (IGFBP1-7) alters the bioavailability of circulating IGFs and locally expressed tissue IGFs. The IGF-IR binds IGF-I and IGF-II with high affinity and can also bind insulin. Hybrid IGF/insulin receptors exist and bind IGFs with high affinity and some bind insulin.[47] The IGF-IR is the primary mediator of the trophic actions of both IGF-I and IGF-II. The insulin receptor mediates the metabolic actions of low physiological levels of insulin. At elevated levels, insulin can have proliferative and anti-apoptotic effects, which may be mediated by IR, IGF-IR, or hybrid IGF-I/IR. The type 2 IGF receptor specifically binds IGF-II and acts as a scavenger to limit IGF-II binding to IGF-IR.[46]

Circulating levels of IGF-I are derived largely from liver (~75%) and are regulated by insulin, caloric intake, and growth hormone (GH).[48] GH engages GHRs on hepatocytes to stimulate IGF-I production in the liver and its secretion into circulation. GH can acts on other organs, including the intestine to stimulate IGF-I synthesis.[49] IGF-I is expressed in most, if not all, non-hepatic tissues.[48] Studies from our lab have demonstrated that IGF-I is synthesized in mesenchymal cells within non-hepatic tissues, including intestine, and has paracrine effects on neighboring epithelium.[48,50] Studies in mouse models suggest that IGF-II is critical for embryonic growth. Imprinting normally silences the maternal IGF-II allele.[51] Loss of imprinting (LOI) for IGF-II increases local IGF-II in the intestine and this is prevalent in patients with adenoma.[52]

The IGF-IR consists of 2 extracellular α subunits and 2 intracellular β subunits. IGF-I or IGF-II bind the extracellular portion of the IGF-IR and induce a conformational change that results in autophosphorylation of the intracellular β subunits.[46,48] This signal is enhanced by phosphorylation of additional tyrosine residues in the β subunit. Downstream docking proteins, such as insulin receptor substrate 1 (IRS-1) and Shc, are recruited to these sites and are subsequently tyrosine phosphorylated. This activates PI-3K/Akt and Ras/Raf/MAPK signaling cascades to induce proliferation, inhibit apoptosis, and regulate differentiation.[46] In vitro studies suggest that IGF-I increases the stability of β-catenin and that IGF-IR signaling components may interact with β-catenin.[53,54] In C10 colorectal cancer cells, IGF-IR and β-catenin co-immunoprecipitated with E-cadherin and this was reduced by IGF-I treatment.[54] In embryonic fibroblasts, IGF-I stimulated IRS-1 translocation to the nucleus and IRS-1 co-localization with β-catenin.[53]

c. IRS-1 mediates the trophic effects of IGF-I

IRS-1, IRS-2, and Shc are the main signaling substrates recruited to the IGF-IR or IR.[46] While IGF-IR and IR can activate both IRS-1 and IRS-2 in cell lines, in vivo evidence suggests that IRS-1 and IRS-2 have distinct roles (Figure 1.4). IRS-$1^{-/-}$ mice show reduced body growth, modest reductions in small intestine weight and impaired glucose tolerance, but do not develop type II diabetes.[55,56] IRS-$2^{-/-}$ mice have normal body and organ growth, but develop type II diabetes.[57,58] Studies from our laboratory using IRS-$1^{-/-}$ mice crossed with mice overexpressing an IGF-I transgene demonstrated that IRS-1 is a major mediator of IGF-I-dependent growth in multiple organs, but the

degree of dependence on IRS-1 is organ-specific.[59] IGF-I-stimulated skeletal muscle growth was the most impaired in IRS-1$^{-/-}$ mice, yet brain growth was not significantly affected.[59] IGF-I induced intestinal growth showed intermediate dependence on IRS-1 indicating that while IRS-1 is necessary for normal IGF-I-induced intestinal growth, other pathways can contribute to growth effects of IGF-I in intestine.[59] Our studies examined the role of IRS-1 in IGF-I transgene-induced growth of colon mucosa or smooth muscle and revealed cell- and process-specific requirements for IRS-1. Enteric smooth muscle growth, induced by two different IGF-I transgenes (MT-hIGF-I or αSMactin-hIGF-I), was unaffected by homozygous disruption of both IRS-1 genes.[60] Disruption of both IRS-1 alleles attenuated IGF-I transgene-induced overgrowth of colon mucosa.[60] This reflected complete loss of the anti-apoptotic effects of IGF-I in IRS-1$^{-/-}$ colon crypts, but only partial loss of IGF-I-induced crypt proliferation suggesting that IRS-1 is a required in vivo mediator of the anti-apoptotic effects, and to a lesser extent proliferative effects, of IGF-I in the colon.[60] IRS-1$^{-/-}$ mice have increased basal and irradiation-induced apoptosis localized to the stem cell region of the crypts.[61] Loss of IRS-1 also protected against tumor development in *Apc*$^{Min/+}$ mice.[61] Given the key role of IRS-1 in mediating IGF-I action in intestine, and the fact that IRS-1 likely mediates the proliferative and anti-apoptotic actions of IGF-II, and possibly insulin, studies in chapter IV used IRS-1$^{-/-}$ *Apc*$^{Min/+}$ mice to test for in vivo interactions of loss of function of the IGF-signaling in intestinal tumorigenesis.

F. IGF-I/insulin family in colon cancer

Compelling evidence supports a role of the IGF-I family in the progression and survival of colorectal cancer (Figure 1.5). Elevated circulating IGF-I or IGF-II is associated with increased risk of colorectal cancer.[62,63,64] Increased plasma insulin is also associated with increased adenoma risk and decreased apoptosis in the normal colon.[65] Loss of imprinting (LOI) of the IGF-II maternal allele elevates local levels of IGF-II and is associated with increased risk of intestinal adenomas.[66,51] A recent meta-analysis containing ten prospective studies showed a modest positive association between circulating IGF-I and colorectal cancer risk.[67] IGF-IR expression is increased in a number of tumors and this correlates with grade and stage of intestinal tumors.[68,69,70] Expression of IGFBPs is often altered in tumors and low IGFBP3 expression in the colon is associated with increased risk of colorectal cancer.[71,72] Recently, a polymorphism in the 5' promoter region of IGF-I in the consensus domain of the Oct1/Oct2 binding transcription factor binding site was associated with a 40% reduction in CRC risk.[73]

Functional studies in mice strongly support a role for the IGF-I family in colorectal cancer. Multiple studies in mouse models demonstrate that exogenous IGF-I increases intestinal length, mucosal mass, and crypt cell proliferation and reduces apoptosis.[59,74,75] Mice with a liver-specific deletion of IGF-I have a 50-70% reduction in circulating IGF-I and, when treated with the carcinogen azoxymethane (AOM), show reductions in tumor multiplicity and tumor size.[76,77] This was associated with increased apoptosis and enhanced proliferation.[76] Other studies have examined the role of LOI of IGF-II in the progression of colorectal cancer. A mouse model of LOI of IGF-II in $Apc^{Min/+}$ mice showed dramatic increases in intestinal tumors compared to littermate

controls.[78] This phenotype could be rescued by the introduction of a transgene expressing soluble full-length IGF-II.[79] These studies suggest that intrinsic mechanisms or therapies that limit the IGF-I family may be useful preventative or therapeutic strategies against colon cancer.

G. SOCS proteins (Adapted from [80])

The family of suppressors of cytokine signaling (SOCS) was discovered in the late 1990s based on their ability to exert negative feedback on cytokine receptor signaling through the Janus Kinase (JAK), signal transducers and activators of transcription (STAT) pathway (Figure 1.6).[81,82, 83,84] This family includes cytokine inducible SH2-containing protein (CIS) and SOCS 1-7.[84] Each SOCS family member shares a similar structural organization containing a COOH-terminal SOCS box, an SH2-domain and an N-terminal domain. The SOCS box is an approximately 40-residue motif showing strong homology across all SOCS family members.[83,85] The SOCS box is essential for interactions with elongins to form an E3 ubiquitin ligase, which targets bound proteins for poly-ubiquitination and proteasomal degradation.[85] The SH2-domain of SOCS proteins is critical for interactions between SOCS and phospho-tyrosine residues of target signaling proteins.[84] The N-terminal domains of the SOCS proteins vary in length and sequence and may impact on specificity or mode of SOCS action.[84]

a. Pleiotrophic effects SOCS on cytokine actions

Consistent with the discovery of SOCS proteins as cytokine-inducible negative feedback inhibitors of cytokine receptor/JAK-STAT signaling, a wealth of evidence suggests that multiple cytokines and associated receptors can induce expression of one or more SOCS mRNAs and proteins.[84] Conversely each particular SOCS can limit the activity of multiple cytokines. However as discussed, mouse models with targeted disruption of SOCS genes indicate specific or preferential roles of some SOCS in limiting particular cytokines and their receptors. This dissertation is focused on SOCS2. The SOCS proteins limit the magnitude or duration of cytokine signaling by multiple mechanisms that depend on the particular SOCS protein induced (Figure 1.6).[85] These include:

a) Direct inhibition of activated JAKs, which has been demonstrated for SOCS1 and SOCS3 and relies on a kinase inhibitory region (KIR) present in these SOCS.
b) Binding of SOCS to the cytoplasmic domain of the cytokine receptor via SH2 domain interactions and subsequent inhibition of JAK activity, which has been demonstrated for SOCS3.
c) Binding to SH2 domains of cytokine receptors and competitively inhibiting STAT binding to the cytokine receptor, which occurs for SOCS2.
d) Recruitment of the E3 ubiquitin ligase complex to the SOCS box, leading to ubiquitination and degradation of receptors/signaling molecules associated with SOCS.

Of the SOCS family members, SOCS1 and SOCS3 are the most structurally and functionally similar.[83] Both contain a kinase inhibitory region (KIR), which enables them

to directly bind and inhibit JAKs.[86] SOCS1 and SOCS3 exhibit some functional redundancy in the modulation of cytokine signaling. SOCS1 is induced by and can modify signaling of multiple cytokines, including interleukin- (IL-4), IL-6, leukocyte inhibitory factor (LIF), and interferon (IFNγ).[82,87,88] SOCS3 is an inhibitor of ligands which activate the family of cytokine receptors that share the gp130 receptor subunit, including IL-6, LIF, and oncostatin M (OSM).[84,89,90]

SOCS2, which is the focus of chapter II, was originally discovered in an EST database search for SOCS-1 related genes.[82] SOCS2 mRNA is expressed during embryogenesis and in a number of adult mouse and human tissues including liver, heart, lung, the small intestine and colon.[91,82] SOCS2 expression is induced in various cell types by several cytokines including IL-6, IL-3, IL-4, granulocyte macrophage colony stimulating factor (GMCSF), IFNγ, LIF, erythropoietin (EPO), IL-1, and GH.[82] Several features distinguish SOCS2 from SOCS3. SOCS2 lacks a KIR and is structurally similar to CIS. While cytokines typically induce rapid and transient expression of SOCS1 and SOCS3, SOCS2 expression gradually increases and persists for over 24 hours.[82] Although SOCS2 is activated by multiple cytokines, it does not seem to have a significant role in immune function.[92] Considerable evidence summarized in detail below indicates a primary role of SOCS2 in regulating GH action via the GH receptor.[93] In addition yeast two hybrid data and work from the Lund laboratory indicate that SOCS2 can bind to and inhibit the IGF-IR.[94,91, 95] Since IGF-IR is a receptor tyrosine kinase, structurally distinct from cytokine receptors, this is a novel action of SOCS2. It is noteworthy that some evidence suggests that SOCS3 can limit signaling via the insulin receptor, which shares structural similarity with IGF-IR.[96]

H. Physiological role of SOCS2 as a negative regulator of GH/IGF-I signaling.

a. Role of SOCS2 in GH signaling

Insights into the physiological role of SOCS2 have come from studies using genetic mouse models. Mice with targeted gene-deletion of SOCS2 ($SOCS2^{-/-}$) have significant increases in post-natal growth and adult mice are, on average, 40% heavier than their WT littermates. The 40% increase in body weight reflects increased organ size although not all organs are affected equally or proportionally with body weight.[92] $SOCS2^{-/-}$ mice also display increases in body length, length of bones in the limbs, and collagen deposition in skin.[92] This phenotype is similar to that seen in GH-transgenic mice.[12,11,67,97] Genetic disruption of GH signaling completely reverses the body overgrowth phenotype seen in $SOCS2^{-/-}$ mice and the phenotype is regained by twice-daily injections of GH.[93] This body overgrowth phenotype is partially mediated by STAT5b as genetic activation of this protein partially reverses the $SOCS2^{-/-}$ phenotype.[93] SOCS2 is localized to the high growth (*HG*) region in mice, humans and pigs, where a breakpoint deletion in the SOCS2 coding sequence has been identified.[98,99] The phenotypes of *HG* mice and $SOCS2^{-/-}$ mice are also very similar. $SOCS2^{-/-}$ mice have normal plasma insulin-like growth factor levels, where both GH-transgenic and *HG* mice have elevated plasma levels of IGF-I.[98,93] This suggests that the SOCS2 phenotype has both GH-dependent and GH-independent effects.[93] Surprisingly, widespread transgenic over-expression of SOCS2 also promotes increases in body weight, but much less as those seen with loss of SOCS2.[100] This finding highlights another distinct feature of SOCS2 signaling, whereby low concentration of SOCS2 are inhibitory and high

concentrations of SOCS2 may, in fact, be stimulatory.[101,100] A model of SOCS2 action on GH signaling is shown in Figure 1.7.

b. Role of SOCS2 in IGF-IR signaling

SOCS2 negatively regulates IGF-IR signaling (Figure 1.7). SOCS2 has also been shown to bind to the IGF-IR by yeast two-hybrid assays and by co-immunoprecipitation.[91,75] Co-transfection of SOCS2 with the IGF-IR and STAT3 in 293T cells demonstrated that SOCS2 inhibits IGF-I-induced STAT3 phosphorylation, but did not have an effect on IGF-I-induced receptor phosphorylation.[102] Similar results were seen in primary embryonic fibroblasts from SOCS2$^{-/-}$ mice.[91] However, further studies in Caco2 cells, a colon cancer cell line, demonstrate that overexpression of SOCS2 attenuates IGF-I induced tyrosine phosphorylation of the IGF-IR, as well as IGF-I-induced tyrosine phosphorylation of IRS-1.[75] These results suggest that SOCS2 may have cell-type specific effects on IGF-IR signaling.

I. Role of SOCS2 in the Intestine (Adapted from [80])

a. Small Intestine

SOCS2 is a negative feedback regulator of GH/IGF-I-induced growth of the small intestine and exerts these effects on both intestinal epithelial cells and myofibroblasts. SOCS2$^{-/-}$ mice have significant although modest ~25% increases in the length and mass of the small intestine, due to enhanced proliferation and reduced apoptosis in the crypts.[75,95] These mice have longer villi and deeper crypts.[75] In Caco2 cells and in IEC-6

cells, a non-transformed intestinal epithelial cell line, SOCS2 overexpression inhibited proliferation and promoted differentiation.[95] Isolated crypts from SOCS2$^{-/-}$ mice showed increased basal proliferation that was further enhanced in response to GH or IGF-I treatment, though IGF-I effects were more pronounced.[95] Treatment in SOCS2$^{-/-}$ mice with exogenous IGF-I promoted further increases in the weight and mass of the small intestine, which was accompanied by enhanced tyrosine-phosphorylation of the IGF-IR.[75] In addition, IGF-I-induced STAT3 DNA binding was also enhanced in the intestine, suggesting that SOCS2 normally limits IGF-I-induced STAT3 activation.[75] Fibroblasts from SOCS2$^{-/-}$ mice show increased GH- and IGF-I-induced proliferation and collagen deposition.[103] In a model of growth hormone excess, loss of one copy of SOCS2 promoted increased body overgrowth and pronounced increases in small intestine weight and proliferation.[49] (Unexpectedly, these mice also developed hyperplastic and lymphoid polyps in their large intestine, representing the first evidence that SOCS2 may normally inhibit tumor development and is discussed in more detail below.[49])

b. Colon

The role of SOCS2 in GH/IGF-I-induced growth in the colon is similar to that in the small intestine, though effects on growth are more pronounced. SOCS2$^{-/-}$ mice have significant increases in colon weight and length and a pronounced thickening of the muscularis layer.[75,95] Like in the small intestine, these effects in the colon are also due to changes in the balance of proliferation/apoptosis in the crypt.[75,95] Infusion of either IGF-I or EGF in SOCS2$^{-/-}$ mice promotes further increases in the weight and mass of the colon, indicating that SOCS2 may normally negatively regulate the actions of EGF as well as

IGF-I.[75] GH-Tg mice have increased colon growth and this is further enhanced by partial loss of SOCS2.[49] Partial loss of SOCS2 in GH-Tg mice produced hyperplastic and lymphoid polyps composed of mostly B cells that were actively proliferating, as indicated by BrdU positive cells in the crypts.[49] It is important to note that hyperplastic and lymphoid polyps are typically considered benign. In addition these polyps developed only in a model of GH excess and haplotype insufficiency of SOCS2. Thus the role of SOCS2 in intestinal tumorigenesis in situations of normal GH states was not defined.

J. SOCS as Tumor Suppressors in Colorectal Cancer

Since their initial discovery as intrinsic mediators of cytokine signaling, SOCS proteins have now been hypothesized to act as tumor suppressors based on their ability to limit the effects of multiple proliferative, growth-promoting pathways. SOCS, namely SOCS1, SOCS2, and SOCS3 proteins are hypermethylated at CpG islands and show reduced expression in a number of cancers, including esophageal, melanoma, hepatocellular carcinoma, and colon cancer.[104,105,106,107,108] The role of SOCS proteins in gastrointestinal cancers has been largely focused on SOCS1, SOCS2 and SOCS3. This introduction will briefly review evidence about SOCS1 and SOCS3 since SOCS2 is the focus of chapter II.

As mentioned previously, patients with inflammatory bowel disease have an increased lifetime risk of developing colorectal cancer.[109,110,111,112] A widely accepted mouse model of inflammation-associated CRC is treatment with azoxymethane/dextran sodium sulfate (AOM/DSS). AOM is administered at the onset of the study followed by

multiple cycles of inflammatory agent dextran sodium sulfate (DSS) given in the drinking water. When this regime was given to transgenic mice lacking SOCS3 in intestinal epithelial cells (IEC), tumor load was increased four-fold compared to controls, with associated increases in STAT3 and NFκB activation.[113] Conversely, SOCS3 over-expression was shown to decrease proliferation in colon cancer cells lines, further supporting the role of SOCS3 as a suppressor of tumor cell growth in this model.[113] Similar to SOCS3, SOCS1 may also have tumor suppressive properties in CRC. A SOCS1$^{-/-}$Tg model, in which mice with global disruption of SOCS1 genes had SOCS1 is restored specifically in T and B cells, was used to study the role of SOCS 1 development of spontaneous colorectal tumorigenesis. Colitis occurred in these mice at 3 months, followed by tumor development in inflamed areas of the proximal colon by 6 months of age, with constitutively active STAT1.[114] Treatment with anti-IFNγ antibody reversed this phenotype. Interestingly, these mice had an increase in STAT3 and NFκB activation attributed to increased local TNFα expression, as well as increased COX-2 and iNOS in tumor-associated macrophages.[114] This model supports the concept that SOCS1 limits IFNγ-dependent tumorigenesis in the colon. It is important to stress that the spontaneous tumor formation in the SOCS1$^{-/-}$Tg model arises from local inflammation, as other reports show that STAT1 does not play a significant role in the *Apc*$^{Min/+}$ model of spontaneous intestinal tumorigenesis.[115]

It is likely that loss of SOCS2 promotes increase risk of colon cancer; however, very few studies have examined the role of SOCS2 in colon cancer. Evidence for such a role has come from several studies in primary tumors of other organs, such as breast, endometrial cancer, and melanoma, which suggest that the promoter of SOCS2 is

hypermethylated in tumors, resulting in a decrease in SOCS2 expression [summarized in Table 1]. Evidence in vivo that SOCS2 may limit intestinal tumorigenesis comes from our studies in SOCS2$^{+/-}$/GH-tg mice, which show a 50% reduction in SOCS2 expression.[49] In addition to enhanced GH-induced increases in body and intestinal weight, these mice also developed lymphoid and hyperplastic polyps in the colon suggesting that small decreases in SOCS2 expression in a model of GH-excess promote abnormal growth.[49] While lymphoid and hyperplastic polyps are not generally considered pre-cancerous, this was the first indication that SOCS2 may limit aberrant growth in the intestine. When SOCS2$^{+/-}$/GH-Tg mice were aged over 250 days, the colonic polyps did not progress into pre-cancerous lesions; however, GH-Tg mice heterozygous for SOCS2 developed duodenal adenomas, which are considered pre-cancerous lesions.[61] Strikingly, the one SOCS2$^{-/-}$/GH-tg generated over 40 litters developed a duodenal polyp that was a pathologically confirmed adenocarcinoma, suggesting a dose-dependent effect of SOCS2 to inhibit tumor growth.[116] While these effects were specific to GH-Tg animals, a model of acromegaly, considering the inhibitory effect of SOCS2 on GH/IGF-I-induced proliferation and cell survival and evidence for a role of IGF-I in colon cancer, we hypothesized that loss of SOCS2 promotes neoplastic growth in setting of spontaneous tumorigenesis. This hypothesis was tested in studies described in chapter 2 and our recent publication.[117]

K. EGF family

The EGF receptor family is composed of 4 related receptors: EGFR (ErbB1/Her1), ErbB2 (Her2/neu), ErbB3 (Her3), and ErbB4 (Her4) which are considered prototypical tyrosine receptor kinases (Figure 1.8).[118,119] Several ligands are members of the EGF-family of growth factors and bind various EGFR receptors with different affinities. EGF, transforming growth factor-α (TGFα), and amphiregulin bind the EGFR (Figure 1.8).[119] The EGFR has a ligand binding domain containing two cysteine rich regions, a transmembrane domain, and a kinase domain that catalyzes the transfer of phosphate molecules from ATP to an active site of the tyrosine kinase.[118] The C-terminal tail is then auto-phosphorylated and allows the binding of proteins with SH2 or phosphotyrosine binding domains (PTB).[118] One exception is the ErbB2 receptor, which does not contain a ligand-binding domain, and instead influences binding affinity of other EGFR family members for their ligands by heterodimerization (Figure 1.8).[120] ErbB3 is an EGFR family member that has a kinase domain, but lacks intrinsic kinase activity due to evolutionary changes in its sequence (Figure 1.8).[121] EGF is known to activate several signaling pathways in response to ligand binding and receptor dimerization. EGFR is known to activate Ras to lead to MAPK/ERK activation, activate JAK/STAT pathways and also activate PI-3K/Akt.[118] Activation of these pathways seems to be context-specific and leads to proliferation, migration, or differentiation of enterocytes.

The growth effects of EGFR activation in the intestine are well documented. EGF is a known mitogen that promotes intestinal adaptation after surgical resection in mice.[122] EGF expression is increased following intestinal resection, with increases in EGF mRNA specifically in the crypt enterocytes.[122] Mice with a mutation in the EGFR kinase domain,

called *waved-2* mice, have impaired kinase activity and show impaired adaptive response following surgical resection.[15] EGF is a potent stimulator of DNA synthesis and proliferation of intestinal epithelial cells in vitro and in vivo.[123,124,122,125,126] EGF promotes wound healing through a Rac-dependent mechanism in mouse colonic epithelial cells.[127] Increased EGF expression is found in mouse models of colitis and treatment with EGF partially restores ion transport in colons of colitis-induced mice.[128,129] When EGF is inhibited in organ cultures of intestinal epithelium, this promotes apoptosis and reduces survival of the intestinal cultures.[130] These studies suggest that EGF normally regulates the maintenance and repair of the intestinal epithelium.

L. EGF family in colon cancer

The EGFR and its ligands are up-regulated in a majority of colon cancers.[24,131] Strong EGFR staining in colorectal tumors is associated with higher tumor stage, shorter duration of disease-free survival, and worsened overall survival.[132] EGFR expression is detected in 65-70% of human colon cancers and is associated with more aggressive disease and poorer prognosis.[133] Studies in mouse models strongly support a role for EGFR signaling in the progression of colon cancer. Genetic or pharmacological inhibition of the EGFR in $Apc^{Min/+}$ mice reduced tumor number by 90% or 60%.[134] In $Apc^{Min/+}$ mice, exogenous EGF treatment increased polyp size in the proximal intestine.[135] In the AOM model, the EGFR tyrosine kinase inhibitor, gefitinib, reduced tumor number by 42% and was associated with decreased proliferation and reduced cyclin D1 and Cox2.[136] Recently, an intestinal-specific deletion of ErbB3 resulted in loss

of ErbB4 expression and increased inflammation in response to DSS-induced intestinal injury.[121] In addition, when these mice were crossed with *Apc*$^{Min/+}$ mice, there was a complete absence of colon tumors and almost complete ablation of tumors in the small intestine, as a result of increased caspase-3-mediated apoptosis.[121] As a result of human expression and mouse studies, several companies have developed targeted therapeutics against EGFR and IGF-IR, which are currently in clinical trials.

As indicated above, loss of function of APC and aberrant transcriptional activation of β-catenin are considered to be key pathways driving tumorigenesis in the colon. Some evidence suggests that EGF activates β-catenin, although this may be indirect, by disassociating β-catenin from adherens junctions.[137,138,139,140] In A431 epidermoid carcinoma cells, treatment with EGF disrupted cell-cell junctions downregulating E-cadherin and caveolin-1 mRNA and increasing β-catenin/TCF transcriptional activity.[137] EGF also disrupts the α-catenin/β-catenin complex at adherens junctions by promoting EGF-induced activation of ERK leading to α-catenin phosphorylation and β-catenin dissociation.[138] Activation and nuclear translocation of β-catenin is dependent on histone deacetylase-6 (HDAC-6) in colon cancer cell lines, where inactivation HDAC6 blocked EGF-induced nuclear localization of β-catenin and proliferation.[139] Recent studies demonstrate that EGF induces β-catenin mediated transcriptional activation of TCF/lef in oral cancer cell lines and cultured dermal fibroblasts.[140,141] Together, these studies support a role for EGF in β-catenin nuclear accumulation and transcriptional activation.

M. Synergistic effects of IGF-I and EGF

Studies in our lab and others show that EGF and IGF-I synergistically stimulate DNA synthesis and proliferation in intestinal epithelial cells greater than either treatment alone.[123,142,143] Studies in our lab using IEC-6 cells, a non-transformed rat intestinal epithelial cell line, showed that while IGF-I or EGF alone stimulated ^{3}H-thymidine DNA synthesis, the combined treatment of both growth factors produced synergistic increases in DNA synthesis.[123] There is a temporal order to this effect where EGF pretreatment followed by IGF-I treatment produces synergistic increases in DNA synthesis; however, IGF-I pretreatment followed by EGF treatment produces effects similar to EGF alone.[123] A small 30-minute pulse of EGF or continuous EGF treatment followed by 48 hour IGF-I treatment in IEC-18 rat intestinal epithelial cells synergistically increased proliferation, while IGF pretreatment followed by 48 hour EGF treatment had no effect.[142] Studies in FHs74 human intestinal epithelial cells also show synergistic activation of DNA synthesis by combined treatment of IGF-I and EGF and this was similar to effects seen with low levels (2%) of serum.[143] One suggested hypothesis for this temporal order is that EGF acts as a competence factor, promoting the transition from G_0 to G_1 and the addition of IGF-I then drives DNA synthesis from G_1 to S phase.[142] However, as each growth factor alone has been shown to promote DNA synthesis and proliferation, with EGF showing more potent effects, other unknown mechanisms are probably responsible for the synergistic mitogenic effects of EGF and IGF-I.[123] Chapter III explores mechanisms underlying synergistic mitogenic effects of IGF-I and EGF in IEC-6 cells.

N. Current tyrosine kinase receptor therapies

There are currently two major classes of inhibitors that target either EGFR or IGF-IR: (1) monoclonal antibodies that bind to the extracellular domain and (2) small molecular tyrosine kinase inhibitors that block binding of adenosine triphosphate to the intracellular tyrosine kinase domain of the receptors.[144,145]

a. EGFR therapeutics in colon cancer

As previously mentioned EGFR inhibitors have been developed to specifically target the EGFR in human colon cancer. Both small molecule inhibitors and monoclonal antibodies have been developed. Panitumab and cetuximab are monoclonal antibody inhibitors that are FDA-approved for treatment against metastatic colon cancer as both have been shown to be effective in at least some patients.[146] Small molecule inhibitors have also been developed, such as gefitinib and erlotinib, though erlotinib has shown more effectiveness and is currently approved for treatment in non-small cell lung cancer (NSCLC).[144] These agents have potent in vitro and in vivo effects to inhibit proliferation and reduce tumor development in experimental settings, but have been effective in only a subset of patients with colorectal cancer.[147,148,149] Patients with breast cancer or NSCLC respond to EGFR inhibitors but develop resistance to these therapies.[150,151] This suggests that additional pathways are mediating tumor survival or growth when EGFR is inhibited.

For this reason, there is increasing interest in combining anti-EGFR inhibitors with other therapies.[131] In vitro studies in breast, prostate and other cancer cell lines suggest that up-regulation and activation of IGF-I/IRS-1 signaling mediates resistance to anti-EGFR therapies.[152,153,151,154,155] Resistance to trastuzumab in breast cancer cells was

shown to be mediated by IGF-I stimulated phosphorylation of ErbB2 (HER-2).[152] Inhibition of the IGF-IR by a specific tyrosine kinase inhibitor decreased ErbB2 phosphorylation.[152] Other studies showed that IGF-I interferes with the growth inhibitory effects of trastuzumab in MCF7/HER2-18 cells, which overexpress ErbB2 receptors.[151] EGF treatment of MCF-7 breast cancer cells enhanced phosphorylation of IRS-1 on Y896, a Grb2 binding site that promotes MAPK signaling.[154] IGF-II treatment induces IRS-1 phosphorylation on Y612, a P13-K recruitment site.[154] When MCF-7 cells were treated with gefitinib, a selective EGFR inhibitor, association between IRS-1/EGFR was reduced and IRS-1/IGFIR interactions were promoted.[154]

b. IGF-IR therapeutics in colon cancer

Inhibitors that specifically target the IGF-IR are currently in Phase I and Phase II clinical trials in breast, lung, and colon cancer.[156] The field was greatly enhanced by the ability to produce inhibitors that specifically target the IGF-IR, while having minimal effects to inhibit related insulin receptors, which may produce undesirable metabolic effects.[145] Preclinical, in vitro studies demonstrate that specific small molecular inhibitors and monoclonal antibodies against the IGF-IR induce apoptosis in colon cancer cells and reduce tumors colon cancer derived-xenografts.[157,158,159,68] The small molecule IGF-IR kinase inhibitor, NVP-AEW541, decreased tumor development in xenograft and in vitro models of colon, pancreatic, esophageal, and liver cancer in a dose-dependent manner and did not influence formation of IGFIR/IR hybrid receptors which may interfere with efficacy of treatment.[158] NVP-AEW541 was also shown to inhibit proliferation of a panel

of colon cancer cell lines and primary cell cultures by inducing apoptosis and cell cycle arrest, leading to an increase in the apoptotic protein Bax, and decrease in the anti-apoptotic Bcl-2.[68] The promising results of these studies have led to the movement of these therapeutics into Phase I and Phase II clinical trials. Dosing studies using monoclonal antibodies that specifically target the IGF-IR, such as CP751,871 and AVE-1642, have shown some measure of toxicity including hyperglycemia, anemia, and thrombocytopenia indicating that even inhibitors specific to the IGF-IR may perturb insulin signaling.[160] Results of current clinical trials will help to elucidate the effectiveness of these molecules in promoting cancer survival and disease remission. In the current study we used $Apc^{Min/+}$ mice with targeted disruption of one IRS-1 allele and treatment with an EGFR inhibitor to explore the in vivo effects of combined loss of function of IGF signaling and EGFR signaling on spontaneous intestinal tumorigenesis.

O. Hypothesis Tested

I. Homozygous deletion of SOCS2 limits intestinal tumorigenesis in the intestine of *Apc*$^{Min/+}$ mice. Studies testing this hypothesis are described in the chapter II.

II. Increased intestinal tumorigenesis in SOCS2$^{-/-}$/*Apc*$^{Min/+}$ mice is due to increased IGF-I signaling and STAT activation. Studies testing this hypothesis are described in Chapter II.

III. Combined IGF-I and EGF activation synergistically promotes increased proliferation by enhancing IGF-IR activation and additive activation of β-catenin. Studies testing this hypothesis are described in Chapter III.

IV. Combined heterozygous deletion of IRS-1 and pharmacological inhibition of EGFR in *Apc*$^{Min/+}$ mice will additively or synergistically reduce intestinal tumor development. Studies testing this hypothesis are described in Chapter IV.

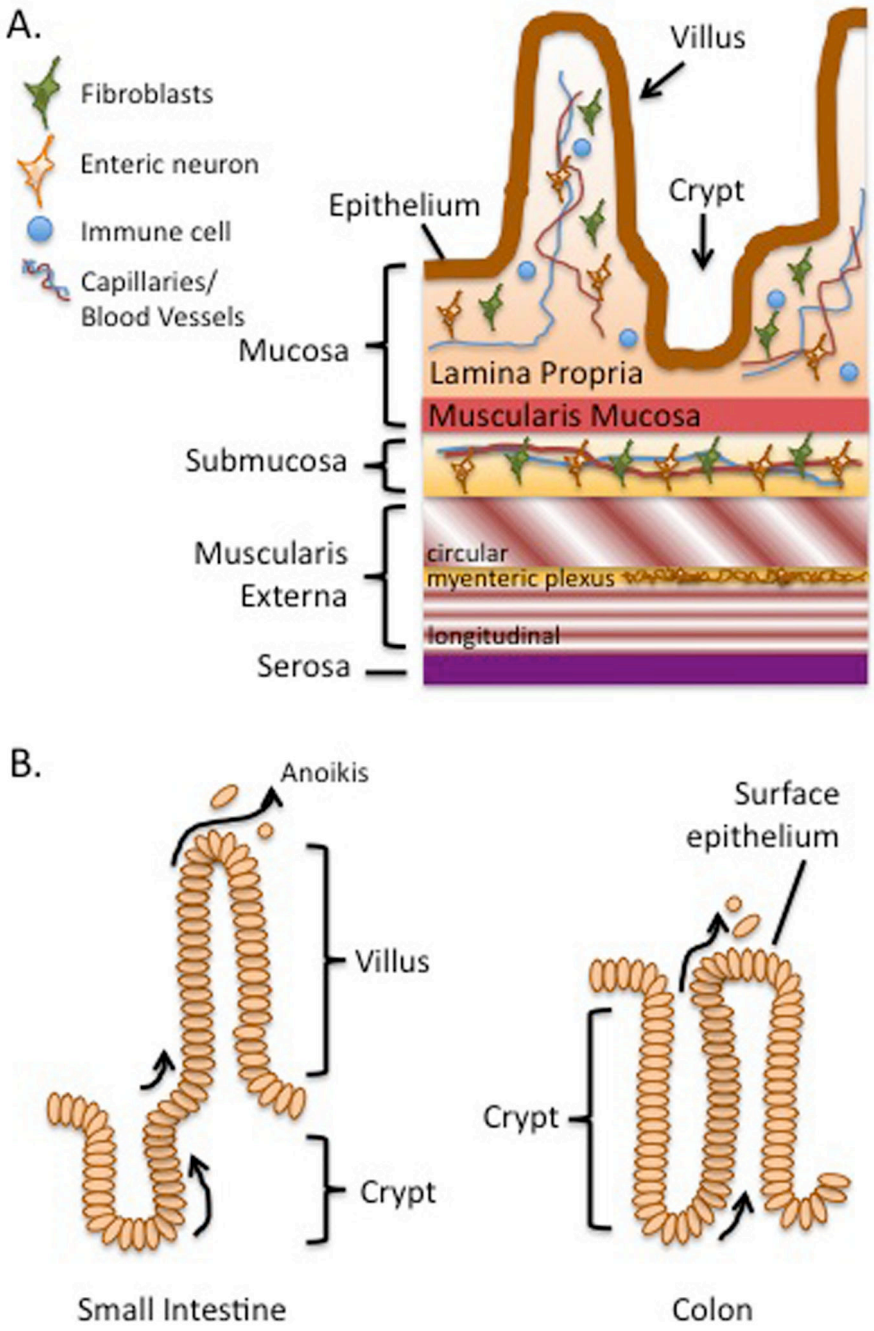

Figure 1.1. Structure of the Intestine. A. Illustration of the structure of the Intestine. B. Crypt-Villus axis in the small intestine (left) and crypt-surface axis in the colon (right).

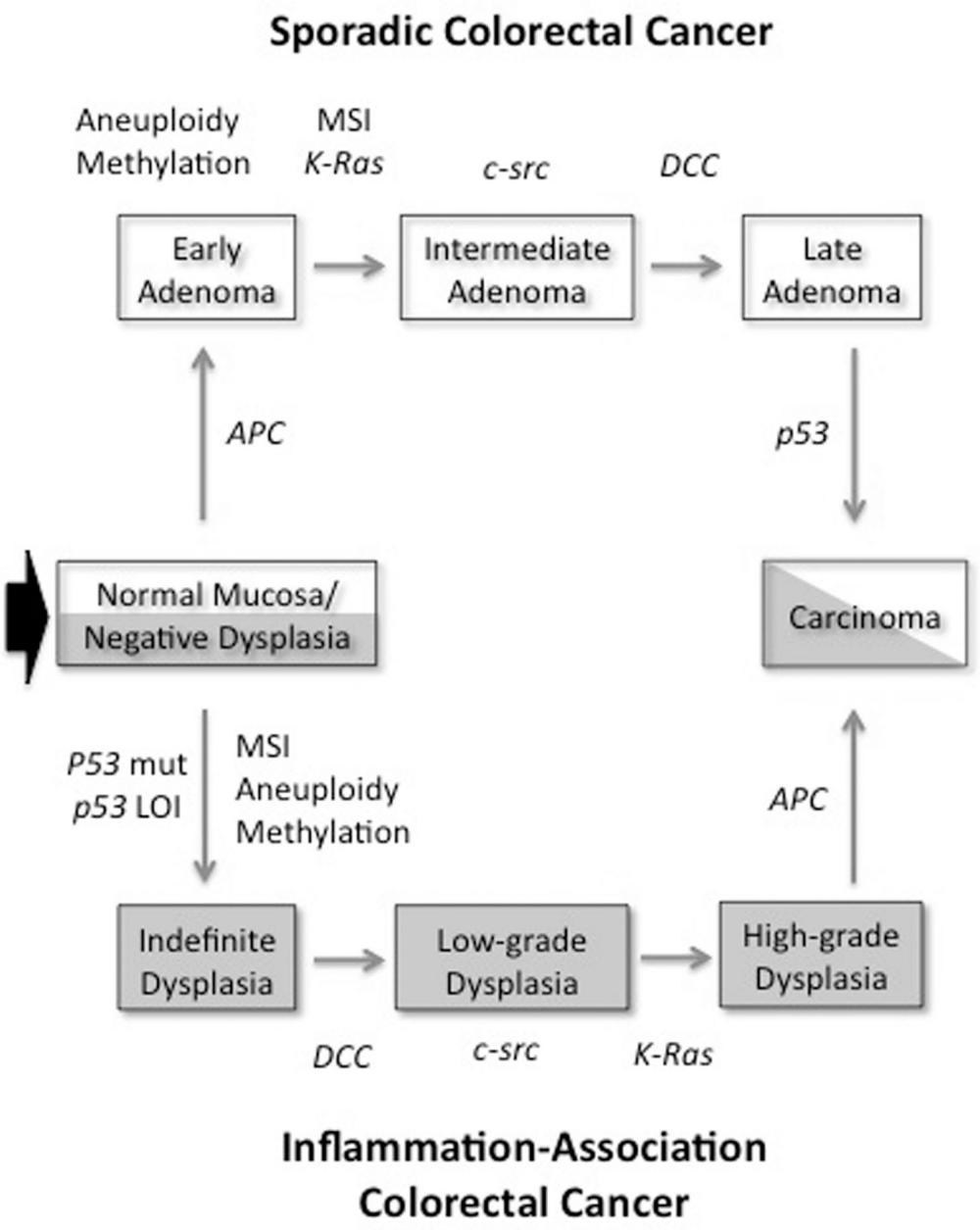

Figure 1.2. Diagram comparing progression sporadic and inflammation-Associated Colorectal Cancer. Adapted from [31]. Sporadic colon cancer progression (white). Inflammation-associated colorectal cancer (grey). MSI = microsatellite instability; LOI = Loss of Imprinting; DCC=deletion in colon cancer, loss of function of chromosome region 18q.

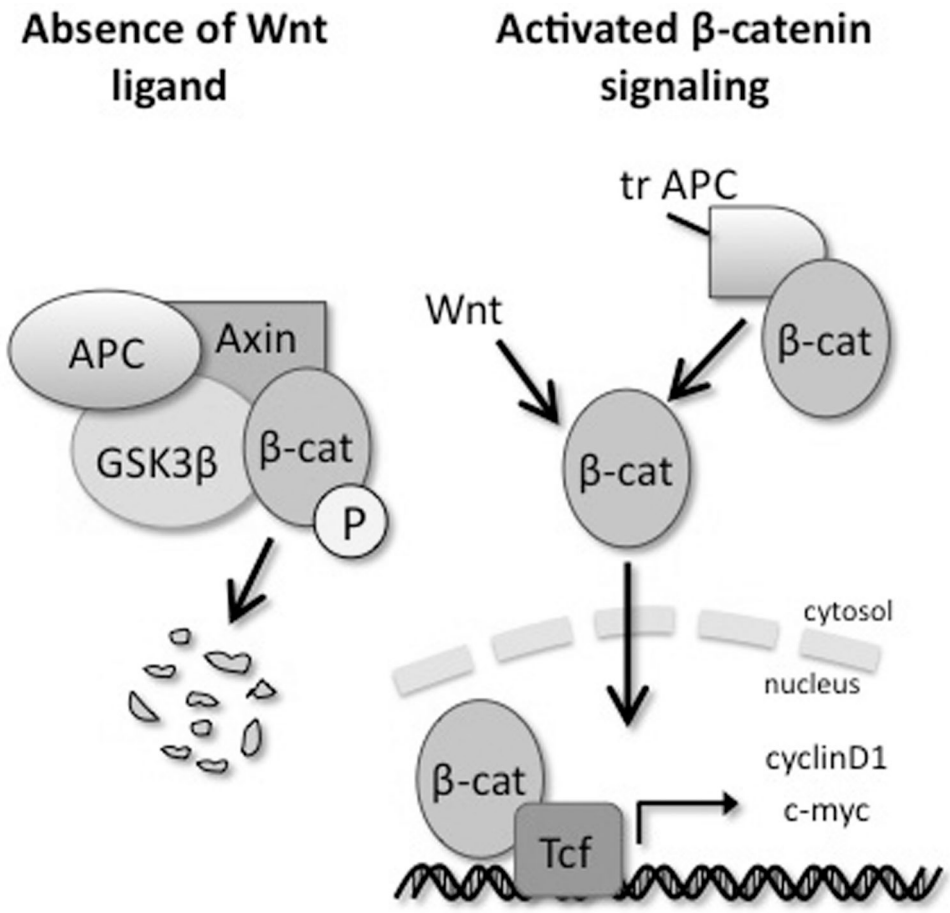

Figure 1.3. Illustration of the Wnt/β-catenin signaling pathway. (Left) In the absence of Wnt ligand or other activating factors, β-catenin is phosphorylated and degraded. (Right) In the presence of Wnt ligand, truncating mutations in *Apc* (tr *Apc*) or other factors, β-catenin is stabilized and translocates into the nucleus where it mediates gene transcription.

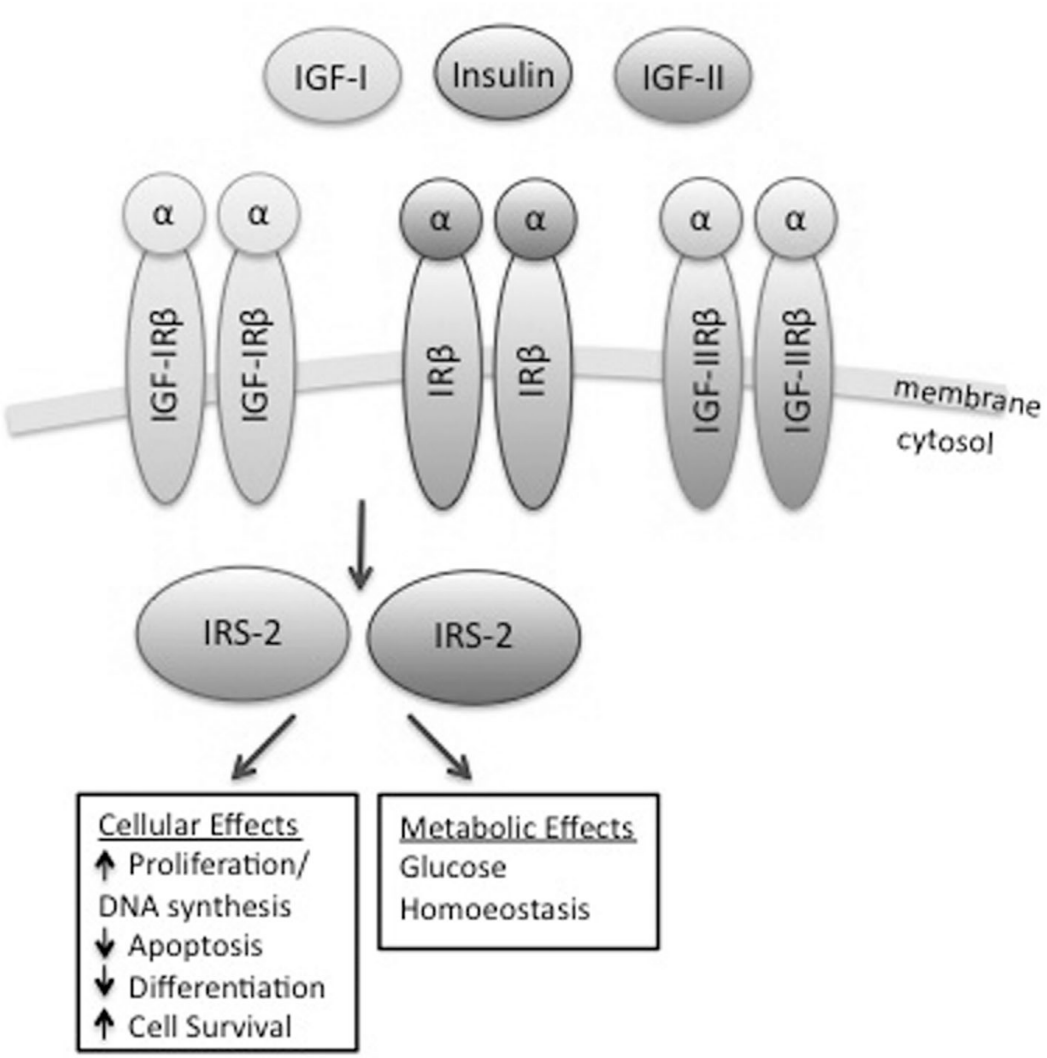

Figure 1.4. Schematic of the IGF-I/Insulin Family of ligands and receptors and effects of IRS downstream signaling.

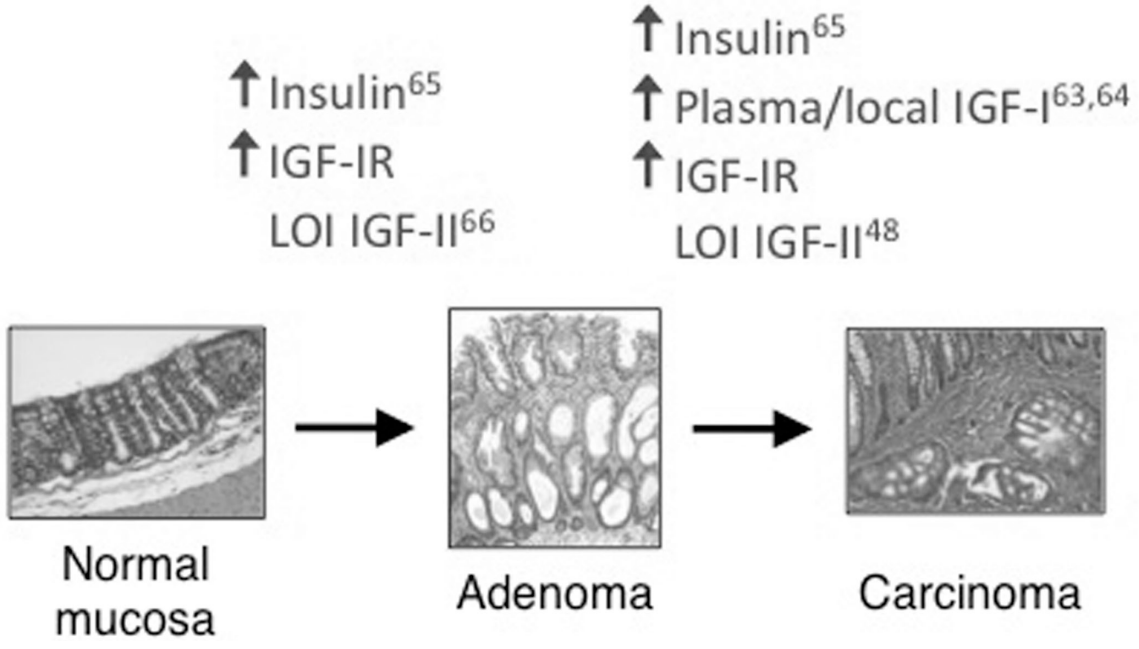

Figure 1.5. Role of IGF-I family in the Progression of Colorectal Cancer. Adapted from "Fearon and Vogelstein model" of colon carcinogenenesis.

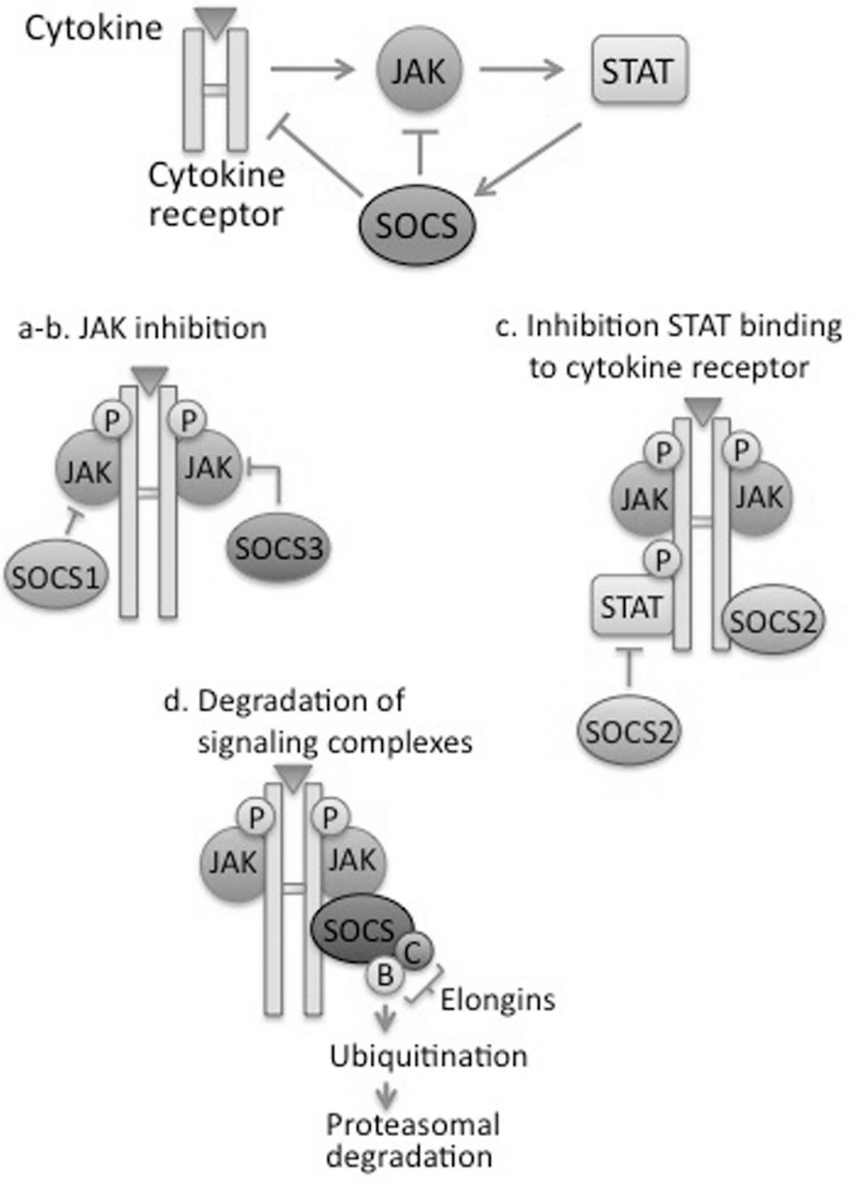

Figure 1.6. Pleiotrophic effects of SOCS to limit receptor signaling. A. Direct inhibition of JAKs. B. Binding to cytokine receptor and inhibiting JAKs. C. Competitively inhibiting STAT binding to cytokine receptors. D. Ubiquitination and proteasomal degradation. Adapted from [80].

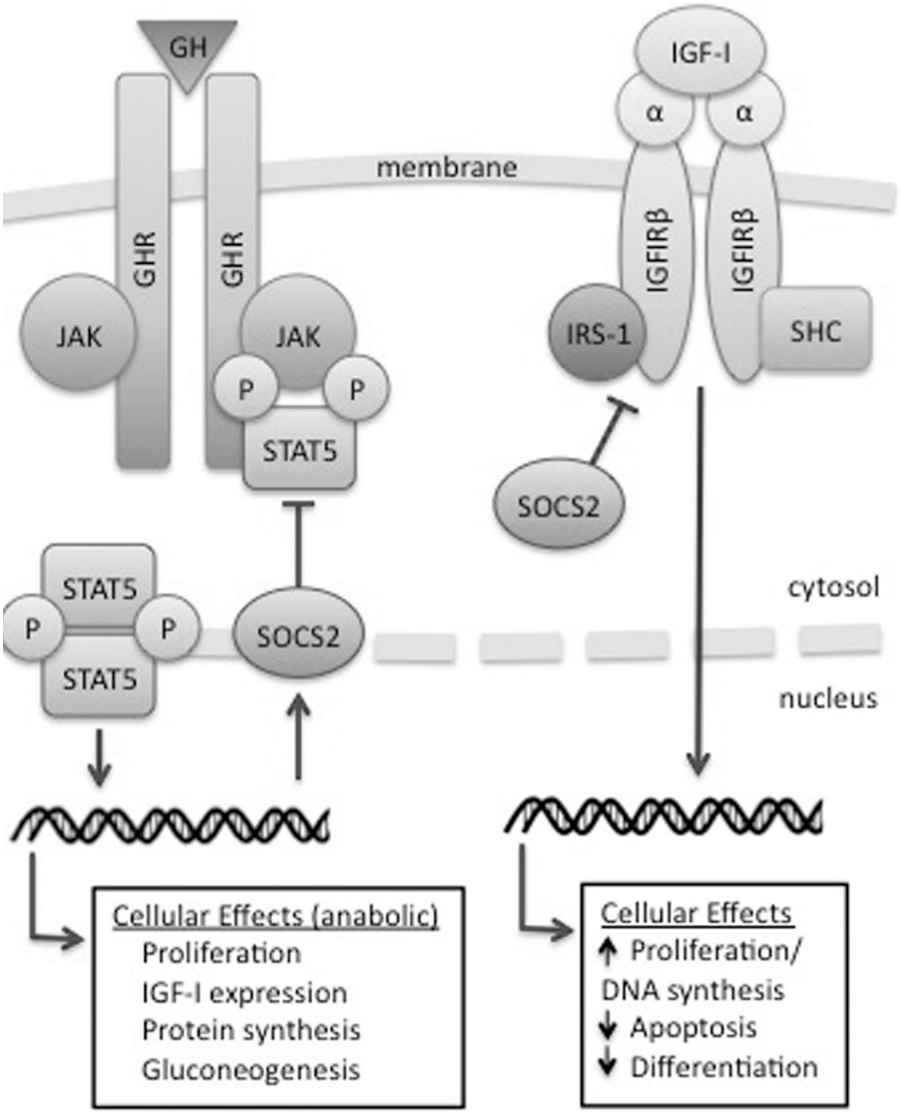

Figure 1.7. SOCS2 is a negative regulator of GHR and IGF-IR signaling pathways. Left, SOCS2 negatively regulates GH signaling to inhibit cellular effects. Right, SOCS2 binds to and inhibits IGF-IR signaling to limit growth-promoting effects. Adapted from [80].

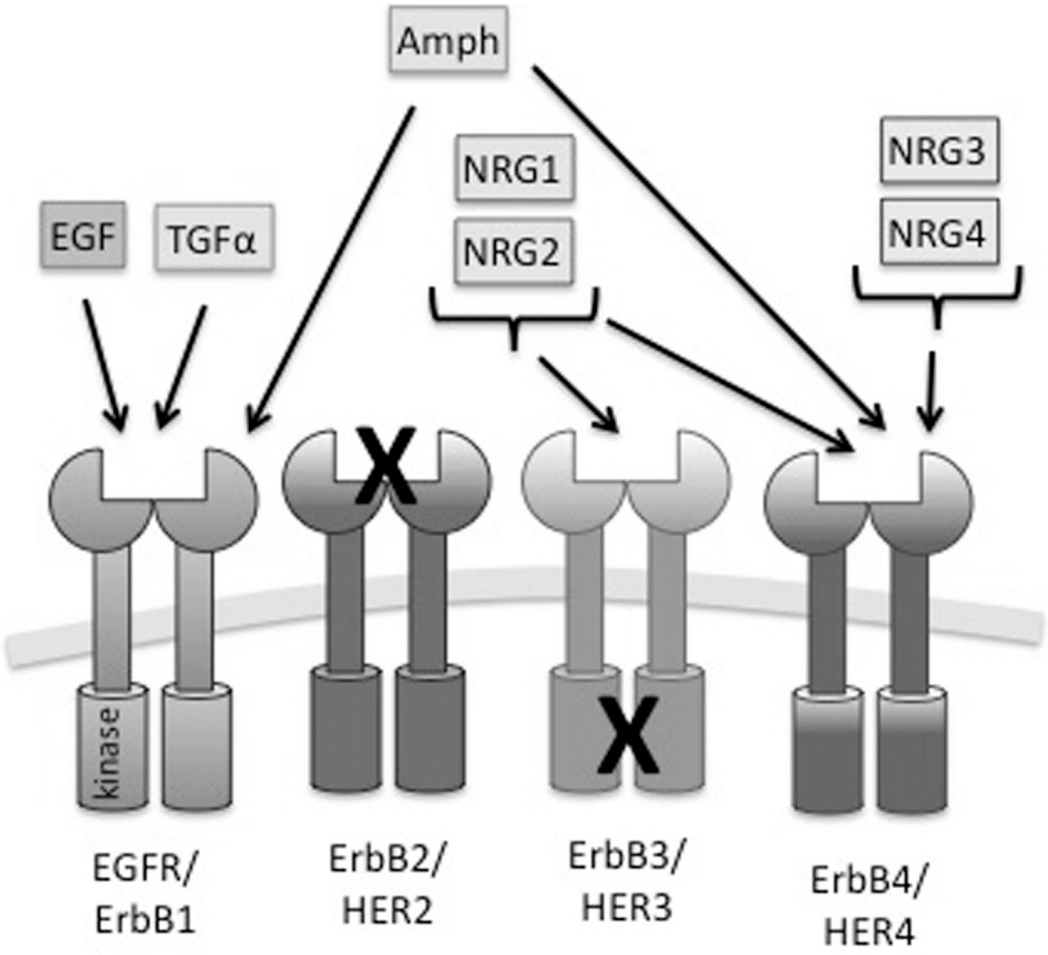

Figure 1.8. EGF Receptor Family: Ligand and Receptor Binding. TGFα=Transforming growth factor α; Amph = Amphiregulin; NRG = neuregulin. X indicates unable to bind ligand or lacks kinase activity.

Table 1.1. Summary of SOCS2 methylation and expression in cancer.

Cancer	Primary Tissue	Expression	Citation
HCC	GNMT-KO mice	Decreased expression Increased promoter methylation	Martinez-Chantar...2008
	1°	80% human HCC & cell lines Correlates with loss of SOCS2 expression	Calrisi.. .2006
Ovarian	Cell lines	Low-moderate expression 4/6 cell lines methylated	Sutherland… 2004
	1°	6/42 1° tumors methylated 10/43 LOH	
Breast	Cell lines	Low expression in some cell lines 8/10 methylated	
	1°	0/48 methylated 7/53 LOH	
	1°	Low protein expression Associated with low proliferation and high differentiation	Farabegoli… 2005
	1°	High expression correlates with increased survival Independent predictor of good prognosis	Haffner…2007
Endometrial	1°	High methylation	Fiegl...2004
Pulminary adenocar-cinoma	1°	Downregulated/low mRNA expression No methylation	Wikman… 2002
CML	Cell lines: Phila-delphia Chrom+	BCR/Ab1 increases SOCS2 expression Downregulated by BCR/Abl inhibitors	Häkansson… 2008
	CD34+ cells	Overexpressed	Zhang… 2006
	Cell lines/CML	Highly expressed in CML cell lines SOCS2 overexpression reduced cell growth Sensitizes to cell death	Schultheis… 2002
	1°	CML patients in blast crisis show high expression	
Melanoma	1° serum	Decreased expression Increased methylation in 2 sites in SOCS2 promoter	Marini…2006
	1° tumors/cell lines	80% 1° tumors/cell lines show methylation	Lie...2008

CHAPTER 2

SUPPRESSOR OF CYTOKINE SIGNALING-2 GENE DISRUPTION PROMOTES $APC^{MIN/+}$ TUMORIGENESIS AND ACTIVATOR PROTEIN-1 ACTIVATION

A. Introduction

The SOCS family of proteins consists of SOCS1-8 and CIS (cytokine-inducible SH2-containing protein).[161] SOCS proteins were initially discovered as negative regulators of cytokine signaling via the janus kinase (JAK) and signal transducers and activators of transcription (STAT) pathway.[161] Activated STATs form homo- and heterodimers and later translocate into the nucleus where they mediate gene transcription, proliferation, cell-survival and transformation.[162] Cytokine receptor-mediated JAK-STAT activation induces the expression of SOCS, which inhibit or terminate cytokine signaling by inhibiting JAK activation, competing with STATs or other signaling proteins for binding sites on cytokine receptors, and by targeting signaling proteins for proteosomal degradation.[161]

SOCS2 has been found to exert negative feedback effects on growth hormone (GH) signaling. $SOCS2^{-/-}$ mice show enhanced body growth that is phenotypically similar to GH-transgenic mice and is reversed when GH is genetically inactivated, demonstrating a key role of SOCS2 in negatively regulating GH-induced body overgrowth.[93] However, our recent studies indicate novel effects of SOCS2 deletion in the intestine whereby $SOCS2^{-/-}$ mice show enhanced growth promoting effects of IGF-I as well as GH. $SOCS2^{-/-}$ mice infused with IGF-I have significantly greater increases in mucosal mass of the small bowel compared with WT littermates, establishing a role for SOCS2 in regulating the potency of action of IGF-I.[75] These studies also show that SOCS2 deficiency enhances the proliferative and anti-apoptotic actions of IGF-I in the small intestine and colon, along with the ability of IGF-I to activate STAT3. Other studies in intestinal epithelial or colon cancer cell lines have demonstrated that SOCS2 directly

binds to the type I IGF-I receptor and limits its activation by IGF-I.[75,95] Disruption of one SOCS2 allele in mice expressing a GH-transgene was found to enhance transgene-induced increases in body weight and mucosal mass, to enhance colon and jejunal crypt proliferation, and to promote the formation of hyperplastic and lymphoid polyps in the colon.[49] Though hyperplastic and lymphoid polyps are generally considered benign, this was novel *in vivo* evidence that SOCS2 may negatively regulate aberrant growth in the intestine in a model of GH and IGF-I excess.[163] However, the role of SOCS2 in development of spontaneous precancerous adenomas in the intestine has not been established.

Limited evidence in cancers of other organs suggests that the SOCS2 gene may be epigenetically silenced by hypermethylation of CpG islands within the promoter.[164,165,166,167,168] In melanoma patients, SOCS2 has been found to be silenced by methylation and its transcription is reduced in primary tumor samples.[165] High SOCS2 expression inversely correlates with tumor grade of breast cancers and favors a good prognostic value.[169] Another study in breast carcinoma samples showed that SOCS2 protein expression is positively correlated with low grade tumors.[167] While these studies in primary human tumors and cancer cell lines suggest a potential role of SOCS2 in suppressing tumor growth, this has not been directly evaluated in an *in vivo* model of intestinal cancer.

The current study tested whether SOCS2 gene disruption enhances spontaneous intestinal tumor formation in $Apc^{Min/+}$ mice. The $Apc^{Min/+}$ mouse model is widely used to study mediators and mechanisms that govern the initiation, establishment and progression of intestinal tumors.[34] $Apc^{Min/+}$ mice are heterozygous for a mutation that results in a

truncated form of APC, a gene frequently mutated in human colon cancer that leads to aberrant activation of the β-catenin pathway.[36] An APC-containing complex normally degrades cytoplasmic β-catenin.[32] In the absence of APC, β-catenin accumulates in the nucleus and coactivates the transcription of proliferative and pro-survival genes in conjunction with T-cell factor/lymphoid enhancer factor transcription factors.[170] Depending on the genetic background, $Apc^{Min/+}$ mice develop tens to hundreds of spontaneous adenomas in the small intestine and fewer adenomas in colon, both of which typically arise due to focal loss of the remaining wild-type *Apc* allele.[34] We cross-bred $SOCS2^{-/-}$ and $Apc^{Min/+}$ mice to test the hypothesis that disruption of one or both SOCS2 alleles will increase tumor number or size in $Apc^{Min/+}$ mice. Studies in our lab have shown that ex vivo treatment with IGF-I in the intestine induces STAT3 DNA binding activity and this effect is enhanced and prolonged in $SOCS2^{-/-}$ mice.[75] Therefore, we postulated that loss of SOCS2 could increase activation of STAT3, which is increasingly linked to normal growth and tumorigenesis in the intestine.[171] STAT3 is activated by tyrosine phosphorylation which permits the formation of STAT3 homo- or heterodimers with other STATs as well as DNA binding activity. STAT3 is also serine-phosphorylated, which has recently been linked to AP-1 activation, cell proliferation, and transformation in cancer cell lines.[172,173,174] Our studies demonstrate that SOCS2 gene deletion in $Apc^{Min/+}$ mice enhanced serine- but not tyrosine-phosphorylation of STAT3, especially in tumors, and this was associated with increased AP-1 but not STAT3 binding activity. Together, these findings provide *in vivo* evidence that SOCS2 deletion promotes $Apc^{Min/+}$ tumorigenesis and this is associated with novel effects on serine phosphorylation of STAT3 and AP-1 activation.

B. Materials and methods

Laboratory animals

Apc$^{Min/+}$ male mice on the C57BL/6 background were purchased from Jackson labs (Bar Harbor, Maine). C57BL/6 mice with targeted disruption of both SOCS2 alleles were developed as previously described and provided by Dr. Christopher Greenhalgh.[92] To generate SOCS2 knockout mice on the *Apc*$^{Min/+}$ background, SOCS2$^{+/-}$ mice were crossbred with *Apc*$^{Min/+}$ mice, and SOCS2$^{+/-}$/*Apc*$^{Min/+}$ male progeny were bred with SOCS2$^{+/-}$ females to generate *Apc*$^{Min/+}$ mice with SOCS2$^{-/-}$, SOCS2$^{+/-}$ or SOCS2$^{+/+}$ genotypes. Genotyping was performed on tail DNA as previously described.[49,134] Age-matched *Apc*$^{Min/+}$ males and females with different SOCS2 genotypes were studied for number and size of tumors. Our studies emphasized female *Apc*$^{Min/+}$ mice because male *Apc*$^{Min/+}$ mice were primarily used as breeders since female *Apc*$^{Min/+}$ mice exhibit complications during pregnancy.[37] The Institutional Animal Care and Use Committee of the University of North Carolina approved all animal studies. Study protocols were in compliance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH).[175]

Sample Preparation, Tumor and Blood Measurements

At least 6 age-matched mice of each genotype were studied at 13-17 weeks of age. Animals were weighed and anesthetized and blood was collected by cardiac puncture. The entire colon was dissected. The small intestine was dissected and separated into 3

segments roughly equal in length. Each intestinal segment was flushed with PBS supplemented with vanadate (2mM), PMSF (1mM), and phosphatase inhibitor cocktail (diluted 1:100, Sigma, P2850). Intestinal segments were splayed open onto 3MM Whatmann paper and fixed in 10% zinc-formalin (Fischer, Pittsburgh, PA) overnight at 4º C, then dehydrated in 70% ethanol. Adenoma number for small and large intestine of each animal was counted under a Leica dissecting scope, using an in-lens micrometer to measure adenoma diameter. A portion (2-3cm) of proximal ileum was flash frozen for RNA isolation. For immunohistochemistry, intestinal segments were rolled into a pinwheel, paraffin-embedded, and sectioned at 5mm. Embedding was performed by the CGIBD Histology Core Facility. The presence and morphology of adenomas were confirmed by hematoxylin and eosin (H&E) staining and by immunohistochemistry for b-catenin. Because $Apc^{Min/+}$ mice develop severe anemia as polyposis progresses, hematocrit was measured as an indirect marker of tumor load and disease severity[176] and was performed by the Animal Clinical Chemistry core facility at UNC.

Immunohistochemistry

De-waxed and rehydrated sections underwent epitope retrieval in Reveal Decloaker (Biocare Medical, Concord, CA) and endogenous peroxidase activity was blocked with 3% H_2O_2. Sections were incubated in normal blocking serum either from a Vectastain ABC kit (Vector laboratories, Burlingame, CA) for rabbit antibodies or from a MOM kit (BD Transduction laboratories, Franklin Lakes, NJ) for mouse antibodies. Sections were incubated overnight with mouse monoclonal antibody to β-catenin (BD Transduction

Laboratories, Franklin Lakes, NJ), rabbit anti-pS727-STAT3 (Cell Signaling, Beverly, MA), or rabbit anti-STAT3 (Cell Signaling, Beverly, MA). Bound antibody was detected with a Vector Elite kit or biotinylated anti-mouse IgG (Vector laboratories, Burlingame, CA), followed by DAB substrate. Tissues were counterstained with hematoxylin. Primary antibody was omitted as a negative control. Sections from both SOCS2$^{+/+}$/*Apc*$^{Min/+}$ and SOCS2$^{-/-}$/*Apc*$^{Min/+}$ mice were stained on the same slide and all slides for a particular antibody were stained in the same batch. Sections were examined on a Zeiss Axio imager A.1. and photographs taken using AxioCam MRc5 and associated AxioVision software (v4.6). Comparisons were made between sections from pairs of SOCS2$^{+/+}$/*Apc*$^{Min/+}$ and SOCS2$^{-/-}$/*Apc*$^{Min/+}$ mice mounted on the same slide. Brightness and contrast of photographs was selected to maximize the clarity for publication.

Plasma IGF-I Assay

Plasma IGF-I was measured by ELISA. ELISA was performed by the CGIBD immunotechnologies core at UNC-Chapel Hill on plasma that was acid-ethanol extracted and cryoprecipitated to remove IGF-I binding proteins (IGFBPs) as previously described.[177,49] ELISA used a Mouse IGF-I Quantikine kit (R&D Systems, Minneapolis, MN) according to maufacturer's protocols, except that samples were diluted 1:500.

Quantitative RT-PCR

Total RNA was extracted from frozen ileum from at least 5 mice per genotype using TRIzol reagents and standard methods (Invitrogen, Carlsbad, CA). Concentration was determined by nanodrop (Thermo Scientific, Willmington, DE). Aliquots of each RNA sample were run on a 1% agarose gel to verify RNA integrity and concentration. RNA (4 μg) was DNAse-treated using the TURBO DNA-*free*™ Kit (Ambion, Foster City, CA) and transcribed into cDNA with AMV-Reverse Transcriptase (Promega, Madison, WI). A no-RT control was also generated for each sample.

To quantify IGF-I mRNA, a SYBR-green-based real-time PCR (SYBR® Green JumpStart™ Taq ReadyMix, Sigma, St. Louis, MO) was used with the following primers: mIGF-I (F: 5'-GTG TGG ACC GAG GGG CTT TTA CTT C-3'; R: 5'-GCT TCA GTG GGG CAC AGT ACA TCT C-3'). To measure *c-Fos* and *c-Jun* mRNA, Taqman Gene Expression Assays (Applied Biosystems, Foster City, CA) were used according to manufacturer's protocol (c-Fos, Mm_00487425_m1; c-Jun, Mm_00495062_s1). Mouse HMBS (hydroxymethylbilane synthase) mRNA was also quantified as an invariant control using mouse SYBR green primers (F: 5'-TGT GTT GCA CGA TCC TGA AAC-3'; R: 5'-CTC CTT CCA GGT GCC TCA GAA-3') or Taqman Assay (Mm_01143545_m1). A standard curve was generated from dilutions ($1x10^7$–$1x10^3$ copies/μL) of a PCR product using conditions optimized for each primer or primer-probe set. DNA sequencing was performed by the UNC-CH genomic analysis facility to confirm correct sequence of PCR product. A no-template control was included in each run as a negative control as well as a standard to normalize the standard curve across runs. A cDNA pool generated from intestine and liver RNA from multiple mice was

added to each run to control inter-run variability. A melt was performed after each run to confirm that a single product was generated. Reactions were run on the Rotor-Gene 2000 (Corbett Research, Sydney, Australia) and analyzed with Rotor-Gene Software 6.0.14. Data for each sample were calculated as absolute copy number based on comparison of test sample data with standard curve and normalized to HMBS.

Nuclear Extracts and Immunoblotting

In a subset of SOCS2$^{+/+}$/*Apc*$^{Min/+}$ and SOCS2$^{-/-}$/*Apc*$^{Min/+}$ mice, tumors were finely dissected under a dissecting scope from the middle third of the small intestine (~12cm in length) and tissue was immediately used for extraction of nuclear proteins. Size-matched biopsies of grossly normal, tumor-free intestinal mucosa were also dissected for comparison. Tumor or non-tumor samples were homogenized using a dounce homogenizer in 1mL of ice-cold 1X Tris-buffered saline supplemented with vanadate (2mM) and PMSF (1mM). Nuclear proteins were extracted as previously described.[178] Concentration was determined by Bradford Assay using Coomasie Plus reagent (Pierce Chemicals, Rockford, IL) and confirmed by examining samples on an SDS-PAGE gel using the NuPAGE gel system (Invitrogen, Carlsbad CA). Gels were visualized and analyzed using the LI-COR Odyssey Infrared Imaging System v3.0 (LI-COR Biosciences, Lincoln, NE).

Western Immunoblotting

Equal amounts of protein (30μg) were size fractionated on 4-12% TEO-CI SDS-PAGE gel (PAGEgel, Fischer Scientific, Pittsburgh, PA) and transferred onto a nitrocellulose membrane (Biorad, Hercules, CA) using a NuPAGE gel system (Invitrogen, Carslbad, CA). After blocking in Blocker™ Casein in PBS (Pierce, Rockford, IL), blots were incubated with primary antibodies against phospho-serine727 STAT3 (Cell Signaling, Beverly, MA), phospho-tyrosine705 STAT3 (Cell Signaling, Beverly, MA), total STAT3 (Santa Cruz Biotechnology Santa Cruz, CA) or Histone H1 (Santa Cruz Biotechnology, Santa Cruz, CA) for 16 hours at 4°C. Blots were washed in PBS containing 0.1% Tween and incubated with secondary antibodies conjugated to Dylight800 for ~1 hour at room temperature. Immunoreactive proteins were visualized and analyzed using the LI-COR Odyssey Infrared Imaging system. Densitometry was performed on visualized bands and normalized to the total protein gel.

Electromobility Shift and Supershift Assays

DNA:Protein binding reactions were carried out in 20μl total volume containing 100 mM NaCl, binding buffer (20% glycerol, 1 mM MgCl2, 1 mM EDTA, 0.5 mM DTT, 0.2 ng poly dI:dC), 20-30μg nuclear protein extract and 100,000–400,000 cpm of a ^{32}P-labeled double-stranded oligomer corresponding to a consensus STAT3 binding site (STAT3-RE; Santa Cruz Biotechnology, Santa Cruz, CA) or consensus AP-1 binding site (AP-1-RE; Promega, Madison, WI). Binding was performed at room temperature for 20 min. For cold competition, an excess of unlabeled oligomer that was specific to either

AP-1 or STAT3, or unrelated oligomer specific to the TCF DNA response element (Integrated DNA Technologies Inc, Coralville, IA; S: 5'- GGT AAG ATC AAA GGG 3').[179] Samples were then loaded onto non-denaturing 4% polyacrylamide gels and electrophoresed in 0.5 × TBE (1X = 89 mM Tris borate pH 8.0, 2 mM EDTA) for 2–3 h. After electrophoresis, gels were dried on 3MM Whatmann paper and exposed to phospho-imager screens. Screens were viewed on the Typhoon 6400 scanner (GE Healthcare, Piscataway, NJ). Intensity of shifted DNA:STAT3 and DNA:AP-1 complexes was quantified using Image-Quant Software v1.2. and expressed as a percentage of values in non-tumor samples for SOCS2$^{+/+}$/*Apc*$^{Min/+}$ mice, which was set as 100%.

Supershift assays were performed to establish the composition of AP-1 binding activity according to previously published methods.[178] Antibodies used for supershift assays were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA). Each supershift reaction included equal amounts of nuclear extract and 6µg of one of the following antibodies: rabbit antibodies specific for c-Jun, JunD, or c-Fos or mouse antibody specific for phospho-c-Jun (p-c-Jun, phosphorylated at serine 63). Nuclear extracts were incubated for 30 minutes at room temperature followed by the addition of γ^{32}P-labeled AP-1 oligomer and further incubated for 20 minutes. Samples were analyzed on 4% non-denaturing polyacrylamide gels as described above for EMSA. Intensities of super-shifted complexes were quantified using ImageQuant Software v1.2.

SOCS2 overexpression in IEC-6 cells

IEC-6 cells infected with SOCS2-expressing adenovirus or empty virus were used as a simple *in vitro* system to confirm *in vivo* findings that SOCS2 impacts on AP-1 binding activity. IEC-6 cells are a non-transformed intestinal epithelial cell line previously shown to exhibit robust AP-1 activation in response to combined IGF-I and EGF.[123] IEC-6 cells at 80% confluence were infected with either FLAG-tagged SOCS2 (Ad-SOCS2) or empty control adenovirus (Ad-Empty) in 10 cm^2 dishes as previously described.[75] Briefly, growth medium+FBS was removed and serum-free medium (SFM) containing the appropriate virus at a multiplicity of 100 viral particles/cell was added. Twenty-four hours after infection, fresh SFM or SFM plus rhIGF-I (20ng/mL, Genetech, San Francisco, CA) and rhEGF (5ng/mL, Sigma, St. Louis, MO) was added for 0, 15, and 30 minutes. Cells were scraped and nuclear extracts prepared as described above. AP-1 binding activity was assessed by EMSA and super-shift as described above. Data were quantified as a percentage of the values in Ad-Empty infected cells in SFM alone.

Statistical analyses

Values for average adenoma number, size, and tumor load and hematocrit were expressed as mean ± SEM. Analysis of variance (ANOVA) was used to compare data derived from $Apc^{Min/+}$ mice with $SOCS2^{+/+}$, $SOCS2^{+/-}$, or $SOCS2^{-/-}$ genotypes to determine if there was a significant effect of SOCS2 deletion. Plasma IGF-I and IGF-I, c-*Jun,* and c-*Fos* mRNA data were compared between $SOCS2^{+/+}/Apc^{Min/+}$ and $SOCS2^{-/-}/Apc^{Min/+}$ mice by student's t-test. Densitometry data for STAT3 and AP-1 DNA binding in tumor versus non-tumor

tissue of SOCS2$^{+/+}$/*Apc*$^{Min/+}$ and SOCS2$^{-/-}$/*Apc*$^{Min/+}$ mice were analyzed by ANOVA. Pair-wise comparisons were performed using Fischer's Exact test. Densitometry on AP-1 DNA binding in *in vitro* studies were compared between Ad-SOCS2 and Ad-Empty infected cells using student's t-test. Statistical tests were performed using Statview 4.1.

C. Results

Disruption of both SOCS2 alleles modestly affects body weight

SOCS2$^{-/-}$ mice have a body overgrowth phenotype.[93] We determined whether SOCS2$^{-/-}$ mice retained this phenotype when crossbred onto the *Apc*$^{Min/+}$ line. SOCS2$^{+/+}$/*Apc*$^{Min/+}$ mice had a final body weight of 21.4±0.4 g at sacrifice. Loss of one SOCS2 allele had no significant effect on body weight (21.8±0.3 g). However, SOCS2$^{-/-}$ /*Apc*$^{Min/+}$ mice had body weights of 27.8±2.6 g, representing a significant 29.6% increase in final body weight ($p<0.05$).

SOCS2 gene disruption dramatically increases *Apc*$^{Min/+}$ tumorigenesis

Small Intestine

Figure 2.1A shows representative images of tumors in small intestine of *Apc*$^{Min/+}$ mice with SOCS2$^{+/+}$, SOCS2$^{+/-}$ or SOCS2$^{-/-}$ genotypes. Quantitative data for tumor number, size or total tumor burden (number x size) are shown in Figure 1 for the entire small intestine (Figure 2.1B) or different regions of small intestine (Figure 2.1C).

Compared with SOCS2$^{+/+}$/*Apc*$^{Min/+}$ mice, SOCS2$^{-/-}$/*Apc*$^{Min/+}$ mice showed significant and dramatic increases in total tumor number and tumor size, together representing a 441% increase in overall tumor burden in the small intestine. *Apc*$^{Min/+}$ mice with disruption of one SOCS2 allele showed an intermediate phenotype with a significant increase in tumor number and tumor size (Figure 2.1B). This suggests that SOCS2 has a gene-dosage effect on *Apc*$^{Min/+}$ tumorigenesis. Examination of different regions of the small intestine revealed that SOCS2$^{-/-}$/*Apc*$^{Min/+}$ mice showed significant increases in tumor number, size, and load in all segments (Figure 2.1C). While a trend for increased tumor number and size was observed in all small bowel regions of SOCS2$^{+/-}$/*Apc*$^{Min/+}$ mice, this was statistically significant only for tumor number in the most distal small bowel segment.

Colon

SOCS2 gene disruption in *Apc*$^{Min/+}$ mice also had a dramatic effect on tumor number and size in colon. By 13-17 weeks of age, 100% of SOCS2$^{-/-}$/*Apc*$^{Min/+}$ mice (6/6) and 71% of SOCS2$^{+/-}$/*Apc*$^{Min/+}$ (5/7) mice had at least one colon tumor, whereas only one of six SOCS2$^{+/+}$/*Apc*$^{Min/+}$ mice studied (17%) had an observable colon tumor. Figure 2.2A shows representative images of colon tumors and Figure 2.2B shows quantitative data for tumor number and size. These analyses revealed significant increases in tumor number, size, and burden in colon of SOCS2$^{-/-}$/*Apc*$^{Min/+}$ mice compared with SOCS2$^{+/+}$/*Apc*$^{Min/+}$. As was seen with the small intestine, SOCS2$^{+/-}$/*Apc*$^{Min/+}$ showed an intermediate phenotype.

We used hematocrit as an indirect measure of tumor burden. Consistent with increases in tumor burden, we found that SOCS2$^{+/+}$/*Apc*$^{Min/+}$ mice had hematocrit within normal range (34.2±5.9%), SOCS2$^{-/-}$/*Apc*$^{Min/+}$ mice had significant reductions in hematocrit (22.4±5.3%, $p<0.05$), and SOCS2$^{+/-}$/*Apc*$^{Min/+}$ mice had an intermediate hematocrit (29.5±7.7%). Further studies compared SOCS2$^{+/+}$/*Apc*$^{Min/+}$ and SOCS2$^{-/-}$/*Apc*$^{Min/+}$ mice, in which the most dramatic changes in tumor number and size were observed.

β-catenin immuno-staining

Tumor development in *Apc*$^{Min/+}$ mice is driven by focal loss of heterozygosity of the *Apc* gene.[34] This leads to translocation of β-catenin from the lateral membranes of the epithelium to the nucleus where it promotes gene transcription. In the normal small intestine of both SOCS2$^{+/+}$/*Apc*$^{Min/+}$ and SOCS2$^{-/-}$/*Apc*$^{Min/+}$ mice, β-catenin was detected at the lateral and basement membranes of epithelial cells along the villi and in cell membranes and some nuclei in the crypts (Figure 2.3A). Adenomas in the small intestine of both SOCS2$^{+/+}$/*Apc*$^{Min/+}$ and SOCS2$^{-/-}$/*Apc*$^{Min/+}$mice showed positive nuclear and cytoplasmic staining for β-catenin in epithelial-like cells of adenomas, but no obvious differences in staining intensity or nuclear localization were observed (Figure 2.3B). Colon adenomas in SOC2$^{-/-}$/*Apc*$^{Min/+}$ mice were positive for nuclear and cytoplasmic β-catenin, indicating activated β-catenin signaling (Figure 2.3C). Since so few SOCS2$^{+/+}$/*Apc*$^{Min/+}$mice develop colon tumors, β-catenin was not analyzed. The data

confirm that increased nuclear β-catenin occurs in adenomas of both SOCS2$^{+/+}$/*Apc*$^{Min/+}$ and SOCS2$^{-/-}$/*Apc*$^{Min/+}$ mice but provide no evidence that loss of SOCS2 enhances nuclear β-catenin accumulation.

SOCS2$^{-/-}$/*Apc*$^{Min/+}$ mice show increased IGF-I expression in the small intestine, but no elevation in plasma IGF-I

We used real-time PCR to assess if the enhanced tumorigenesis in small intestine of SOCS2$^{-/-}$/*Apc*$^{Min/+}$ mice was associated with increases in local IGF-I mRNA. Figure 2.4A shows that compared to the liver, which is the major target of GH-induced IGF-I expression, the small intestine expresses relatively low amounts of IGF-I mRNA. As shown in Figure 2.4B, SOCS$^{-/-}$/*Apc*$^{Min/+}$ mice had small but statistically significant 1.5 fold increases in IGF-I mRNA in the ileum. In contrast, plasma IGF-I levels did not differ significantly in SOCS2$^{+/+}$/*Apc*$^{Min/+}$and SOCS2$^{+/+}$/*Apc*$^{Min/+}$ mice (Figure 2.4C). These data indicate that disruption of both SOCS2 alleles in *Apc*$^{Min/+}$ mice promotes increased local intestinal IGF-I expression, without affecting circulating IGF-I.

SOCS2 gene disruption promotes serine-phosphorylation but not tyrosine phosphorylation of STAT3 in *Apc*$^{Min/+}$ mice.

In normal intestine, SOCS2 gene disruption has previously been linked to enhanced IGF-I-induced STAT3 DNA binding activity.[75] Therefore, we examined whether grossly normal mucosa (non-tumor) or dissected tumors of SOCS2$^{+/+}$/*Apc*$^{Min/+}$ or SOCS2$^{-/-}$/*Apc*$^{Min/+}$ mice showed altered levels of nuclear tyrosine-phosphorylated STAT3 (pY705), which is integral to STAT3 DNA binding activity, and compared this with levels of serine-phosphorylated STAT3 (pS727). Surprisingly, levels of pY705-STAT3 did not differ in tumors or non-tumor tissue from SOCS2$^{-/-}$/*Apc*$^{Min/+}$ versus SOCS2$^{+/+}$/*Apc*$^{Min/+}$ mice (Figure 2.5). However, the immuno-reactive pY705-STAT3 in SOCS2$^{-/-}$/*Apc*$^{Min/+}$ tumors was an obvious doublet, most likely due to concurrent serine-phosphorylation. Western immunoblot of the same samples revealed that pS727-STAT3 was upregulated in non-tumor tissue from SOCS2$^{-/-}$/*Apc*$^{Min/+}$ mice and especially tumors of SOCS2$^{-/-}$/*Apc*$^{Min/+}$ mice, showing an overall 2.5±0.45 fold increase in pS727-STAT3 ($p<0.05$) (Figure 2.5). When analyzed across multiple blots, total STAT3 did not significantly differ in tumor and non-tumor tissues between SOCS2$^{+/+}$/*Apc*$^{Min/+}$ and SOCS2$^{-/-}$/*Apc*$^{Min/+}$ mice (Figure 2.5).

To confirm increased serine phosphorylation of STAT3 in the tumors of SOCS2$^{-/-}$/*Apc*$^{Min/+}$ mice, we performed immunohistochemistry on small intestine and colon. In SOCS2$^{+/+}$/*Apc*$^{Min/+}$ mice, pS727-STAT3 immunoreactivity was barely detectable except at the base of the crypts (Figure 2.6A). Immunostaining revealed dramatic increases in pS727-STAT3 in the nucleus and cytoplasm of crypt and villus epithelial cells in the normal small intestine of SOCS2$^{-/-}$/*Apc*$^{Min/+}$ mice (Figure 2.6A). Serine-phosphorylated-

STAT3 was detectable in the adenomas of SOCS2$^{+/+}$/*Apc*$^{Min/+}$ mice. However, small intestine adenomas of SOCS2$^{-/-}$/*Apc*$^{Min/+}$ mice showed dramatic increases in pS727-STAT3 in the cytoplasm and nucleus (Figure 2.6B). Intense staining of pS727-STAT3 was also detected in colon adenomas from SOCS2$^{-/-}$/*Apc*$^{Min/+}$ (Figure 2.6C). We also examined total STAT3 by immunohistochemistry and detected strong staining throughout the epithelium in the normal intestine that did not differ between SOCS2$^{+/+}$/*Apc*$^{Min/+}$ and SOCS2$^{-/-}$/*Apc*$^{Min/+}$ (Figure 2.6D). Compared to non-tumor tissue, tumors from both SOCS2$^{+/+}$/*Apc*$^{Min/+}$ and SOCS2$^{-/-}$/*Apc*$^{Min/+}$ mice showed stronger cytosolic and nuclear STAT3 immunostaining but, in contrast to pS727-STAT3 immunostaining, there was no dramatic difference in total STAT3 in SOCS2$^{-/-}$/*Apc*$^{Min/+}$ versus SOCS2$^{+/+}$/*Apc*$^{Min/+}$ tumors (Figures 2.6E and F). These studies suggest that deletion of SOCS2 genes promotes serine-727 phosphorylation of STAT3 in intestinal epithelium and that this is further enhanced in small intestine and colon tumors.

SOCS2 deficiency enhances AP-1 but not STAT3 DNA binding.

Since serine phosphorylation of STAT3 was increased in the tumors of SOCS2$^{-/-}$/*Apc*$^{Min/+}$ mice, we tested whether this was associated with changes in STAT3 DNA binding activity. STAT3 DNA binding activity was evaluated in nuclear extracts from pooled tumors and matched biopsies of grossly normal intestine from SOCS2$^{+/+}$/*Apc*$^{Min/+}$ and SOCS2$^{-/-}$/*Apc*$^{Min/+}$ mice. As shown in Figure 2.7A, tumor or non-tumor samples from SOCS2$^{+/+}$ and SOCS2$^{-/-}$ carrying the *Apc*$^{Min/+}$ mutation had multiple STAT3 DNA

binding complexes. Tumors from both SOCS2$^{+/+}$/*Apc*$^{Min/+}$ and SOCS2$^{-/-}$/*Apc*$^{Min/+}$ mice showed a trend for increased STAT3 DNA binding activity relative to non-tumor tissue, but this achieved statistical significance only when comparing tumor and non-tumor tissues of SOCS2$^{-/-}$/*Apc*$^{Min/+}$ mice (Figure 2.7B). Surprisingly, however, there was no difference in STAT3 DNA binding activity in tumor or non-tumor tissues of SOCS2$^{-/-}$/*Apc*$^{Min/+}$ mice compared with SOCS2$^{+/+}$/*Apc*$^{Min/+}$ mice (Figure 2.7B). Thus, loss of SOCS2 did not significantly increase overall STAT3 binding activity as might have been expected.

Because phosphorylation of serine 727 of STAT3 has been linked to AP-1 activation in tumor cell lines, we analyzed whether SOCS2 deletion affected nuclear AP-1 DNA binding. This revealed enhanced AP-1 binding activity in non-tumor and particularly tumor tissue of SOCS2$^{-/-}$/*Apc*$^{Min/+}$ compared to SOCS$^{+/+}$/*Apc*$^{Min/+}$ (Figure 2.8A). The results of three independent experiments were quantified using densitometric volume analysis and demonstrated significant increases in AP-1 DNA binding activity in the tumors of the SOC2$^{-/-}$/*Apc*$^{Min/+}$ mice compared to both tumor and non-tumor tissue from SOCS2$^{+/+}$/*Apc*$^{Min/+}$ mice (Figure 2.8B).

To verify specificity of enhanced AP-1 DNA binding activity in SOCS2$^{-/-}$/*Apc*$^{Min/+}$ mice, we performed supershift analysis for c-Jun, JunD, phosphorylated c-Jun and c-Fos, as well as competition with unlabeled AP-1 oligomer versus an unrelated oligomer (TCF consensus sequence). As shown in figure 2.8C, AP-1 DNA binding complexes in tumors from SOCS2$^{-/-}$/*Apc*$^{Min/+}$ mice showed supershifted complexes with JunD, p-c-Jun, and c-Jun antibodies and showed specific competition with AP-1, but not

TCF oligomers. Antibodies to c-Fos did not elicit a supershifted complex or major shift-inhibition (i.e. reduction in intensity of DNA:protein complex). Thus, the enhanced AP-1 DNA binding activity in tumors from SOCS2$^{-/-}$/*Apc*$^{Min/+}$ mice contains primarily p-c-Jun, JunD and c-Jun. Supershifted complexes were lower in intensity in non-tumor samples, yet qualitatively similar in composition (Figure 2.10).

To assess if the enhanced AP-1 DNA binding activity reflected increased expression of *c-Jun* or *c-Fos*, we quantified mRNA using quantitative real-time PCR. This revealed no significant difference in *c-Fos* or *c-Jun* between SOCS2$^{-/-}$/*Apc*$^{Min/+}$ (*c-Fos,* 1.20 ± 0.45 ; *c-Jun*, 1.09 ± 0.10) versus SOCS2$^{+/+}$/*Apc*$^{Min/+}$ (*c-Fos*, 1.0 ± 0.3; *c-Jun* 1.0 ± 0.1).

SOCS2 overexpression reduced AP-1 DNA binding in IEC-6 cells

Previous studies from our lab have demonstrated that IGF-I and EGF act synergistically to promote proliferation of IEC-6 cells and induce AP-1 transcriptional activity greater than either alone.[123] We therefore used this *in vitro* system to directly test if SOCS2 affects AP-1. As shown in Figure 2.9, IGF and EGF induced AP-1 binding activity in IEC-6 cells and SOCS2 overexpression significantly attenuated growth factor-induced AP-1 DNA binding (Figure 2.9A-B). This complements the *in vivo* data in SOCS2$^{-/-}$/*Apc*$^{Min/+}$ mice, providing additional evidence that SOCS2 affects AP-1 transcriptional activity in intestinal epithelial cells. Super-shift analysis demonstrated that the AP-1 complex in untreated or IGF/EGF treated IEC-6 cells contained c-Jun, JunD, and p-c-Jun, as observed in SOCS2$^{-/-}$/*Apc*$^{Min/+}$ tumors. Growth factor treatment increased

the intensity of p-c-Jun supershifted complex (Figure 2.9C). Super-shifted complexes were uniformly lower in intensity in SOCS2 overexpressing cells (Figure 2.9C) but the relative percentage of AP-1 binding represented by JunD, phosphorylated c-Jun and c-Jun was similar between empty vector and SOCS2 overexpressing cells (Figure 2.9C). This indicates that SOCS2 down-regulates all Jun isoforms within AP-1 DNA binding complexes to an approximately similar extent.

D. Discussion

Our studies show that disruption of both SOCS2 alleles in $Apc^{Min/+}$ mice dramatically increases tumor number and tumor size in the small intestine and colon. We found this to be associated with increased local intestinal IGF-I expression. Serine phosphorylation of STAT3 was increased in the tumors of $SOCS2^{-/-}/Apc^{Min/+}$ mice and, to a lesser extent, in grossly normal intestine of $SOCS2^{-/-}/Apc^{Min/+}$ mice. Neither tyrosine phosphorylation of STAT3 nor STAT3 DNA binding activity was enhanced by SOCS2 gene deletion. The pro-tumorigenic effects of SOCS2 gene disruption were, however, associated with enhanced AP-1 DNA binding activity in the tumors of $SOCS2^{-/-}/Apc^{Min/+}$ mice, where the AP-1 complex contained c-Jun, phosphorylated c-Jun, and JunD. Overexpression of SOCS2 in IEC-6 cells reduced AP-1 DNA binding, with approximately equal reduction in c-Jun, phosphorylated c-Jun, and JunD.

The increases in tumor number in $SOCS2^{-/-}/Apc^{Min/+}$ mice occurred across all regions of the small intestine, with maximal effects in the most distal regions. Effects of

SOCS2 deletion on tumor size were less dramatic, but represent a significant finding since most modifiers of $Apc^{Min/+}$ tumorigenesis alter tumor number, but not size.[180] Our findings, therefore, suggest that loss of SOCS2 impacts the rate of tumor growth as well as the number of initiated tumors or their survival. This is strongly supported by data in the colon of SOCS2$^{-/-}$/$Apc^{Min/+}$mice. Very few SOCS2$^{+/+}$/$Apc^{Min/+}$mice developed colon tumors at the time studied. In contrast, 100% of SOCS2$^{-/-}$/$Apc^{Min/+}$mice and 71% of SOCS2$^{+/-}$/$Apc^{Min/+}$mice developed colon tumors by 13-17 weeks, and tumor size was increased in colon as observed in small intestine. The current findings in the $Apc^{Min/+}$ model are consistent with our prior findings that loss of one SOCS2 allele in GH-transgenic mice promotes benign colon polyps.[49] However, to our knowledge, this current study is the first to demonstrate that deletion of one or both SOCS2 alleles promotes spontaneous pre-cancerous lesions that are driven by aberrant β-catenin activation, a pathway that is disregulated in many human intestinal tumors. This suggests that epigenetic SOCS2 silencing may be relevant to colon cancer risk in humans.

A number of studies have shown that SOCS2 genes are epigenetically inactivated by hypermethylation of CpG islands within the promoter region of biopsies of primary human tumors or cancer cell lines from a number of organs, including prostate, melanoma, breast, ovary, and endometrium and this correlates with reduced SOCS2 expression.[165,164,167,166,168] Other SOCS genes, including SOCS1 and SOCS3, have also been shown to be hypermethylated in Barrett's adenocarcinoma.[104] SOCS1 is hypermethylated in breast cancer biopsies and in glioblastoma cells.[181,182] SOCS3 is also methylated in glioblastoma cells and hypermethylation of SOCS3 is associated with

unfavorable clinical outcome.[181] However, relatively few studies have shown a functional role for SOCS in tumor development or growth *in vivo*. Mice with SOCS3 deleted specifically in liver parenchymal cells exhibited increased tumor development when treated with the carcinogen diethylnitrosamine.[183] Deletion of SOCS3 specifically in intestinal epithelial cells (IEC) led to greater tumor load in the azoxymethane/dextran sodium sulfate (AOM/DSS) mouse model of inflammation-induced colon cancer.[113] This indicates a role for SOCS3 in normally limiting inflammation-associated colon cancer. Our current data in SOCS2$^{-/-}$/*Apc*$^{Min/+}$mice indicate that endogenous SOCS2 normally limits the development of tumors that derive from disregulated β-catenin, a pathway that is integral to the development of many human colon cancers. To date, the relative roles of SOCS2 compared with SOCS1 or SOCS3 in tumor development have not been formally compared in the same animal model of intestinal tumorigenesis. In this regard, preliminary findings in the AOM/DSS model provided no evidence that SOCS2 gene disruption affects tumor number or load (SOCS2$^{+/+}$ = 2.2 ± 0.6, SOCS2$^{-/-}$ = 2.9 ± 0.4; n=5) in this model of inflammation-associated colon cancer. While more study is needed, this would suggest that SOCS2 and SOCS3 should be compared as potentially useful biomarkers of the risk of human colon tumors driven by two distinct and major initiating factors, activation of β-catenin versus inflammation.

Our findings suggest some novel regulatory mechanisms by which SOCS2 deletion may promote *Apc*$^{Min/+}$-mediated intestinal tumorigenesis. We initially assessed local IGF-I because SOCS2 gene deletion was shown to increase local IGF-I gene expression in some tissues and to increase GH-stimulated, local IGF-I expression in the intestine.[49,75,92] Our findings demonstrate a modest upregulation of local IGF-I expression

in SOCS2$^{-/-}$/*Apc*$^{Min/+}$intestine, which may contribute to enhanced tumor load. IGF-I signaling has been reported to enhance stability, nuclear localization and transcriptional activation of β-catenin.[53,54] This is thought to occur through insulin receptor substrate-1 (IRS-1), which is an immediate downstream mediator of IGF-I receptor signaling.[53,54] However, observations that tumors from SOCS2$^{+/+}$/*Apc*$^{Min/+}$and SOCS2$^{-/-}$/*Apc*$^{Min/+}$mice show comparable increases in β-catenin accumulation argue against a major effect of SOCS2 deletion or accompanying increases in local IGF-I on β-catenin activation or accumulation at least at the stage of tumor development examined. We note that increased local IGF-I expression in the intestine of SOCS2$^{-/-}$/*Apc*$^{Min/+}$ was not accompanied by an elevation in plasma IGF-I. This is consistent with prior findings in SOCS2$^{-/-}$/*Apc*$^{Min/+}$ mice and suggests that SOCS2 gene disruption impacts preferentially on local intestinal IGF-I expression rather than circulating IGF-I levels.[49,93]

We examined the STAT3 pathway because of our prior findings that SOCS2$^{-/-}$ mice show enhanced IGF-I-induced STAT3 activation in normal intestine.[75] STAT3 activation is frequently associated with colon cancer in a setting of chronic inflammation.[184,185,186] Recent studies demonstrated that specific STAT3 deleted in intestinal epithelial cells reduces tumor multiplicity in *Apc*$^{Min/+}$ mice.[187] Phosphorylation of STAT3 at tyrosine residue 705 is required for dimerization, nuclear translocation and DNA binding.[173,188] Unexpectedly, we found similar levels of tyrosine-phosphorylated STAT3 in normal small intestine tissue and tumors from SOCS2$^{-/-}$/*Apc*$^{Min/+}$compared to SOCS2$^{+/+}$/*Apc*$^{Min/+}$mice. Instead, our studies revealed an increase in serine 727-phosphorylated STAT3 in SOCS2$^{-/-}$/*Apc*$^{Min/+}$ mice. Serine-phosphorylated STAT3

localized to epithelial cells in normal intestine and to tumors in SOCS2$^{-/-}$/*Apc*$^{Min/+}$ mice. The increased pS727-STAT3 in SOCS2$^{-/-}$/*Apc*$^{Min/+}$ compared to SOCS2$^{+/+}$/*Apc*$^{Min/+}$ observed by western immunoblot was independently confirmed by immunohistochemistry. This is intriguing because a series of recent papers has demonstrated upregulation of pS727-STAT3 in mammary gland tumors[189] and in a number of tumor cell lines including ras-transformed fibroblasts, bladder and lung tumor cell lines,[174] and medullary thyroid tumor cell lines.[172] Studies using prostate cancer cell lines have shown that a phosphomimetic mutation of serine727 to a glutamine promoted anchorage-independent growth, invasion, and increased tumor number in xenograft models.[190] These effects were reversed by an un-phosphorylatable STAT3 mutant.[190] In another study, mutation of serine727 in STAT3 to an alanine decreased post-natal growth and survival in mice lacking one functional STAT3 allele.[191] This correlated with reduced circulating GH and IGF-I.[191] Together, these studies provide evidence that serine phosphorylation of STAT3 may regulate normal and neoplastic growth. Our current findings add novel evidence that loss of SOCS2 preferentially enhances pS727-STAT3 in the intestine *in vivo* and this is associated with dramatically increased intestinal tumor development and growth in animals carrying an inactivating *Apc* mutation.

The role of pS727-STAT3 in DNA binding and transcriptional activation of STAT3 is not fully defined. Prior studies have linked pS727-STAT3 to reduced tyrosine phosphorylation and transcriptional activation of STAT3.[192,193] Other reports indicate that serine phosphorylation of STAT3 does not impact DNA binding.[194] More recent studies indicate that there are context-, cytokine- and growth factor-dependent differences in serine phosphorylation versus tyrosine phosphorylation of STAT3.[195,172,173,196,197]

Importantly, a recent report in medullary thyroid carcinoma cell lines demonstrated that pS727-STAT3 is essential to transcriptional activation of AP-1 DNA binding activity.[172] This is consistent with the current observations that nuclear AP-1 DNA binding activity is dramatically upregulated in tumors from SOCS2$^{-/-}$/*Apc*$^{Min/+}$ mice. Our findings that SOCS2$^{-/-}$/*Apc*$^{Min/+}$ mice show enhanced AP-1 DNA binding activity, but no detectable increase in c-*Fos* or c-*Jun* mRNA indicates that loss of SOCS2 promotes activation of AP-1 rather than increased synthesis of AP-1 components. Futhermore, supershift data indicate that the AP-1 binding complex in SOCS2$^{-/-}$/*Apc*$^{Min/+}$ mice contains primarily c-Jun, and particularly phosphorylated c-Jun and JunD. These results were complemented with *in vitro* studies in IEC-6 cells, demonstrating that adenoviral overexpression of SOCS2 reduced basal and growth factor-stimulated AP-1 DNA binding activity with approximately equal inhibitory effects on c-Jun, phosphorylated c-Jun, and JunD. This is intriguing since recent studies suggest that Jun proteins are strongly expressed in tumors of mice with specific activation of β-catenin in intestinal epithelium.[198] Activation of c-Jun N-terminal kinase (JNK), which mediates c-Jun phosphorylation at serine 63, was also shown to activate TCF4/β-catenin gene targets and increase crypt proliferation.[199] These studies provide indirect evidence to support a concept that the enhanced AP-1 activity observed here in SOCS2$^{-/-}$/*Apc*$^{Min/+}$ may contribute to the increased tumorigenesis. Additional studies will be required to fully delineate the mechanisms by which loss of SOCS2 expression promotes serine phosphorylation of STAT3 and AP-1 DNA binding activity, whether these two effects are causally connected, and their functional roles in enhanced tumorigenesis in SOCS2$^{-/-}$/*Apc*$^{Min/+}$ mice. However, our findings provide novel evidence that SOCS2 normally serves as a suppressor of *Apc*$^{Min/+}$

tumorigenesis in intestine and acts on pathways other than tyrosine-phosphorylation of STAT3.

In summary, the results from our study suggest that SOCS2 gene deletion promotes tumor initiation and progression in the $Apc^{Min/+}$ model of spontaneous intestinal cancer driven by β-catenin activation and this is associated with local increases in IGF-I, serine phosphorylation of STAT3, and AP-1 activation but not increased tyrosine phosphorylation of STAT3. These studies provide novel evidence that SOCS2 normally limits tumor growth in both the small intestine and colon and suggest that reduced expression or epigenetic silencing of SOCS2, and activation of pS727-STAT3 or AP-1 may be potential biomarkers for intestinal tumor development and growth in a setting of APC mutation or β-catenin activation.

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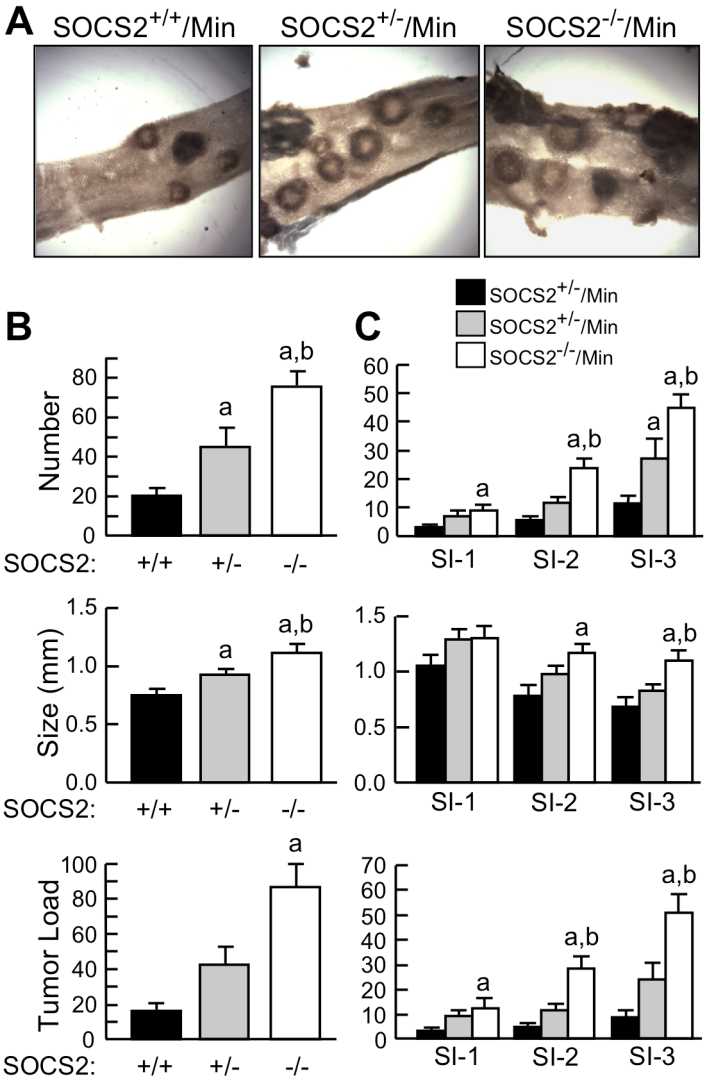

Figure 2.1. SOCS2 gene disruption in *Apc*$^{Min/+}$ mice increases tumor number and size in the small intestine. A. Representative gross images of adenomas in small intestine of SOCS2$^{+/+}$/*Apc*$^{Min/+}$, SOCS2$^{+/-}$/*Apc*$^{Min/+}$, and SOCS2$^{-/-}$/*Apc*$^{Min/+}$ mice taken at 12.3x under a dissecting scope with white light optics underneath the specimen. B. Histograms show mean ± SE for tumor number (*top*), size (*middle*), and load (number x size)(*bottom*) in entire small intestine. C. Mean ± SE for tumor number, size, and load in different regions of the small intestine. SI-1 is the first third of the small intestine that contains the duodenum and is most proximal, whereas SI-3 contains the ileum and is most distal. a = $p < 0.05$ vs. SOCS2$^{+/+}$/*Apc*$^{Min/+}$ mice and b = $p < 0.05$ vs SOCS2$^{+/-}$/*Apc*$^{Min/+}$.

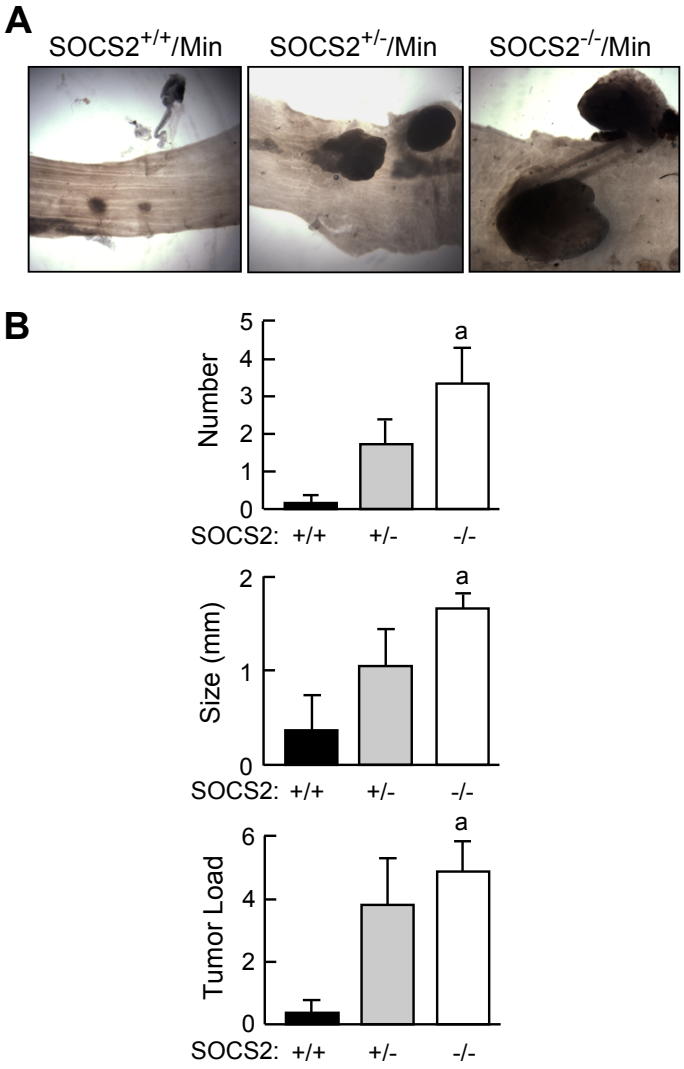

Figure 2.2. SOCS2 gene disruption in *Apc*$^{Min/+}$ mice increases tumor number and size in the colon. A. Representative gross images of colon adenomas of female SOCS2$^{+/+}$/*Apc*$^{Min/+}$, SOCS2$^{+/-}$/*Apc*$^{Min/+}$, and SOCS2$^{-/-}$/*Apc*$^{Min/+}$ mice at 13-17 wks of age taken at 12.3x under a dissecting scope. Colon tumors from female mice are shown since they demonstrated the greatest increases in tumor size compared to age-matched female SOCS2$^{+/+}$/*Apc*$^{Min/+}$. At this time, 17% of age- and sex-matched SOCS2$^{+/+}$/*Apc*$^{Min/+}$mice had colon tumors, whereas 71% of SOCS2$^{+/-}$/*Apc*$^{Min/+}$ and 100% of SOCS2$^{-/-}$ /*Apc*$^{Min/+}$mice had tumors. The small lesions visible in SOCS2$^{+/+}$/*Apc*$^{Min/+}$ colon were determined to be lymphoid aggregates. B. Histograms show mean ± SE for tumor number (*top*), tumor size (*middle*), and tumor load (*bottom)* in colon. a = $p<0.05$ vs. SOCS2$^{+/+}$/*Apc*$^{Min/+}$ mice by ANOVA.

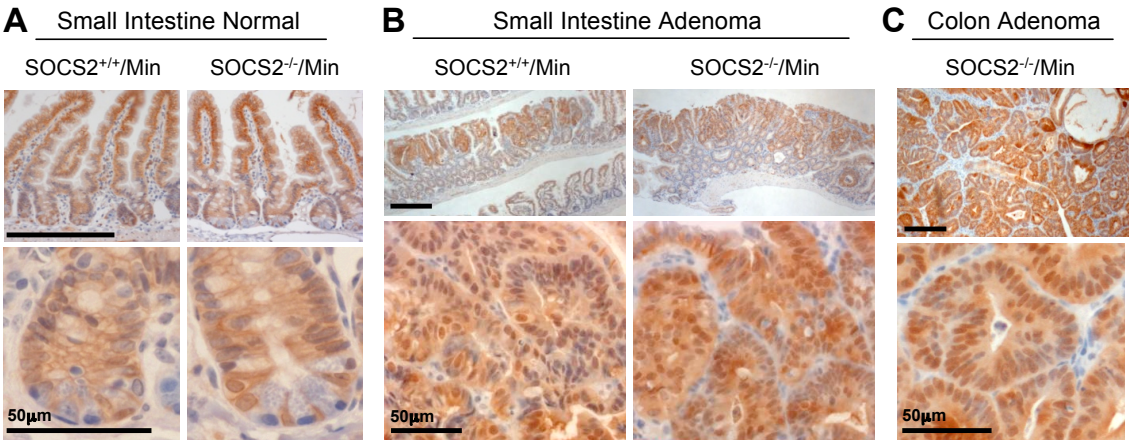

Figure 2.3. Tumors of SOCS2$^{+/+}$/*Apc*$^{Min/+}$ and SOCS2$^{-/-}$/*Apc*$^{Min/+}$ mice are positive for nuclear β-catenin. Representative histological images of A. grossly normal small intestine of SOCS2$^{+/+}$/*Apc*$^{Min/+}$ and SOCS2$^{-/-}$/*Apc*$^{Min/+}$ mice immunostained for β-catenin taken at 20X (*top)* and 40X *(bottom)*. B. β-catenin positive adenomas in the small intestine of SOCS2$^{+/+}$/*Apc*$^{Min/+}$ and SOCS2$^{-/-}$/*Apc*$^{Min/+}$ mice at 5X *(top)* and 40X *(bottom),* C. adenoma in the colon of a SOCS2$^{-/-}$/*Apc*$^{Min/+}$ mouse positive for β-catenin immunostaining at 5X (*left)* and 40X *(right)*. Scale bar indicates 200 µm unless otherwise noted.

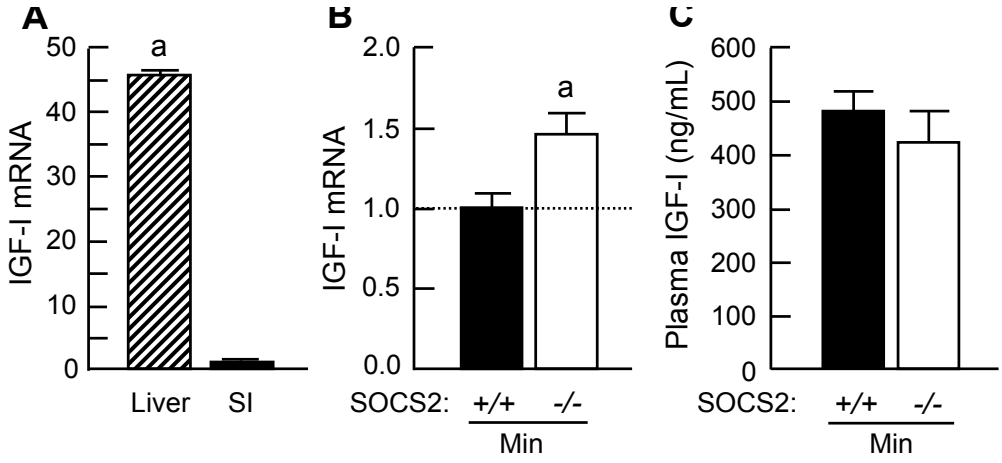

Figure 2.4. Homozygous SOCS2 gene disruption increases local intestinal IGF-I expression, but not plasma IGF-I. A-B. Real-time PCR was performed on RNA extracted from WT liver and small intestine (ileum) or entire ileum from SOCS2$^{+/+}$ or SOCS2$^{-/-}$ mice carrying the *Apc*$^{Min/+}$ mutation. Absolute copy number was calculated from Ct values for either IGF-I or HMBS using a standard curve. Histograms show the relative expression (mean ± SE) of three runs for IGF-I/HMBS. A. Histogram shows fold change of IGF-I expression in the liver relative to the small intestine. n ≥ 3. Note expression in the liver is ~45 fold higher compared to the small intestine. a = $p < 0.05$ vs SOCS2$^{+/+}$/WT. B. Histograms show IGF-I expression in ileal RNA from SOCS2$^{-/-}$/*Apc*$^{Min/+}$ mice relative to SOCS2$^{+/+}$/*Apc*$^{Min/+}$ mice analyzed in the same run. a = $p < 0.05$ versus SOCS2$^{+/+}$/*Apc*$^{Min/+}$. C. ELISA for plasma IGF-I levels (mean ± SE) in SOCS2$^{+/+}$/*Apc*$^{Min/+}$ and SOCS2$^{-/-}$/*Apc*$^{Min/+}$ mice. Binding proteins were removed prior to detection of IGF-I. Samples were run in duplicate, n > 4.

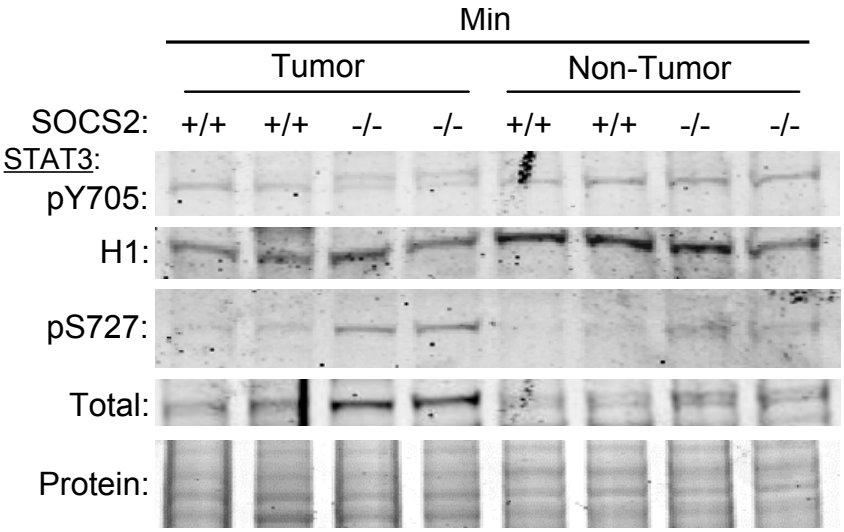

Figure 2.5. Tumors of SOCS2$^{-/-}$/*Apc*$^{Min/+}$ mice show increased serine phosphorylation of STAT3. Western blots on nuclear extracts from either normal intestine or a pool of tumors from the small intestine of SOCS2$^{+/+}$/*Apc*$^{Min/+}$ or SOCS2$^{-/-}$/*Apc*$^{Min/+}$ mice. Duplicate blots were immunoblotted for pY705-STAT or pS727-STAT3. Blots were then reprobed for H1, a nuclear protein used as a loading control, or total STAT3. Shown are reprobes for H1 on the pY705 blot and total STAT3 on the pS727-blot. Protein stain of nuclear proteins is shown in the bottom panel to demonstrate equal loading. A ~86kDa band was detected by both the pY and pS-STAT3 antibodies, although a doublet was apparent in the tumor samples with the pY705 antibody, most likely due to serine phosphorylation of STAT3. Overall, no difference in pY705-STAT3 was detected between SOCS2$^{+/+}$/*Apc*$^{Min/+}$ (100 ± 9%) and SOCS2$^{-/-}$/*Apc*$^{Min/+}$(108 ± 10%) (values normalized to H1 control). SOCS2$^{-/-}$/*Apc*$^{Min/+}$ mice had a significant increase in serine phosphorylation (normalized to total STAT3) compared to SOCS2$^{+/+}$/*Apc*$^{Min/+}$ mice (258 ± 45% versus 100 ± 23%, $p<0.05$). Although total STAT3 appeared elevated in tumors of SOCS2$^{-/-}$/*Apc*$^{Min/+}$ on this representative blot, across multiple blots and independent samples (n = 5) there was no significant difference in total STAT3 between SOCS2$^{+/+}$/*Apc*$^{Min/+}$ and SOCS2$^{-/-}$/*Apc*$^{Min/+}$ mice (tumor: 1.13 ± 0.06 versus 1.15 ± 0.06; non-tumor: 1.00 ± 0.05 versus 1.00 ± 0.08).

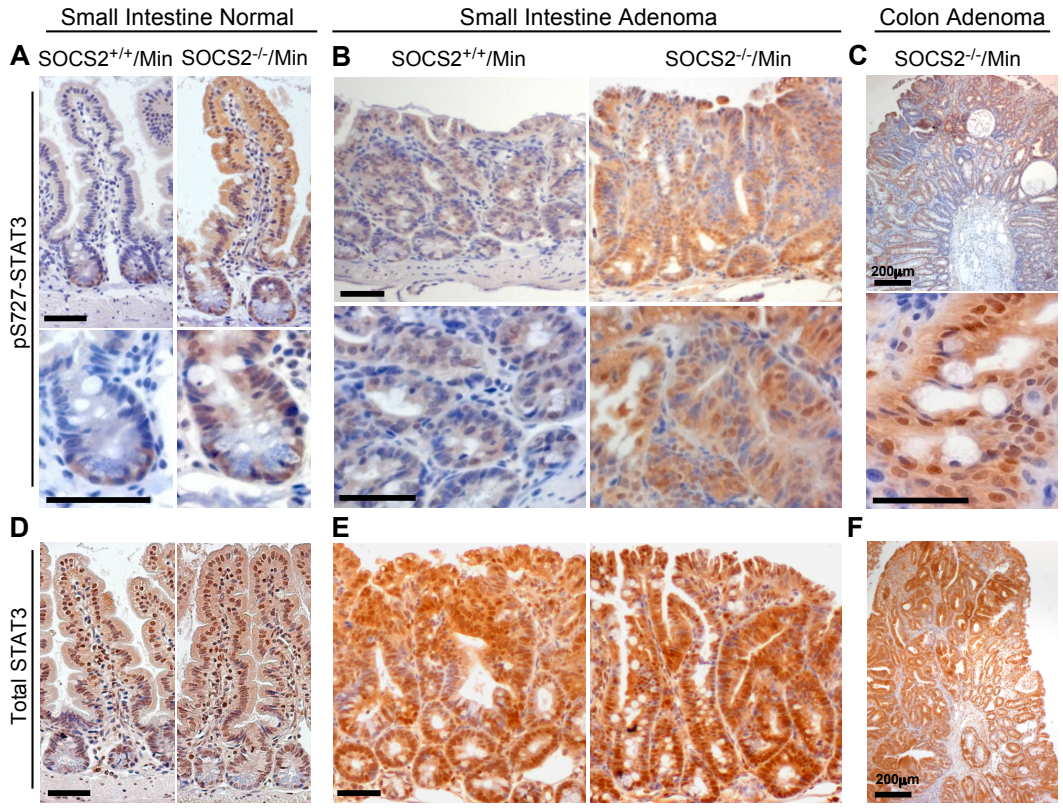

Figure 2.6. Increased pS727-STAT3 but not total STAT3 immunostaining in SOCS2$^{-/-}$/*Apc*$^{Min/+}$ mice. Representative images of tissue sections from SOCS2$^{+/+}$/*Apc*$^{Min/+}$ and SOCS2$^{-/-}$/*Apc*$^{Min/+}$ mice immunostained for pS727-STAT3 (A-C) or total STAT3 (D-F). A-C show immunostaining for pS727-STAT3 in: A. Normal small intestine from SOCS2$^{+/+}$/*Apc*$^{Min/+}$ or SOCS2$^{-/-}$/*Apc*$^{Min/+}$ mice at 20X *(top)* and 40X *(bottom)*, B. Small intestine adenoma from SOCS2$^{+/+}$/*Apc*$^{Min/+}$ and SOCS2$^{-/-}$/*Apc*$^{Min/+}$ mice at 10X *(top)* and 40X *(bottom)*, or C. Colon adenoma from SOCS2$^{-/-}$/*Apc*$^{Min/+}$ mouse at 5X *(top)* and 40X *(bottom)*. D-F show immunostaining for total STAT3 in D. Jejunum (20X), E. Small intestine adenoma (10X) from SOCS2$^{+/+}$/*Apc*$^{Min/+}$or SOCS2$^{-/-}$/*Apc*$^{Min/+}$mice, or F. Colon adenoma from SOCS2$^{-/-}$/*Apc*$^{Min/+}$ mouse (5X). Scale bars for all images indicate 50 μm unless otherwise noted

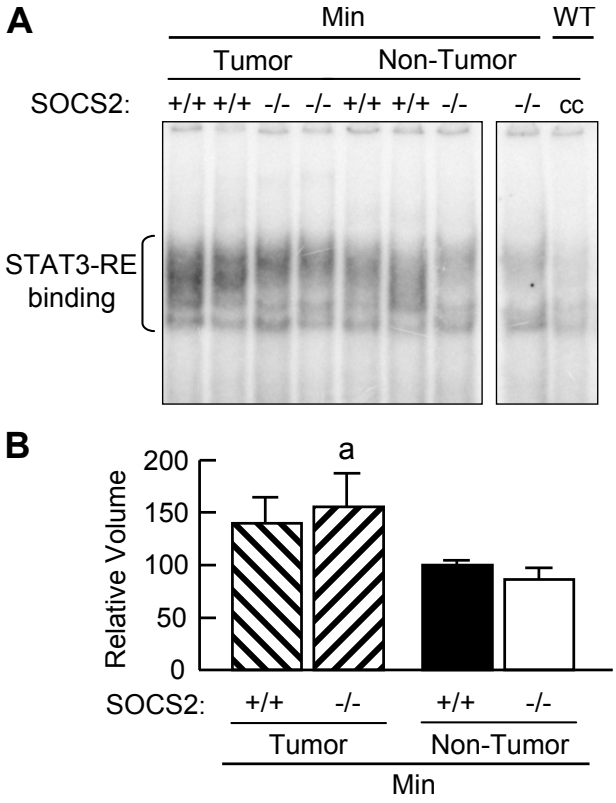

Figure 2.7. SOCS2 gene disruption does not increase nuclear STAT3 DNA binding activity in *Apc*$^{Min/+}$ mice. A. Autoradiogram of EMSA for nuclear binding activity to a STAT3 DNA binding sequence in either tumors or grossly normal small intestine of SOCS2$^{+/+}$/*Apc*$^{Min/+}$ and SOCS$^{-/-}$/*Apc*$^{Min/+}$ mice. cc, inhibition of DNA:protein binding complexes by an excess of unlabeled oligomer. Both panels are from the same blot. Multiple DNA:protein binding complexes were detected with the STAT3 response element. B. Densitometric analysis of STAT3 DNA binding complexes. Results show mean ± SE for intensity of DNA:protein complexes from 3 independent experiments expressed as a percentage of the SOCS2$^{+/+}$/*Apc*$^{Min/+}$ non-tumor samples. a = $p < 0.05$ vs non-tumor tissue from SOCS2$^{-/-}$/*Apc*$^{Min/+}$ mice.

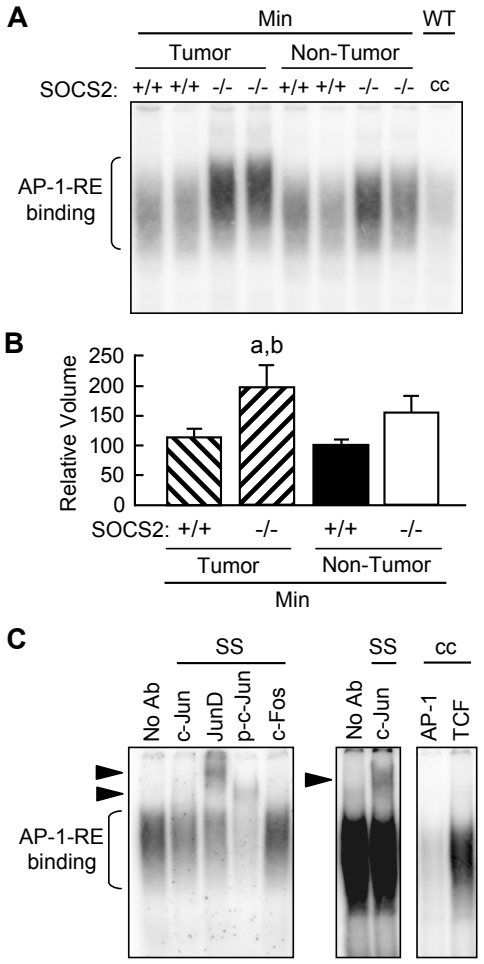

Figure 2.8. SOCS2 gene disruption promotes increased nuclear AP-1 DNA binding activity in *Apc*$^{Min/+}$ mice. A. Autoradiogram of EMSA for nuclear binding activity to an AP-1 DNA binding sequence in either tumors or grossly normal small intestine of SOCS2$^{+/+}$/*Apc*$^{Min/+}$ and SOCS2$^{-/-}$/*Apc*$^{Min/+}$ mice. cc, inhibition of shifted complexes by an excess of unlabeled oligomer. B. Densitometric analysis of AP-1 DNA Binding complex. Results show mean ± SE for intensity of AP-1:DNA binding complexes expressed as a percentage of the SOCS2$^{+/+}$/*Apc*$^{Min/+}$ non-tumor samples run on the same gel (n = 3 independent experiments). a = $p < 0.05$ vs SOCS2$^{+/+}$/*Apc*$^{Min/+}$ non-tumor tissue and b = $p < 0.05$ vs SOCS2$^{+/+}$/*Apc*$^{Min/+}$ tumor tissue by ANOVA. C. Autoradiograms of supershift asays and cold competition specificity controls on pooled tumors from SOCS$^{-/-}$/*Apc*$^{Min/+}$ mice. SS, indicates supershift reactions. Triangles indicate super-shifted complexes. *Left*, super-shifted complexes for JunD, p-c-Jun, c-Jun. *Middle*, image intensity was increased to visualize c-Jun super-shifted complex. *Right*, shows cold competition with excess of unlabeled AP-1 oligomer and no inhibition of AP-1 binding with an unrelated oligomer corresponding to the TCF binding site.

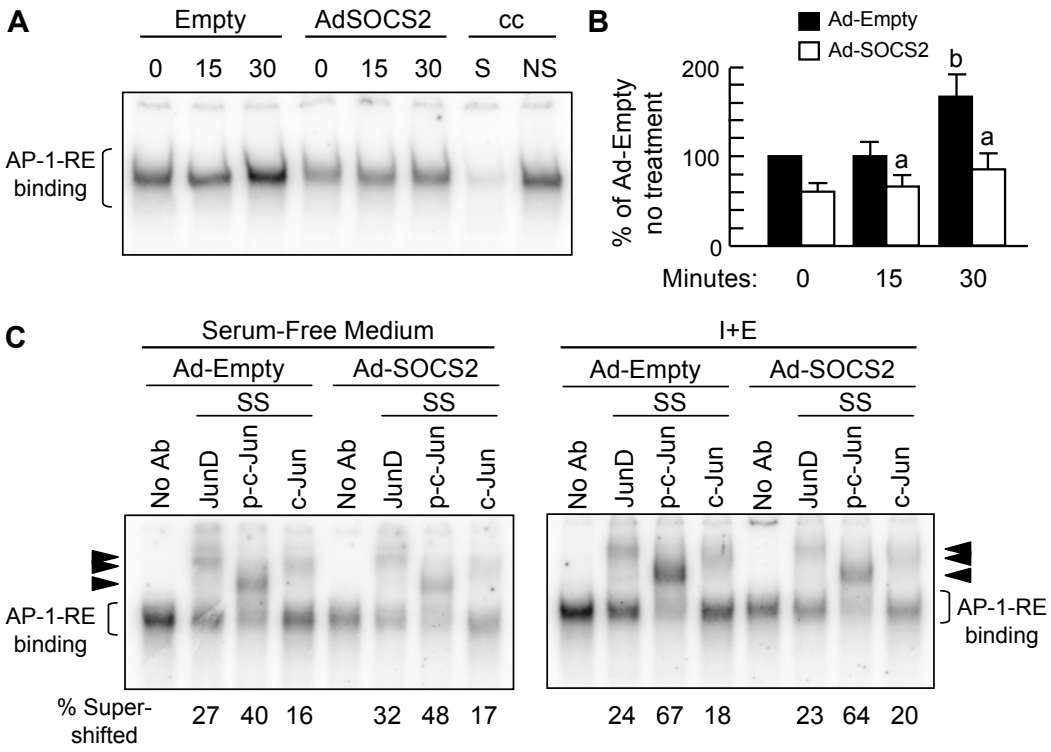

Figure 2.9. Overexpression of SOCS2 reduces nuclear AP-1 DNA binding activity in IEC-6 cells. A. Representative autoradiogram of EMSA with radiolabeled AP-1 oligomer on nuclear extracts from IEC-6 cells infected with either empty adenovirus (Ad-Empty) or adenovirus containing FLAG-tagged SOCS2 (Ad-SOCS2). Cells were treated with serum-free medium (SFM) alone (0) or combined rhIGF-I (20ng/mL) and rhEGF (5ng/mL) (I + E) for 15 or 30 minutes. cc, cold-competition. S, unlabeled AP-1 oligomer. NS, unrelated TCF oligomer. B. Densitometric anlaysis of AP-1 DNA binding complex. Results show mean ± SE for intensity of AP-1 DNA binding complex expressed as a percentage of the Ad-Empty in SFM alone (n = 3 independent experiments). a = $p<0.05$ vs Ad-Empty; b = $p<0.05$ vs no treatment. C. Autoradiogram of super-shift assays on extracts from Ad-Empty or Ad-SOCS2 infected IEC-6 cells treated with SFM (left) or IGF-I and EGF for 30 minutes (I + E; right). Triangles indicate super-shifted complexes with JunD, p-c-Jun, and c-Jun antibodies relative to no antibody (no Ab) control. Numbers at the bottom indicate the percent JunD, p-c-Jun, or c-Jun in the AP-1:DNA protein complex (assessed as intensity super-shifted band/intensity shifted complex in no antibody control x 100) in each treatment group. Note that while the intensity of super-shifted complexes is lower in Ad-SOCS2 vs Ad-Empty cells, the relative proportions of JunD, p-c-Jun, or c-Jun in AP-1 complexes are similar. Thus SOCS2 overexpression reduces all Jun isoforms within AP-1 complexes.

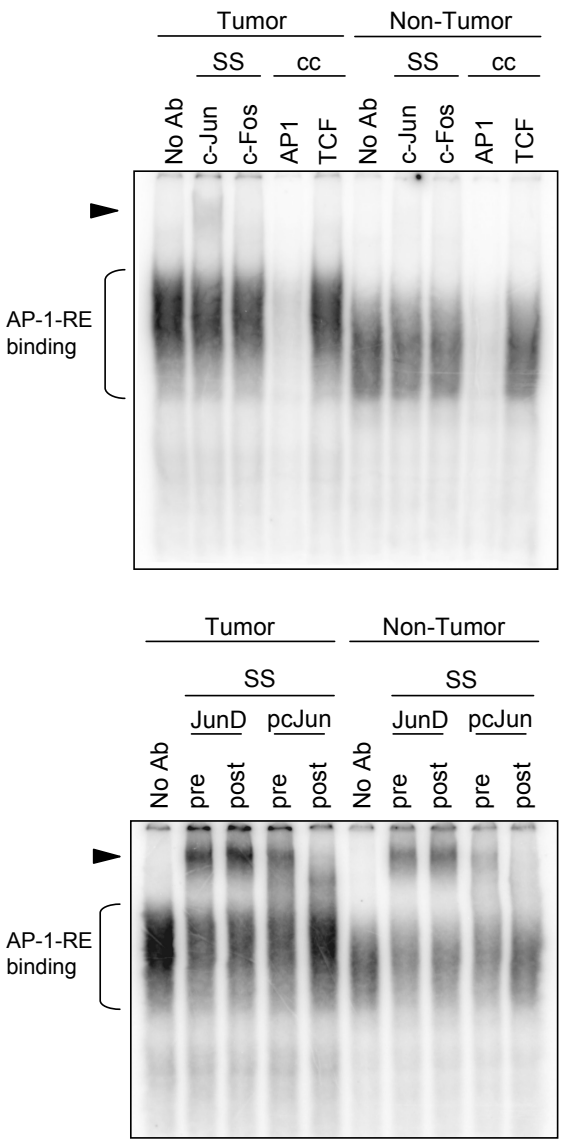

Figure 2.10. Supplemental Figure. Autoradiogram of pooled tumor and non-tumor tissue from SOCS2$^{-/-}$/*Apc*$^{Min/+}$ mice showing super-shifted complexes for c- Jun (top) and JunD and p-c-Jun (bottom). Pre and post indicate whether antibody was incubated before (pre) or after (post) the addition of labeled-oligomer. SS, super-shifted reactions. Triangles indicate super-shifted complexes. Note that the shifted and super-shifted complexes are reduced in intensity in non-tumor tissue compared to tumor. Relative levels of c- Jun, JunD or p-c-Jun (calculated as intensity of super-shifted complex/intensity of shifted complex in no Ab control x 100) were approximately equal in tumor and non-tumor samples (data not shown).

CHAPTER 3

INSULIN-LIKE GROWTH FACTOR-I (IGF-I) AND EPIDERMAL GROWTH FACTOR (EGF) PROMOTE SYNERGISTIC INCREASES IN PROLIFERATION, ENHANCES IGF-IR ACTIVATION, AND PROMOTES β-CATENIN ACTIVATION IN INTESTINAL EPITHELIAL CELLS

A. Introduction

The intestinal epithelium is constantly renewed by proliferation of crypt stem and progenitor cells. Many factors including nutrient intake, hormones and growth factors can influence the rate of proliferation and apoptosis of the crypt epithelial cells.[48] Growth factor-stimulated proliferation of normal crypt IEC can have beneficial effects to increase the mass of the intestinal epithelium, but may also contribute to tumor development and growth.

It is well-established that insulin-like growth factor I (IGF-I) and epidermal growth factor (EGF) stimulate growth of intestinal epithelium in vivo.[125,67,200,201] Previous studies have shown that combined treatment of IGF-I and EGF synergistically increases proliferation in IEC-6 cells.[142,123,143] IEC-6 cells are a non-transformed intestinal epithelial cell line that were derived from rat small intestine and retain the characteristics of normal intestinal crypt cells.[123,202] In these studies, EGF or IGF-I were each shown to stimulate DNA synthesis and proliferation, but EGF was more potent than IGF-I. Importantly, EGF in combination with IGF-I synergistically increased IEC proliferation.[142,123] A temporal order to this interaction was identified such that EGF pretreatment, followed by IGF-I produced synergistic increases in DNA synthesis and proliferation, yet this effect was not seen with IGF-I pretreatment followed by EGF.[142,123] The mechanisms underlying this synergy are not fully characterized. IGF-I and EGF act via the type 1 insulin-like growth factor receptor (IGF-IR) or epidermal growth factor receptor (EGFR), which are both receptor tyrosine kinases. IGF-IR and EGFR can activate common downstream mediators of proliferation and cell survival, including PI-

3K or MAPK/ERK, although the degree to which these mediators couple to IGF-IR or EGFR may depend on cell type or context.[203,204,205,126,206]

Both IGF-I and EGF have been implicated in regulating the Wnt/β-catenin pathway. The Wnt/β-catenin pathway is important for the maintenance of the intestinal epithelium and aberrant activation of this pathway is key in the progression of colorectal cancer.[28,27,32] Mutations in this signaling cascade are considered some of the first that occur in the transition from normal mucosa to precancerous lesions in colorectal cancer.[27,34] Normally β-catenin is found at cell-cell junctions in complex with E-cadherin and α-catenin.[32] In the cytosol, a complex containing Adenomatous Polyposis Coli (APC) and glycogen synthase kinase3-β (GSK3β) targets β-catenin for degradation by phosphorylating β-catenin at the N-terminus.[32,33] In the presence of Wnt ligand or other factors, GSK3β is inhibited, thus stabilizing β-catenin and allowing its nuclear translocation. In the nucleus, β-catenin binds to TCF/lef transcription factors and this transcription complex regulates expression of genes involved in proliferation.[170] IGF-I has been shown to stabilize β-catenin protein in the cytosol and promote β-catenin nuclear translocation in colon and liver cancer cell lines, as well as intestinal smooth muscle cells.[207,208,209,53,210,211] In some cases, this resulted in increased TCF/lef transcriptional activity.[54,207,53,211] These effects have been shown to be dependent on activation of PI-3K/Akt pathways and MAPK pathways, though it seems PI3-K/Akt pathways have more potent effects.[207,208,53,210,212] EGF has also been shown to have a role in β-catenin activation, whereby in oral cancer and other cell lines EGF treatment increases β-catenin protein and induces transcriptional activity.[140,213,139,141] EGF disrupts E-cadherin and β-catenin at cell-cell junctions, releasing β-catenin into the cytosol,

increasing the pool of β-catenin.[139,214,215,137,216,217] What is not known is whether IGF-I and EGF pathways interact to additively or synergistically promote β-catenin activation in normal intestinal epithelial cells.

Many colon cancer cell lines secrete ligands for EGFR or IGF-IR, which act in an autocrine manner on cell proliferation and survival. This is illustrated by the fact that dual inhibition of EGFR and IGFR more effectively reduces proliferation and survival of colon cancer cells to a greater extent than inhibition of either receptor alone.[218] Recent studies demonstrate overexpression and co-expression of IGF-IR and EGFR in a large proportion of human CRC in situ, and that high IGF-IR expression predicted poor prognosis.[219] However, mechanisms underlying the functional interaction of EGF and IGF-I signaling in normal IEC or CRC are not well defined.

This study examined the mechanisms of IGF-I and EGF synergy in IEC-6 cells. We hypothesized that EGF alters IGF-I receptor expression or activation to facilitate synergistic increases in DNA synthesis and proliferation when subsequently exposed to IGF-I. In addition, we also hypothesized that the differential or additive effects of EGF and IGF on PI3-K, MAPK or β-catenin contribute to their synergy. We used IEC-6 cells because they are non-transformed IECs, which exhibit little to no expression of endogenous IGF-I or EGF or other EGF family members, allowing us to specifically test the effects of exogenous EGF and IGF-I.[123] We found synergistic mitogenic effects of EGF and IGF-I involved EGF induction of IGF-IR and required EGF-induced activation of both MAPK and PI3K, but IGF-I activation of only PI-3K. Combined IGF-I and EGF had additive effects on nuclear β-catenin and β-catenin/TCF transcriptional activation.

These finding provide novel evidence that IGF and EGF interact at multiple levels to stimulate synergistic proliferative responses of intestinal epithelial cells.

B. Materials and Methods

Cell Culture and Reagents

IEC-6 cells were obtained from American Type Culture Collection (CRL 1592) and were cultured as previously described.[123] Briefly, cells were grown in 75-100 cm^2 flasks in 37°C with 95% air and 5% CO_2. Cells were propagated in growth medium: dulbecco's minimal essential medium with high glucose, 4.5 g/L (DMEM-H, GIBCO, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS, GIBCO), 10μg/mL insulin, 5μg/mL transferrin, 0.67μg/mL sodium selenite (I.T.S. GIBCO), 50U/mL penicillin, and 50μg/mL of streptomycin (GIBCO). Cells were passaged weekly and experiments conducted on cells between passages 10-20. Growth factors were purchased as follows: EGF (R&D Systems, Minneapolis, MN) IGF-I (Genentech, S San Francisco, CA). Antibodies were purchased from the following vendors: Anti-IGF-IRβ from Santa Cruz Biotechnology (sc-713, Santa Cruz, CA); Anti-phosphotyrosine (PY20, BDTransduction Laboratories, San Jose, CA, and 4G10, Millipore, Billerica, MA). InfraRed (IR) secondary antibodies (for use with LiCor Odyssey IR technology) were purchased from Pierce Biochemicals (Dylight800-conjugated goat anti-rabbit or Dylight680 conjugated-goat anti-mouse).

EGF and IGF-I treatments

For all assays of EGF/IGF-I interaction, cells were grown to 50-70% confluence in growth medium, switched to serum-free medium (SFM) for at least 16h and then treated with/without growth factor(s) for defined times. To examine EGF/IGF-I interaction, doses of EGF and IGF-I shown previously to elicit maximal effects (EGF 5ng/mL; IGF-I 20ng/mL) were used in two types of experiments. In one, cells were treated with EGF, IGF-I or both growth factors combined. In other "pretreatment" experiments, cells were treated with one growth factor for varying time periods as specified and then the growth factor removed and replaced with fresh SFM plus or minus a second growth factor.

^{3}H-thymidine assay

^{3}H-thymidine incorporation into DNA was used as a measure of proliferative response. Assays were conducted as previously described using 2μCi/mL ^{3}H-thymidine (3000 Ci/mmol, Perkin Elmer, Boston, MA) added for the last 16 hours of incubation, followed by TCA precipitation of DNA and scintillation counting (Wallac Model 1414 LSC, PerkinElmer, Waltham, MA).

PI3-Kinase and MAPK inhibitors

To assess the role of PI-3K or MAPK in synergistic responses to EGF and IGF-I, PI-3K inhibitor LY294002 (14μM, Sigma-Aldrich, St Louis, MO) or MAPK inhibitor

PD98059 (20μM, Sigma-Aldrich) were incubated with cells during pretreatment or treatment with EGF or IGF depending on the experiment.

Analysis of IGF-I receptor

^{125}I-IGF-I binding: Binding of ^{125}I-IGF-I to cell membranes was used as a measure of IGF-IR. For these assays, ^{125}I-IGF-I (10^6 cpm/mL/well) was added to serum-deprived, IGF-I-treated or EGF-treated cells. Lysates were collected and counted in a Packard Cobra II D5005 automatic gamma counter (PerkinElmer, Waltham, MA). Specificity of ^{125}I-IGF-I binding was confirmed by demonstrating that unlabeled IGF-I, but not insulin, decreased ^{125}I-IGF-I binding in a dose-dependent manner.

***Immunoprecipitation and Western blotting of IGF-IR*:** Cells were grown to 50-70% confluency, serum-starved in SFM for 24 hours, then switched to SFM±EGF for 18 hours. Media was removed and cells were treated with SFM±IGF-I for 3 minutes. Whole cell extracts were prepared in lysis buffer containing 50mM HEPES (pH7.4), 150mM NaCl, 20mM Na pyrophosphate, 100mM NaF, 1.5% Triton X-100, and 100mM EDTA, supplemented with phosphatase and protease inhibitors (1μg/mL aprotinin, 1mM PMSF, and 2mM vanadate). Lysates were centrifuged at 12,000 x g for 1 minute and supernatants collected. Lysate concentrations were determined by BCA (bicinchoninic acid) assays (Pierce, Thermofisher Scientific, Rockford, IL) and lysate integrity was verified by PAGE followed by Coomasie Blue staining.

IGF-I receptor was immunoprecipitated by incubating 1000 ug of cell lysate with 2μg of anti-IGF-IR β (sc-713, Santa Cruz Biotechnology, Santa Cruz, CA) with mixing at 4°C for 18 hours. 20μL of 50% slurry of Protein A (Sigma-Alrich) was added for a 1-hour incubation. Following centrifugation and washing with lysate buffer (recipe above), immunoprecipitates were disrupted in gel loading buffer [95'C, 3 minutes, 10% glycerol, 1% SDS, 30mM Tris-HCl (pH 6.M), 2.5% b-mercaptoethanol], then size-fractionated on an 8.5% SDS-polyacrylamide gel run at 32mA for 3 hours, transferred onto nitrocellulose membrane (BioRad, Hercules, CA) and blocked in Blocker™ Casein in PBS (Pierce, Rockford, IL) overnight. Membranes were immunoblotted with antibodies against phospho-tyrosine (mouse monoclonal antibodies PY20, BD Transduction Laboratories, San Jose, CA, and 4G10, Millipore, Billerica, MA) and rabbit anti-total IGF-IR (sc-713, Santa Cruz Biotechnologies, Santa Cruz, CA) at 4°C for 16 hours. Blots were washed in PBS containing 0.1% Tween and incubated with secondary antibodies conjugated to Dylight800 (Pierce, goat anti-rabbit) or Dylight680 (goat anti-mouse) for 1 hour at room temperature. Immuno-reactive proteins were visualized and quantitated using the LI-COR Odyssey infrared imaging system (Version 3, Li-Cor, Lincoln, NE). Densitometric data are expressed as fold-change versus the no treatment control.

Immunoblot for Nuclear β-catenin

IEC-6 cells were grown as described above. Cells were grown media plus FBS in 150 mm dishes to 70-80% confluency, then switched to SFM. After 18-24 hours, fresh SFM was added with or without EGF (5ng/mL) for another 18-24 hours. IGF-I

(20ng/mL) was then added for 10, 30, 60, or 180 minutes. Nuclear proteins were isolated as previously described[178], concentration determined by BCA (bicinchoninic acid) assay, (Pierce, ThermoFisher Scientific, Rockford, IL) and verified by PAGE followed by Coomasie Blue staining. Equal amounts of protein (20 ug) were size-fractionated on 4-12% SDS-PAGE gel (PAGEgel, Fischer Scientific, Pittsburgh, PA) and transferred onto a nitrocellulose membrane (Biorad) using a NuPAGE gel system (Invitrogen). After blocking in blocking buffer, blots were incubated with primary antibodies against β-catenin (BD Biosciences, San Jose, CA) for 16 hours at 4°C. Blots were washed in PBS containing 0.1% Tween and incubated with secondary antibodies conjugated to Dylight800 for ~1 hour at room temperature. Immuno-reactive proteins were visualized and analyzed using the LI-COR Odyssey Infrared Imaging system. Densitometry was performed on visualized bands and normalized to the total protein gel. Values are expressed as fold change versus the no treatment control.

TOPflash Luciferase Reporter Assay

10^6 cells were co-nucleofected (Nucleofector, Lonza Cologne AG, Koln, Germany) with 3.5 ug of TOPflash plasmid (TCF reporter plasmid, Millipore, Temecula, CA) and 0.5μg of TK renilla plasmid as control for tranfechon efficiency (Clontech, Montain View, CA). Cells were then seeded into 24-well plates for overnight growth. Cells were serum-starved (SFM) for 24 hours, then switched to SFM containing EGF, IGF-I, or IGF plus EGF for 24 hours. After washing, cells were collected in passive lysis buffer (Promega Corporation, Madison, WI). Luciferase assay was performed using a

Luciferase Reporter Assay (Promega, Madison, WI) according to manufacturer's protocol. Luciferase activity was read on Molecular Dynamics LMax luminometer. Data are expressed as fold-change versus the no treatment controls.

Statistical Analysis

Data are expressed as mean $\pm$ S.E.M or mean $\pm$ S.E.M of fold change in each sample versus the mean value in control cells treated with SFM alone. Data were analyzed by ANOVA followed by post-hoc tests for pair-wise comparisons. $P<0.05$ were considered statistically significant.

C. Results

Combined IGF-I and EGF treatment synergistically increases ^{3}H-thymidine incorporation

^{3}H-thymidine incorporation into DNA was used as a measure of the proliferative responses to EGF and IGF-I alone or in combination. As shown in Figure 3.1A and consistent with prior data, treatment with IGF-I produced small, but significant increases in DNA synthesis (Figure 3.1A). EGF had more potent effects and combined treatment with EGF plus IGF-I promoted synergistic increases in DNA synthesis (Figure 3.1A). In previous studies, we demonstrated a temporal order to this effect such that pretreatment with EGF followed by IGF-I synergistically increases DNA synthesis, yet pretreatment with IGF-I followed by EGF treatment produced effects no different from EGF alone.[123]

Here, we examined the minimum time required for EGF pretreatment and demonstrated that 3 hours of EGF pretreatment is sufficient to permit synergistic effects upon subsequent treatment with IGF-I (Figure 3.1B). Longer duration of EGF pretreatment followed by IGF-I resulted in similar synergistic effects (Figure 3.1B).

EGF pretreatment enhances IGF-I receptor levels and activation

Radio-ligand binding assays and western immunoblot were used to test if EGF pretreatment affected IGF-IR. As shown in Figure 3.2A, ^{125}I-IGF-I specifically bound to the IGF-I receptor, since un-labeled IGF-I dose-dependently decreased binding of ^{125}I-IGF-I, while insulin, at similar molar concentrations, had little or no effect (Figure 3.2A). We found that compared to no treatment controls, IGF-I significantly reduced ^{125}I-IGF-I binding. In contrast, EGF significantly increased ^{125}I-IGF-I binding indicative of increased IGF-I receptor (Figure 3.2B). Western immunoblots were used to confirm this observation

Figure 3.3A shows results for immunoprecipitation of IGF-IR followed by western immunoblot for total IGF-IR or phosphotyrosine. Consistent with the ^{125}I-IGF-I binding assays, pretreatment with EGF significantly increased total IGF-IR with a mean increase of 2.3 $\pm$ 0.2 fold compared to cells treated with IGF-I alone (Figure 3.4B). Figure 3.3B shows immunoblots for tyrosine phosphorylated receptor to demonstrate that this effect was associated with dramatic increases in IGF-I-stimulated, but not basal levels of tyrosine phosphorylated IGF-IR. Levels of IGF-I-stimulated tyrosine phosphorylation of IGF-IR were 3.3 $\pm$ 0.7 fold greater in EGF pretreated cells given

subsequent IGF-I compared with cells treated only with IGF-I. Together, these data suggest that EGF increases the levels of IGF-I receptor leading to enhanced IGF-I-induced tyrosine phosphorylation of IGF-I receptor. It is important to note that EGF pretreatment alone is not sufficient to induce tyrosine phosphorylation of IGF-IR without subsequent addition of exogenous IGF-I (Figure 3.3B).

Differential roles of MAPK and PI-3K in EGF/IGF interaction

To assess the role of MAPK or PI3-K pathways in IGF-I/EGF interactions, IEC-6 cells were treated with PD98059 or LY294002 during pretreatment with SFM or EGF or during subsequent treatment with IGF-I or SFM. Both inhibitors significantly reduced basal DNA synthesis observed in the absence of growth factors (Figure 3.4). The stimulatory effects on DNA synthesis observed with EGF pretreatment for 7 hours were significantly inhibited by PD98059 or LY294002 (Figure 3.4). In contrast, the modest but significant effect of IGF-I or DNA synthesis was significantly inhibited by LY294002, but not PD98059 (Figure 3.4). Effects of these inhibitors on the synergistic responses to EGF pretreatment followed by IGF-I demonstrate different roles of these pathways. Use of either PD98059 or LY294002 during EGF pretreatment inhibited the synergistic response by approximately equal degrees (Figure 3.4). In contrast, when added only during IGF-I treatment of EGF pretreated cells, only LY294002 and not PD98059 attenuated the synergistic proliferative responses. When PD98059 was given during both EGF pretreatment and subsequent treatment with IGF-I, the inhibitory effect was similar to when PD98059 was given orally during EGF pretreatment. In contrast, when

LY294002 was given during both EGF treatment and subsequently IGF-I, its effects were more potent than when administered only during EGF pretreatment. However, this was not significantly different from effects of LY294002 during EGF pretreatment only. Together, these findings support a model proposed in the schematic in Figure 3.4, whereby mitogenic effects of EGF pretreatment requires both MAPK and PI3-K pathways, but responses to IGF-I alone or synergistic responses to IGF-I in EGF-pretreated cells are dependent only on PI3-K pathways.

Additive effects of EGF and IGF-I on β-catenin/TCF pathways

Immunoblot for nuclear β-catenin and TCF luciferase reporter assays were used to assess if EGF and IGF-I interact to regulate this key growth-promoting pathway that is relevant to early stages of intestinal cancer. To assess nuclear β-catenin, we pretreated cells with SFM or EGF for 18 hours followed by subsequent treatment with SFM or IGF-I for 10 – 180 minutes. Neither EGF pretreatment alone, nor 60 minutes treatment with IGF-I alone significantly increased nuclear β-catenin, although there was a trend for a modest increase in nuclear β-catenin in EGF pretreated cells (Figure 3.5A). EGF treatment followed by 60 or 180 minutes IGF-I treatment lead to significant increases in nuclear β-catenin (Figure 3.5A). Figure 3.5B shows controls to illustrate that the cytosolic marker tubulin is absent from nuclear extracts but is detected in whole cell extracts and that the nuclear marker Histone H1, is present in nuclear extracts.

We then assessed whether IGF-I or EGF, alone or in combination, enhance the transcriptional activity of β-catenin, using a TCF-luciferase reporter assay.[170] IEC-6 cells

were pretreated with either IGF-I or EGF and followed by IGF-I or EGF treatment. IGF-I or EGF treatment produced approximately two-fold increases in TCF-reporter activity compared to no treatment controls (Figure 3.6). This activity was increased, at least additively, by combined EGF and IGF-I treatment, producing a four-fold increase in TCF promoter activity (Figure 3.6). These results suggest that while either IGF-I or EGF alone can induce TCF promoter activity, combined treatment of EGF and IGF-I significantly and additively increased TCF promoter activity. Together with immunoblots results, these studies suggest that EGF and IGF-I interact to promote increases in nuclear β-catenin and enhanced β-catenin/TCF transcriptional activation.

D. Discussion

Our studies show that IGF-I and EGF interact to promote synergistic increases in DNA synthesis and β-catenin activity in IEC-6 cells. Two independent assays, ^{125}I-IGF-I binding and western blot, demonstrate that EGF pretreatment increased IGF-IR. In addition, EGF pretreatment enhanced IGF-IR and IGF-I-induced receptor phosphorylation. Maximal synergistic effects of EGF and IGF-I on proliferation required both MAPK and PI-3K during EGF pretreatment, but only PI-3K during subsequent IGF-I treatment. We also demonstrate that combined treatment of IEC-6 cells with IGF-I and EGF in IEC-6 cells promotes significant increases in nuclear β-catenin and enhanced TCF transcriptional activation greater than treatment with either growth factor alone. These studies define novel mechanisms of EGF and IGF-I interaction and demonstrate that these two growth factors interact to promote enhanced activation of the key growth

promoting TCF/β-catenin pathway, which is implicated in early stages of tumorigenesis in the intestine.

Our findings that EGF alone is a more potent mitogen than IGF-I, and the synergy between EGF and IGF-I confirm and extend previous studies in our lab and others.[123, 142] Other studies in IEC-6 cells showed that continuous IGF-I treatment produced non-significant increases in cell number, while continuous EGF treatment promoted increased cell numbers.[142] However, combined treatment with IGF-I and EGF over 48 hours produced synergistic increases in cell number and were recapitulated when a short pulse of EGF was given prior to IGF-I treatment.[142] The reverse is not true because IGF-I pretreatment does not lead to synergistic effects in response to subsequent addition of EGF.[123] This and our prior study indicate that EGF is able to 'prime' IEC-6 cells for a synergistic response to IGF-I. One potential mechanism we considered is that EGF may serve as a competence factor promoting transition from $G_0 \rightarrow G_1$ phase and that IGF-I acts as a proliferative agent promoting transition from $G_1 \rightarrow$ S phase, where DNA synthesis occurs as this interaction has been demonstrated in fibroblasts.[123,142] However, in IEC-6 cells, this does not appear to be a likely explanation since both growth factors promote DNA synthesis, a biomarker of transition into S phase, and EGF alone has more potent effects than IGF-I alone.[123] Thus, in IEC-6 cells, it appear that there are different mechanisms of interaction whereby both EGF and IGF-I each promote S phase and act in a synergistic manner when combined or when cells are first exposed to EGF and then IGF-1.

Our prior and current studies suggest that one level of interaction between EGF and IGF-I resides at the level of IGF-IR expression. Our previous report demonstrated

that EGF and IGF-I interact at the level of IGF-IR mRNA.[123] EGF alone did not affect IGF-IR mRNA, but when in combination with IGF-I prevented a dramatic down-regulation of IGF-IR mRNA by IGF-I.[123] In the current study, we show that in contrast to the IGF-I mRNA data, EGF pretreatment up-regulates IGF-IR as assessed by I^{125}–IGF-IR binding assays or by western blot. In turn, this permits more dramatic tyrosine phosphorylation of IGF-IR when cells are subsequently exposed to IGF-I. This interaction appears not to reflect trans-activation of IGF-IR by EGF/EGFR because cells pretreated with EGF did not show tyrosine phosphorylation of IGF-IR until exposed to IGF-I. Thus, the current data suggest that EGF pretreatment acts at a protein level to increase the number of available IGF-I receptors for subsequent activation by IGF-I. Precisely how EGF increases IGF-IR protein will require additional studies, but this could involve either recruitment or maintenance of IGF-IR at the plasma membrane. Whatever the mechanism, this is relevant to combinatorial effects of EGF and IGF-I to more potently promote growth of normal IEC in vivo or cancer cells. Prior studies have shown that in normal or irradiated intestine in vivo EGF more potently promotes mucosal growth or crypt mitosis than IGF-I.[75,125] To our knowledge, no studies have tested the effects of combined treatment with EGF and IGF-I on intestinal growth and repair. However, emerging evidence in the literature documents additive or synergistic effects of EGFR combined with IGF-IR inhibitors in colon cancer cells, which are known to express endogenous ligands for the two receptors.[220,218,221] Our documentation of synergy between exogenous EGF and IGF-I in non-transformed IEC-6 cells suggests that situations where both growth factors might be upregulated, i.e. early stage transformation of normal IEC towards tumor phenotype, may permit the two growth factors to interact to

synergistically expand aberrant precancerous cells. This is speculative at present but ongoing studies in our laboratory are addressing effects of combined inhibition or genetic loss of EGFR and IGF-IR signaling in spontaneous intestinal tumor models and will be discussed in chapter IV.

Our studies addressed the role of two key downstream pathways, MAPK and PI-3K, in mediating the synergistic responses to EGF and IGF-I. These studies revealed that the proliferative effects of EGF or its ability to promote synergistic proliferative responses with subsequent IGF-I treatment required both MAPK and PI-3K pathways. In contrast, only PI-3K and not MAPK inhibitors affected the synergistic response when added only during IGF-I treatment following EGF. The predominant role of PI-3K versus MAPK in mediating IGF-I action in this setting is consistent with findings in other systems which show a major role for PI-3K in growth promoting effects of IGF-I.[222,205,204] It is noteworthy that a number of studies indicate that IGF-I activation of PI-3K is critical for anti-apoptotic actions of IGF-I.[46] In IEC-6 cells under the conditions and time course of growth factor treatment analyzed, there is essentially no detectable apoptosis. Thus, in this situation, PI-3K appears essential to the proliferative rather than anti-apoptotic action of IGF-I and importantly, to the synergistic interaction between EGF and IGF-I in mediating IEC proliferation.

Both IGF-I and EGF have been implicated in the activation and regulation of the APC/β-catenin pathway. Our studies show that IGF-I treatment alone did not promote increases in nuclear β-catenin protein and EGF alone had a non-significant trend to increase nuclear β-catenin. When IEC-6 cells were pretreated with EGF and followed by IGF-I, nuclear β-catenin was enhanced at 60 minutes above either treatment alone and

this increase was sustained. Previous studies have shown that either IGF-I or EGF induced nuclear β-catenin activation in other cell types.[207,208,209] In primary oligodendrocytes and oligodendrocyte cell cultures, IGF-I increased β-catenin protein and mRNA levels and increased transcription of β-catenin target gene cyclin D1.[207] SiRNA against β-catenin reversed IGF-I-induced cell survival.[207] In mouse embryonic fibroblasts overexpressing the IGF-IR, IGF-I treatment induced nuclear translocation of β-catenin that was dependent on a downstream mediator of IGF-I action, insulin receptor substrate-1 (IRS-1).[53] IRS-1 co-localized with β-catenin in the nucleus. This co-immunoprecipitation of IRS-1 and β-catenin indicates that these proteins may interact to promote activation of β-catenin target genes.[53] With regards to EGF, studies in other systems indicate that EGF can induce the nuclear translocation and increase β-catenin transcriptional activity in oral and colon cancer cells.[139,140,223,213] Recently, studies in oral cancer cells show that EGF increases the amount of nuclear β-catenin while reducing β-catenin localized to plasma membranes and also increased β-catenin transcriptional activity.[140] In colon cancer cell lines, EGF increased β-catenin protein and nuclear localization through a mechanism involving HDAC-6.[139] Our studies did not demonstrate an increase in nuclear β-catenin with IGF-I or EGF alone, but both growth factors were required. Previous studies primarily used transformed cancer cell lines, which are known to express endogenous growth factors, and may have contributed to the ability of EGF or IGF-I alone to promote nuclear translocation of β-catenin. Since IEC-6 cells express little or no endogenous EGFR ligands, our studies in these cells allowed us to test the individual effects of IGF-I and EGF, with minimal effect of endogenous growth factors.

Using a TCF luciferase reporter assay, which reflects functional effects on transcription. IGF-I or EGF treatment in IEC-6 cells did increase in transcriptional activation of a β-catenin/TCF reporter, which is consistent with previous models.[211,140] Additional studies in C10 cells, a colorectal cancer cell line, and oesophageal cell lines found that IGF-I alone did not induce TCF transcriptional activity, indicating that IGF-I regulation of β-catenin activation may occur in a cell- or context-specific manner.[224,54] In one previous study, IEC-6 cells treated with EGF did not show an increase in nuclear β-catenin or an effect on transcriptional activity; however, these studies were done in the presence of low serum and may not adequately reflect effects due solely to EGF.[124]

We found that EGF pretreatment, followed by IGF-I treatment significantly increased nuclear β-catenin and dramatically enhanced transcriptional activity greater than either treatment alone. This suggests that these growth factors interact to promote enhanced β-catenin signaling and represents, to our knowledge, the first evidence that these growth factors interact to additively increase transcriptional activation of this key pathway. One obvious future direction is to examine β-catenin gene targets and define specific genes whose transcription is induced by EGF, IGF-I or most importantly, genes that show additive or synergistic responses to combined EGF and IGF-. Ongoing gene microarray analyses are addressing this issue. Few studies have examined activation of β-catenin by IGF-I or EGF in combination with each other or additional growth factors. In IEC-6 cells, 2% FBS plus EGF had no effect on β-catenin activation, yet increased proliferation greater than EGF treatment alone or with 2% FBS.[124] EGF given in combination with LiCl, an inhibitor of GSK3β, led to increased cytoplasmic pools of β-catenin and decreased pools at the membrane.[223] IGF-I treatment has been given in

combination with LiCl. In one study, IGF-I increased the number of surviving colon cancer cells greater than LiCl treatment alone, where combined treatment did not lead to a further increase.[207] When β-catenin siRNA was given, this reversed the effect of IGF-I on cell survival.[207] Combined LiCl and IGF-I treatment was shown to increase β-catenin transcriptional activity above either treatment alone.[54] These effects of combined treatment highlight, not only the effects on β-catenin activation, but also support our previous results where combined growth factor treatments synergistically enhance proliferation. These studies also provide support for further study of interactions of EGFR and IGF-IR signaling pathways. This becomes especially important for cancer prevention and treatment.

Both IGF-I and EGF are implicated as risk factors for colorectal cancer.[62,134,219,225,52] Both IGF-IR and EGFR are overexpressed in colorectal cancer and preclinical models have demonstrated a role for each of these receptors or associated signaling pathways in colon and other cancers.[61,78,77,133,134,136] In response, a number of inhibitors have been developed to target these pathways in human cancers and are currently in clinical trials.[226,227,158,68] EGFR inhibitors have potent in vitro and in vivo effects to inhibit proliferation and reduce tumor development in experimental settings, but have been effective in only a subset of patients with colorectal cancer.[147,221,149,228,229] Patients with breast and NSCLC respond to EGFR inhibitors but develop resistance to these therapies.[150,137,230] Recent studies provide evidence that activation of the IGF-IR signaling pathway may contribute to resistance to EGFR inhibitor.[220,154,231,151] In human glioblastoma multiform cell lines, IGF-I expression significantly correlated with resistance of erlotinib (an EGFR inhibitor).[232] IGF-I and IGF-I-induced Akt pathways

were found to be activated in erlotinib resistant cancer cells and in epithelial cells, activation of both EGFR and IGF-IR induced Akt signaling.[221] This has lead to studies of combined EGFR and IGF-IR inhibitors in several in vitro and a few in vivo studies.[218,220,221,233,234,235] Combined siRNA knock-down of both IGF-IR and EGFR in colorectal cancer cell lines significantly decreased proliferation and induced apoptosis greater than inhibition of either receptor alone.[218] Additional studies in breast, sarcoma, and colon cancer cells using pharmacological inhibition of IGF-IR and EGFR, show synergistic reductions in proliferation and cell survival.[218,231] The findings of Kaulfuss et al[218] are particularly relevant. This study revealed that synergistic reduction in proliferation of colon cancer cells by combined EGFR and IGFR inhibitors led to more effective inhibition of both Akt and MAPK pathways.[218] Knockdown of IGF-IR alone affected Akt, but not EGF-induced MAPK. Akt is downstream of PI-3K and, thus, these results are consistent with our findings in non-transformed cells that EGF activation of both MAPK and PI-3K but IGF activation of only PI-3K are required for synergy. Xenograft models of breast and lung cancer cells demonstrate in vivo that combined treatment of an anti-tumor IGF-IR human antibody (h7C10) with either an anti-EGFR antibody (225) or a chemotherapeutic agent almost completely inhibited tumor growth.[234] These studies highlight the importance of defining mechanisms of EGF/IGF-I interactions and furthering examining the effects and mechanisms underlying more effective anti-tumor effects of combined EGFR and IGF-IR inhibitors.

Together, these studies show that interactions between IGF-I and EGF to promote synergistic increases in IEC proliferation require both PI-3K and MAPK and enhanced IGF-IR upregulation and activation. These studies also demonstrate novel effects of EGF

and IGF-I to enhance nuclear β-catenin and synergistically increase TCF/lef transcriptional activity. These effects are important in understanding the role of multiple growth factors in growth of normal intestinal epithelium and provide support for use of combined inhibitors of IGF-IR and EGFR in treating cancer.

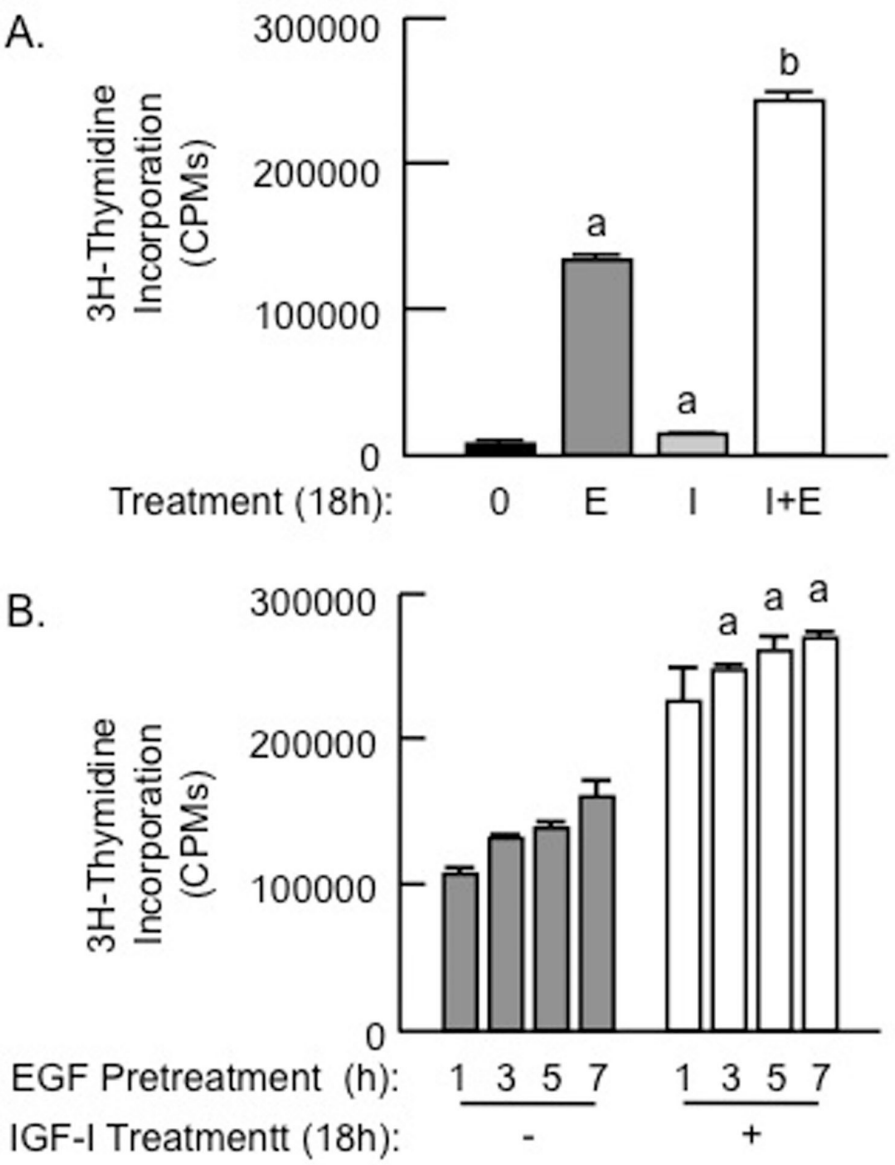

Figure 3.1. EGF pretreatment followed by IGF-I treatment synergistically increases DNA synthesis. A-B. Histogram shows mean ± SEM for ^{3}H-thymidine incorporation in IEC-6 cells, shown as total counts per minute (CPM). A. Cells were treated with EGF (5ng/mL), IGF-I (20ng/mL), or EGF and IGF-I combined at the same time for 18 hours. Untreated cells were used as a control. B. Cells were pretreated with EGF for 1, 3, 5, or 7 hours, media removed and subsequently treated with IGF-I for 18 hours. a=$p<0.05$ vs. no treatment, b=$p<0.05$ vs. additive effects of IGF-I + EGF

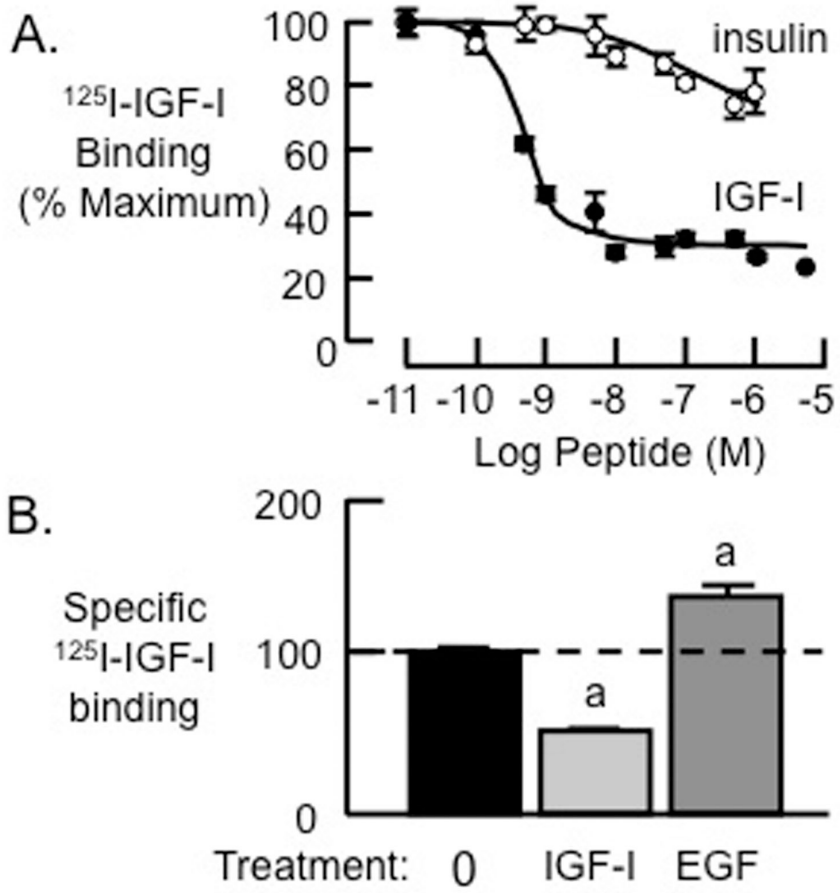

Figure 3.2. EGF pretreatment increases competitive IGF-IR binding activity in IEC-6 cells. A. Competitive radio-ligand binding assay of ^{125}I-IGF-I with increasing concentrations of unlabeled (cold) IGF-I (closed circles) or cold-insulin (open circles). Assay shows specificity for IGF-I as cold IGF-I, and not insulin, compete for binding to IGF-IR. B. IEC-6 cells were treated with IGF-I or EGF for 18 hours, washed and incubated with ^{125}I-IGF-I. ^{125}I-IGF-I was measured from isolated membranes. Mean $\pm$ S.E.M of IGF-I receptor evaluated by direct assays of specific ^{125}I-IGF-I to isolated membranes. a=$p<0.05$ vs. No treatment.

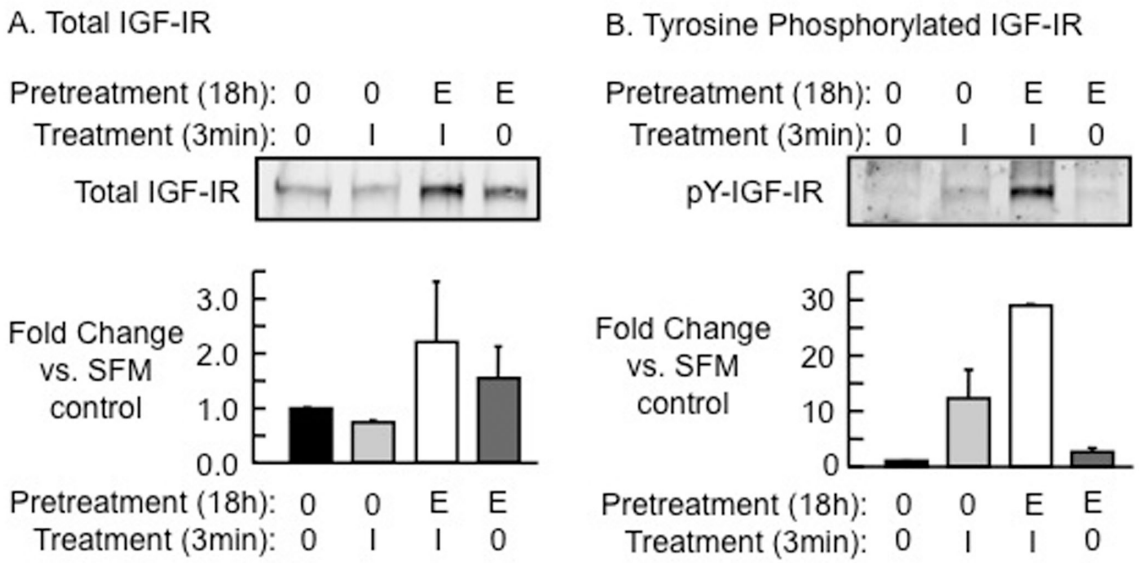

Figure 3.3. EGF pretreatment upregulates IGF-IR and IGF-I-induced receptor phosphorylation. Representative Western immunoblots of A. total IGF-IR or B. tyrosine phosphorylated IGF-IR in IEC-6 cells pretreated with EGF followed by IGF-I treatment (3 min, 25ng/μL) and immunoprecipitated for total IGF-IR. Note EGF treatment alone increases total IGF-IR. Below each immunoblot, densitometric analysis of immunoblots showing change in both total- and tyrosine-phosphorylated-IGF-IR. Results show mean $\pm$ S.E.M for two independent experiments, a=$p<0.05$.

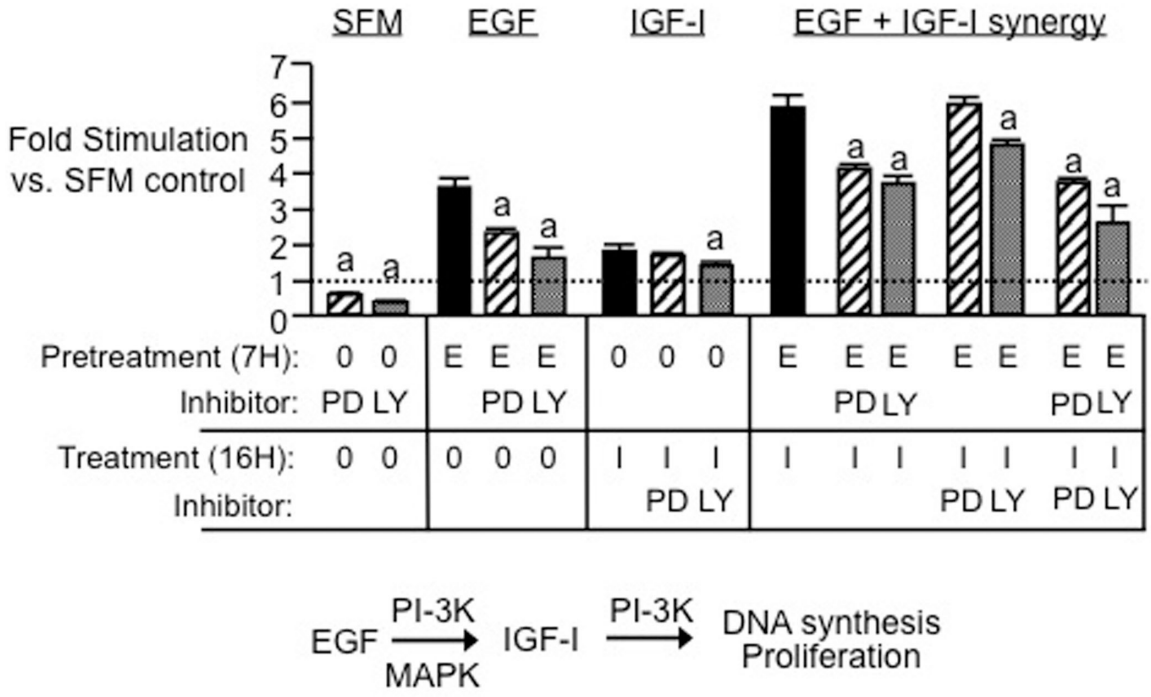

Figure 3.4. MAPK and PI-3K are required during EGF pretreatment but PI-3K is required during IGF-I treatment. *Top*. IEC-6 cells were pretreated with EGF (5ng/mL) and subsequently treated with IGF-I (20ng/mL). Either a MAPK inhibitor (PD98059) or a PI-3K inhibitor (LY294002) was given during the indicated treatment. Histograms show mean ± S.E.M ^{3}H-thymidine incorporation as a measure of synergistic effects on DNA synthesis and expressed as fold change vs. the no-treatment control. a = $p<0.05$ versus treatment without inhibitor. *Bottom*. A diagram summarizing the results.

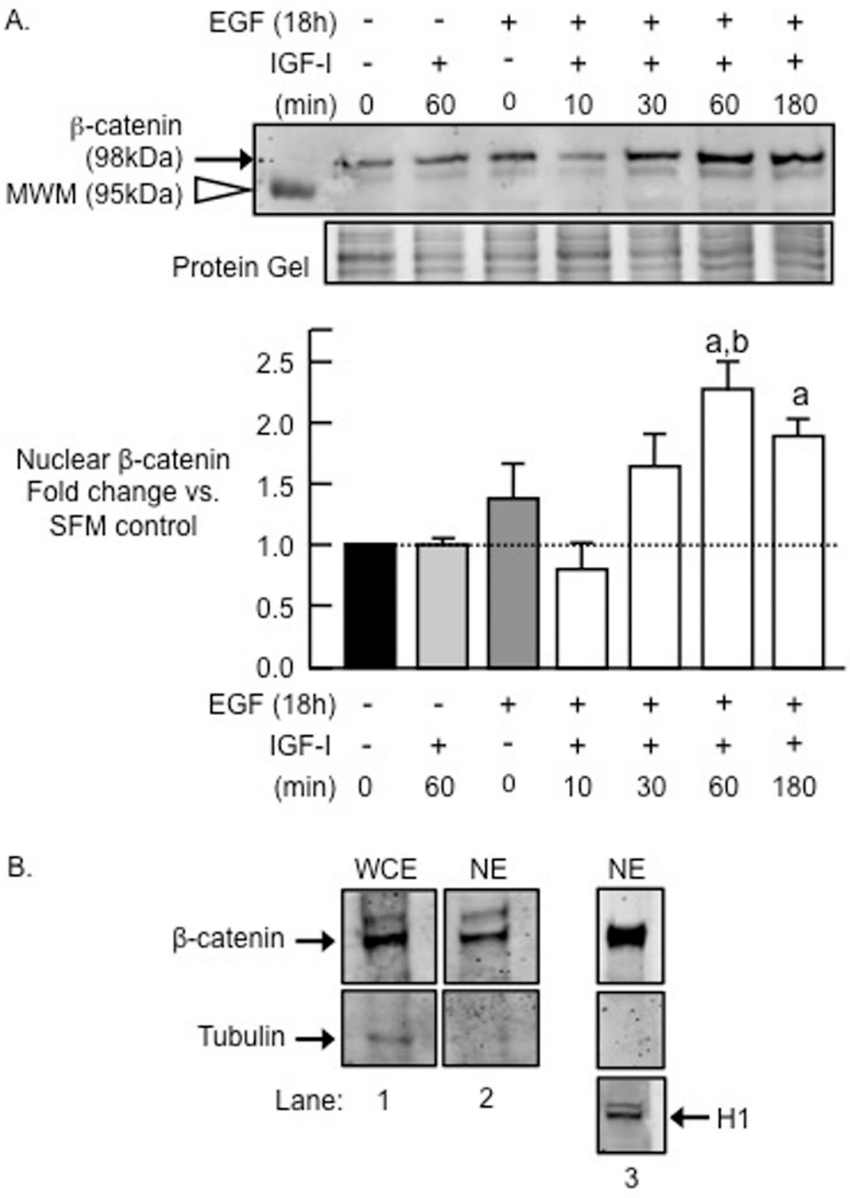

Figure 3.5. EGF promotes IGF-I-induced nuclear β-catenin in IEC-6 cells. IEC-6 cells were pretreated with EGF (5ng/mL) for 18 hours or SFM alone, media removed, and subsequently treated with SFM alone or IGF-I (20ng/mL) for indicated times. Nuclear proteins were isolated and immunoblotted for β-catenin. A. *Top.* Representative immunoblot of nuclear β-catenin in IEC-6 cells treated with EGF and/or IGF-I. Shown underneath is the corresponding total protein gel. *Bottom.* Histogram showing densitometric analysis of β-catenin, expressed as fold change versus no treatment. $n \geq 4$ blots. a = $p<0.05$ versus no treatment; b = $p<0.05$ versus EGF alone. B. Representative immunoblots showing β-catenin following combined IGF-I + EGF treatment in whole-cell extracts (WCE, lane 1) and nuclear extracts (NE, lanes 2-3). Lanes 1 and 2 are from the same blot and lane 3 from a separate blot. Membranes were first immunoblotted for β-catenin then reprobed for either tubulin or histone H1. Note that tubulin appears only in WCE and not NE.

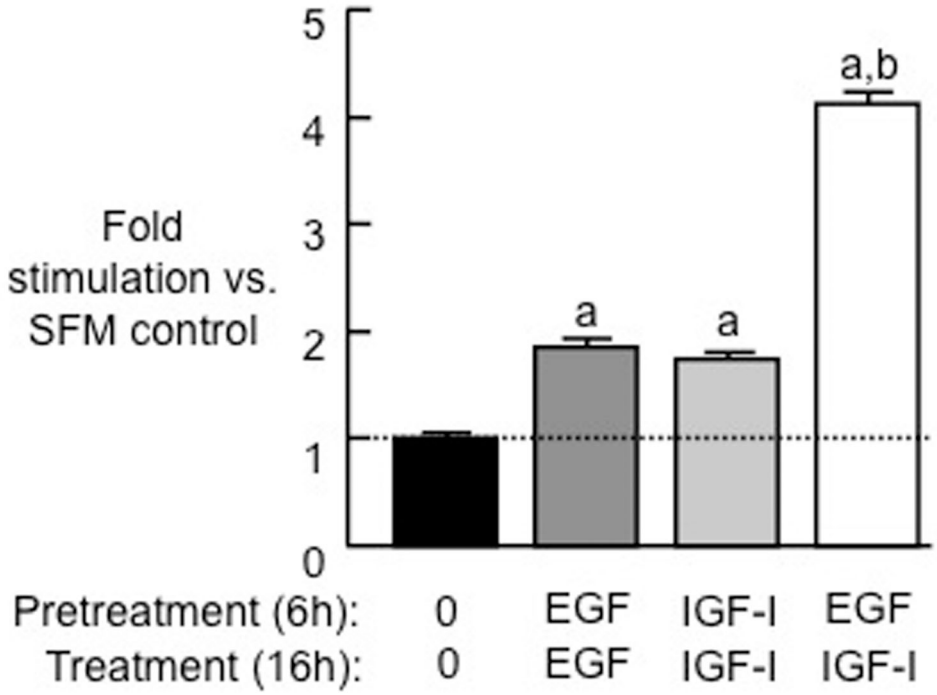

Figure 3.6. EGF promotes synergistic increases in IGF-I-induced TCF/lef promoter activity by TOP-Flash luciferase assay. IEC-6 cells were nucleofected with a TCF/lef luciferase reporter (TOP-GAL) and TK-renilla to measure transfection efficiency. Cells were pretreated with either EGF (5ng/mL) or IGF-I (20ng/mL) for 6 hours, SFM removed, then subsequently treated with EGF or IGF-I for 16 hours to allow for luciferase expression. Histogram shows resulting luciferase activity expressed as fold stimulation versus the SFM control. $p < 0.05$ versus no treatment, b = $p < 0.05$ versus additive effects of IGF plus EGF.

CHAPTER 4

HETEROZYGOUS DELETION OF IRS-1 COMBINED WITH PHARMACOLOGICAL INHIBITION OF EGFR REDUCES TUMOR DEVELOPMENT IN THE COLON OF FEMALE BUT NOT MALE $APC^{MIN/+}$ MICE

A. Introduction

Colorectal cancer is the second leading cause of cancer-related deaths among men and women combined, with an overall 5.2% lifetime risk of developing colorectal cancer.[19] The progression of colorectal cancer is well-documented where genetic mutations in the normal epithelium accumulate, leading to the formation of precancerous adenomas.[26] Further allelic loss or mutations promotes additional neoplastic changes that lead to carcinoma.[26] One of the most frequent mutations in the initial stages of sporadic colorectal cancer occurs in the APC tumor suppressor gene, which is mutated in over 85% of sporadic colorectal cancer.[32] Mutational inactivation of this gene promotes aberrant activation of the β-catenin pathway.[27] The $Apc^{Min/+}$ mouse is a mouse model of APC inactivation, which develops tens to hundreds of adenomas in the small intestine and, depending on the genetic background, develops adenomas in the colon as well.[37,36,34] In addition to APC/β-catenin pathway, other signaling pathways are known to influence the initiation and progression of colorectal cancer.

The IGF-I and EGF family are both implicated in the initiation and progression of colorectal cancer. Both IGF-IR and EGFR are frequently up-regulated in primary colon adenoma and adenocarcinoma samples.[236,69,70,132,219,133] Increased circulating IGF, IGF-II or insulin are associated with increased risk of colorectal cancer.[237,63,238,62,65,239] Studies in mice strongly support a role for IGF-IR and EGF in tumor development or progression in the intestine. Mice homozygous for a hypomorphic $Egfr^{wav2}$ allele have a 90% reduction in receptor kinase activity and when, bred to the tumorigenic $Apc^{Min/+}$ background have a 90% decrease in tumor number.[134] Pharmacological inhibition of EGFR in $Apc^{Min/+}$ mice also leads to a reduction in tumor number, though this is not as dramatic as in mice with

Egfr^{wav-2} mice.[134] Mice with a liver-specific deletion of IGF-I have a 50-75% reduction in circulating IGF-I, and exhibit reduced number and size of colon tumors after treatment with AOM.[76,77] These results have lead to the development of targeted therapeutics that specifically inhibit these signaling pathways.

Small molecules or monoclonal antibodies that specifically target and inhibit the EGFR or IGF-IR are currently in clinical trials, with EGFR inhibitors being the most studied of the two. Agents specifically targeting the EGFR have shown potent effects to inhibit tumorigenesis in preclinical in vitro and in vivo models.[147,148,149] However, when these studies were translated to humans, only a subset of patients responded to EGFR inhibitors and patients treated with these agents also develop resistance to these therapies.[150,151,240,228] These resistance mechanisms may be mediated by increases in other growth promoting pathways, such as the IGF-IR pathway. In breast, prostate, and liver cancer cells, resistance to gefitinib, a small molecule EGFR inhibitor, was associated with increased activation of the IGF-IR and increased sensitivity to IGF-IR inhibitors.[153,212,150] Increased IGF-IR signaling interferes with effects of trastuzumab, an anti-HER2/neu receptor monoclonal antibody, to reduce proliferation.[151] These studies suggest that combined inhibition of IGF-IR and EGFR pathways would be more effective at reducing colon tumor development, greater than inhibition of either pathway alone.

Several in vitro studies demonstrate that combined inhibition of the IGF-IR and EGFR synergistically decreases proliferation and increases apoptosis greater than activation of either receptor alone.[218,221] In colon cancer cells, combined siRNA knockdown of IGF-IR and EGFR inhibited proliferation and increased apoptosis greater than knockdown of individual receptors.[218] Similar effects were seen with combined

treatment of the small molecule IGF-IR inhibitor, NVP-AEW541, and the EGFR inhibitor erlotinib.[218] Combined inhibition of IGF-IR and EGFR additively inhibited the growth of colon cancer cells.[220] To date, in vivo studies investigating the therapeutic potential of combined IGF-IR and EGFR inhibition have been confined to xenograft tumor models.[234] These studies show that combined treatment with the anti-IGF-IR antibody h7C10 (and an anti-EGFR inhibitor) almost completely inhibited tumor growth and prolonged survival.[234] While these studies show promise, further in vivo investigation in models of spontaneous intestinal tumors is needed to fully determine the effects of combined IGF-IR and EGFR signaling and ultimately address their roles at different stages in the colon cancer pathway.

We hypothesized that combined inhibition of IGF-IR and EGFR signaling will additively or synergistically decrease tumor development in the $Apc^{Min/+}$ model of spontaneous tumorigenesis. To test this hypothesis, we used mice with genetic disruption of the IRS-1 gene. We reasoned that loss of IRS-1 signaling would inhibit growth promoting effects of all IGF-I/insulin family ligands. Our previous studies in $Apc^{Min/+}$ mice demonstrated that mice heterozygous or homozygous for IRS-1 gene disruption had significantly reduced tumor load in both the small intestine and colon, thus demonstrating gene dosage effects of IRS-1.[61] The current study treated IRS-$1^{+/-}$/$Apc^{Min/+}$ mice with EGFR inhibitor to test combinational effects. We had aimed also to test EGFR inhibitor in IRS-$1^{-/-}$/$Apc^{Min/+}$mice. However this has proved impractical because of extremely low frequency of birth or survival of IRS-$1^{-/-}$/$Apc^{Min/+}$ mice. In addition, we reasoned that heterozygous deletion of IRS-1 may reflect more physiological reductions in IRS-1 expression. We therefore treated either IRS-$1^{+/+}$/$Apc^{Min/+}$ or IRS-$1^{+/-}$/$Apc^{Min/+}$ mice with a

specific EGFR inhibitor AG1478, which has previously been shown to inhibit cancer cell growth and to be effective at reducing tumor burden in $Apc^{Min/+}$ mice.[241] We found that heterozygous deletion of IRS-1 or pharmacological inhibition of EGFR reduced tumor development in the small intestine and colon; however the small intestine did not show additive effects to reduce tumor development when both pathways were inhibited. The colon showed dramatic reductions in tumorigenesis when IRS-1 deletion and EGFR inhibition are combined. Interestingly, these effects in the colon were confined to female $Apc^{Min/+}$ mice and were not seen in male $Apc^{Min/+}$mice.

B. Materials and Methods

Laboratory Animals

$Apc^{Min/+}$ mice on the C57BL/6J background were purchased from Jackson laboratories (Bar Harbor, ME). IRS-$1^{+/-}$ mice were generated on a purebred C57BL6 background as previously described.[56] and provided by Dr. Ronald Kahn (Harvard, Cambridge, MA). We used a two-step breeding process as previously described to generate IRS-$1^{+/+}$ /$Apc^{Min/+}$, IRS-$1^{+/-}$/$Apc^{Min/+}$, or IRS-$1^{-/-}$/$Apc^{Min/+}$.[61] Because IRS-$1^{-/-}$ mice were born at a very reduced frequency, we were unable to generate IRS-$1^{-/-}$/$Apc^{Min/+}$ mice across more than 40 litters. Therefore, we confined our studies to wild-type or heterozygous IRS-1 mice on the $Apc^{Min/+}$ background. Genotyping was performed on tail DNA as previously described.[134,59] All animal studies were approved by the Institutional Animal Care and Use Committee of the University of North Carolina. Study protocols were in compliance with the NIH Guide for the Care and Use of Laboratory Animals.[175]

AG1478 diet treatment

Tyrophostatin (AG1478, LC Laboratories, Woburn, MA) was kindly provided by Dr. David Threadgill and mixed with AIN-93G (Research Diets, Inc, location) at a concentration of 144mg/kg. (Research Diets, Inc, New Brunswick, NJ). Starting at weaning (3 weeks of age), mice were given either diet containing AG1478 or control diet (AIN-93G alone). Mice remained on diet continuously for 12 weeks. At this time (15 weeks of age), mice were sacrificed and small intestine and colon examined

Tissue Dissection and Adenoma Counts

Animals were weighed and anesthetized. Blood was collected by cardiac puncture. Entire small intestine and colon were dissected and flushed with 1 X PBS supplemented with 2mM vanadate 2mM, 1mM PMSF, and 100mM NaF. Small intestine was divided into 3 segments, roughly equal in length and a 0.5cm segment of proximal ileum was flash frozen in liquid nitrogen for RNA/protein analysis. The remaining small intestine segments, as well as the entire colon, were splayed open on filter paper. Tissues were fixed in 10% zinc-formalin overnight at 4°C then dehydrated in 70% ethanol. Number of adenomas in the small and large intestine of each animal were counted under a Leica dissecting scope, using an in-lens micrometer to measure adenoma diameter. Tumor load was calculated as number x size.

Statistical Analysis

Tumor number, size, and load are expressed as mean ± S.E.M. Two-way ANOVA was performed to determine if there were main effects of genotype, inhibitor, or importantly an interaction between genotype and inhibitor, which would suggest an effect of loss of IRS-1 on efficacy of EGFR inhibitor. Since small intestine shows differences in tumor number across different segments, with distal segments showing the greatest number of tumors, we analyzed data for segment-specific differences in effects of IRS-$1^{+/-}$ genotype, AG1478 treatment, or both conditions in $Apc^{Min/+}$ mice. Pair-wise comparisons were performed using Fischer's PLSD post-hoc test. In addition, we expressed tumor number, size, or load in each individual animal as a percentage of the mean of IRS-$1^{+/+}$/$Apc^{Min/+}$ mice on vehicle diet and then subtracted this from 100 to establish a percent reduction in tumor number, size, or load due to loss of one IRS-1 allele alone, AG1478 alone, or both combined. These values were compared by one-way ANOVA followed by Tukey's test to identify interactions. We also examined effects of IRS-$1^{+/-}$, AG1478, or both on tumor incidence and relative frequency of small versus large tumor number or size. All analysis was conducted on measurements with both sexes combined and also in males or females separately to test for gender-specific differences in efficacy of EGFR inhibitor or loss of IRS-1.

Limitations to the study and statistical analysis

1. Two-way ANOVA revealed main effects of IRS-1 genotype and AG1478, but not a significant interaction in small intestine or colon, or when broken down into males and females. This may not be the optimal test.

2. In pair-wise comparisons, instances where there is a significant difference between IRS-$1^{+/-}$/$Apc^{Min/+}$ vehicle-treated mice and IRS-$1^{+/-}$/$Apc^{Min/+}$ mice given AG1478 demonstrate that EGFR is effective at further reducing tumor number or size in mice with loss of one IRS-1 allele. However, in no instance did we achieve a statistically significant difference between IRS-$1^{+/+}$/$Apc^{Min/+}$ mice and IRS-$1^{+/-}$/$Apc^{Min/+}$ mice treated with AG1478 to statistically verify additional effects of adding loss of IRS-1 allele to EGFR inhibitor treatment.

3. The trends observed for interactions between loss of IRS-1 and EGFR inhibition between males and females are illustrated in the tables and figures and described in the results and discussion.

4. Additional different statistical tests or increases in n (sample size) of particular groups may be required to confirm these trends.

C. Results

Partial Loss of IRS-1 or EGFR inhibition does not affect body weight

Complete loss of IRS-1 in mice results in post-natal growth retardation.[55] In C57BL/J6 mice, chronic exposure to the EGFR inhibitor, AG1478, resulted in reduced

weight gain.[242] We therefore determined whether heterozygous deletion of IRS-1, EGFR inhibitor, or both had an effect on final body weight in *Apc*$^{Min/+}$ mice. As shown in Table 4.1, neither disruption of one IRS-1 allele nor EGFR inhibitor treatment significantly affected mean body weight, nor did the two conditions combined.

In the small intestine and colon, inhibition of IRS-1 or EGFR show gender-specific effects in efficacy to reduce tumor number and combined loss of IRS-1 and EGFR inhibitor are more effective, especially in females.

Table 4.2 summarizes the percent inhibition of tumor number in the small intestine and colon as a result of disruption of one IRS-1 allele, AG1478 treatment, or both. Table 4.3 and 4.4 present the means for tumor number, size and load across these groups. The data reveal some interesting effects. Taking males and females together, in small intestine loss of IRS-1 and EGFR inhibition both decrease tumor number (Table 4.2, Table 4.3). The decrease is greater with both loss of one IRS-1 allele and EGFR inhibitor combined although the effects are not statistically additive and maximum inhibition of tumor number is 65.5 $\pm$ 4.8%. The data become more interesting when separated by gender. They reveal that, in males, loss of IRS-1 leads to slightly greater reductions in tumor number than with EGFR inhibitor, while in females, EGFR is considerably more effective than haploid insufficiency for IRS-1 (Table 4.2, Table 4.3). However, combined effects of IRS-1$^{+/-}$ genotypes and EGFR inhibitor are similar between the two sexes in *Apc*$^{Min/+}$ mice.

In the colon, gender effects on tumor number are more striking. In males, loss of IRS-1 has considerably greater effects than AG1478; in females, both are very effective (Table 4.2, Table 4.4). Maybe most interesting is that in females, and not in males, combined IRS-$1^{+/-}$ genotype and AG1478 almost completely inhibits tumor development (Table 4.2, Table 4.4). This is further emphasized in Figure 4.1, which shows tumor incidence in colon. In females, loss of IRS-$1^{+/-}$ or EGFR inhibition alone each reduces tumor incidence, but when combined, they dramatically reduced tumor incidence to 18% of animals, essentially 2 out of 11 animals having a detectable tumor.

Loss of IRS-1 and EGFR inhibition has gender-specific effects in efficacy to reduce tumor number and tumor load in all regions of the small intestine.

Data for tumor number, size, and load in different small intestine segments are illustrated in figure 4.2 and also demonstrate gender-specific differences in efficacy of IRS-$1^{+/-}$ genotype or AG1478 treatment in *Apc*$^{Min/+}$ mice. In female mice, loss of IRS-1 alone had no significant effect on tumor number in any segment. EGFR inhibitor alone significantly reduced tumor number in two of three segments and tumor number was lowest in all segments when inhibitor was combined with loss of IRS-1 allele (Figure 4.2A). In males, all segments showed reduced tumor number as a result of loss of one IRS-1 allele, while EGFR inhibitor effects did not reach statistically significance when considered in separate segments (although means were lower) (Figure 4.2A). Effects of combined IRS-$1^{+/-}$ genotype and AG1478 treatment were not significantly greater than IRS-$1^{+/-}$ genotype alone.

Combined heterozygous deletion of IRS-1 and EGFR inhibition dramatically reduces tumor size in colon of female *Apc*$^{Min/+}$ mice.

These gender effects in colon were also apparent when considering tumor size (Table 4.4, Table 4.5, Figure 4.3). Examining mean tumor size in the small intestine, EGFR inhibitor treatment reduced tumor size while loss of IRS-1 had no effect alone or combined with EGFR inhibitor in both sexes (Table 4.3, Table 4.5). In the colon of male *Apc*$^{Min/+}$ mice, neither loss of one IRS-1 allele nor AG1478 dramatically affected mean size. Combined effects of loss of IRS-1 and EGFR inhibitor treatment did not differ from EGFR inhibitor alone (Table 4.4, Table 4.5). However, in female *Apc*$^{Min/+}$ mice, loss of IRS-1 or EGFR inhibition tended to reduce mean tumor size while combined their effect was much greater (Table 4.4, Table 4.5). Since mean values can be disproportionately affected by a single, these data we presented differently in Figure 4.3, which plots the percent of colon tumors of different size in females and males. In females, 100% of detected tumors were very small (<1mm) when IRS-1$^{+/-}$ mice were given EGFR inhibitor. In contrast, in males distribution of tumor size was similar in all groups except that mice given AG1478 alone had fewer tumors greater than 2mm in size and only IRS-1$^{+/+}$/*Apc*$^{Min/+}$ mice given vehicle had tumors greater than 3mm in size. Thus, together these data suggest that combined loss of EGFR and IGF-IR/IRS-1 signaling more profoundly affects both number and size of colon tumors in females than loss of either IRS-1 or EGFR signaling alone

D. Discussion

Our studies showed that combined heterozygous deletion of IRS-1 and pharmacological inhibition of EGFR had region- and sex-specific effects to additively reduce tumor development in $Apc^{Min/+}$ mice. Inhibition of IRS-1 and/or EGFR alone did not have an effect to reduce body weight (Table 4.1). In the small intestine, although heterozygous deletion of IRS-1 or inhibition of EGFR reduced tumor number and tumor load, there was not an additive decrease with combined inhibition (Table 4.2) In the small intestine, EGFR inhibition had greater efficacy in females, while IRS-1 deletion had a greater effect in males.(Table 4.3, Figure 4.2). In the colon, combined inhibition of IRS-1 and EGFR significantly reduced tumor number, size and load greater than inhibition of either signaling molecule alone and this effect was unique to the colon of females (Table 4.3). Female IRS-$1^{+/-}$/$Apc^{Min/+}$ mice given EGFR inhibitor showed at least additive reductions in tumor incidence and smaller tumors compared to either IRS-$1^{+/-}$/$Apc^{Min/+}$ mice given vehicle or IRS-$1^{+/+}$/$Apc^{Min/+}$ mice given EGFR inhibitor or vehicle diet (Figure 4.2, Figure 4.3). In contrast, male $Apc^{Min/+}$ mice did not show a combined effect for reduction in tumor development since tumor load and tumor incidence were similar between IRS-$1^{+/-}$/$Apc^{Min/+}$ mice given vehicle-diet or EGFR-inhibitor diet. Together, these studies suggest that combined loss of both IRS-1 and EGFR signaling has the most significant effects to reduce tumor development in the colon and in females, making a case analyzing gender- and tissue-specific effects of pharmacological treatments against colorectal cancer.

Our studies here demonstrated that heterozygous deletion of IRS-1 and/or chronic EGFR inhibition did not have significant effects to reduce body weight in female or male

mice. Though, previous studies in IRS-$1^{-/-}$ mice showed post-natal growth retardation, IRS-$1^{+/-}$ mice did not show an appreciable decrease in body weight compared to IRS-$1^{+/+}$ mice.[55,56] Studies in C57BL/J6 mice show that chronic treatment of the EGFR inhibitor AG1478 reduces body weight.[242] In our studies, chronic EGFR inhibition on the C57BL/J6 background did not affect body weight, possibly due to use of the genetic *Apc*$^{Min/+}$ background.

We hypothesized that combined heterozygous deletion of IRS-1 and inhibition of EGFR in *Apc*$^{Min/+}$ mice would additively or synergistically decrease tumor development in the small intestine, the dominant area of tumor development in *Apc*$^{Min/+}$ mice.[37,36] Partial deletion of IRS-1 significantly decreased tumor number and tumor load, consistent with our previous studies.[61] Inhibition of the EGFR reduced tumor number and tumor load, as previously reported.[134] The combined inhibition of both IRS-1 and EGFR pathways was more effective at decreasing tumor number in males or females than either alone. However in males, loss of IRS-1 alone is more effective than in females and the effects of loss of IRS-1 on tumor number are greater than the effects of EGFR inhibitor. Our studies showed that treatment with the EGFR inhibitor AG1478 reduced tumor size in the small intestine of both sexes. The effect of EGFR inhibition on tumor size seems dependent on the inhibitor and model used. Treatment of *Apc*$^{Min/+}$ mice with EKI-785 did not show an effect on tumor size.[134] Studies in xenograft models and those using a pan-EGFR inhibitor in *Apc*$^{Min/+}$, which targets all EGFR members, show decreased tumor size.[243,244] We used partial deletion of IRS-1 to mimic effects the likely physiological effects of reduced expression as opposed to complete genetic deletion. Previous studies have reported that up-regulation and activation of the IGF-IR pathway, of which IRS-1 is

a mediator, contributes to resistance to EGFR inhibitors.[154,221] In epithelial cell lines, treatment with erlotinib, a small molecule EGFR inhibitor, promotes IGF-I-induced Akt activity by promoting IRS-1 activation.[221] This suggests that the remaining IRS-1 allele could be activated during EGFR inhibition to prevent strictly additive effects in the small intestine. To that end, there was a trend for IRS-$1^{+/-}$/$Apc^{Min/+}$ mice given AG1478 to have further reductions in tumor number and tumor load that were greater than IRS-$1^{+/-}$/$Apc^{Min/+}$ mice on vehicle diet or IRS-$1^{+/-}$/$Apc^{Min/+}$ mice given AG1478. This suggests that complete inhibition of IRS-1 in conjunction with EGFR inhibition will be more potent and more effectively reduce tumor development in the small intestine.

In contrast to the small intestine, the combined inhibition of IRS-1 and EGFR significantly reduced tumor development in the colon and only combined inhibition significantly reduced tumor size.(Table 4.4, Table 4.5, Figure 4.1) Surprisingly this effect was specifically confined to females. Female IRS-$1^{+/-}$/$Apc^{Min/+}$ mice given EGFR inhibitor show dramatic reductions in tumor number, size, load and incidence while these measurements were similar between male IRS-$1^{+/-}$/$Apc^{Min/+}$ mice given EGFR inhibitor or vehicle (Table 4.2, Table 4.4, Table 4.3, Figure 4.1). Female IRS-$1^{+/-}$/$Apc^{Min/+}$ mice given EGFR inhibitor developed few, if any, tumors and those tumors that developed were small, whereas EGFR inhibitor given to male IRS-$1^{+/-}$/$Apc^{Min/+}$ mice had no such effect (Figure 4.1, Figure 4.3). These studies are significant because they establish that combined partial loss of IRS-1, and hence IGF-IR, signaling, and inhibition of EGFR additively reduced tumor development in the colon. Previous studies have demonstrated that combined inhibition IGF-IR and EGFR signaling synergistically reduced cell growth in vitro and xenograft tumor growth in vivo due to increased apoptosis.[218,220,154,221,234] Our

studies used a model of spontaneous tumor development rather than growth of established cancer cell lines in immuno-deficient mice. Prior studies have examined effects of targeting the IGF-IR specifically, yet several IGF-I/insulin ligands or receptor family members may still be capable of activating tumor-promoting pathways when IGF-IR is targeted. Our studies focused on IRS-1 since this is a downstream mediator of the anti-apoptotic effects of IGF-I in the small intestine and colon and mediates signaling of several IGF-IR family members, such as the type II IGF-IR (IGF-IIR) and the insulin receptor as well.[60,61,59] The additive decrease in tumor incidence and shift towards smaller tumors with haplotype insufficiency of IRS-1 combined with EGFR inhibition suggest that IRS-1 may promote the survival of tumors and when inhibited together with EGFR, dramatically reduces the survival and progression of tumors. Our findings also adds support to an emerging hypothesis that resistance to EGFR inhibitor may be mediated at least in part by IGF-IR signaling and extend this to suggest that IRS-1 is a mediator of these effects. These results also suggest that complete inhibition of IRS-1 in $Apc^{Min/+}$ mice combined with EGFR inhibition will have dramatic effects to reduce tumor development and we predict that no tumors will develop in the colon of IRS-$1^{-/-}$/$Apc^{Min/+}$ mice given EGFR inhibitor. However, this proved practically impossible to test.

One surprising observation was the difference in tumor phenotype in the colon between male and female IRS-$1^{+/-}$/$Apc^{Min/+}$ mice given EGFR inhibitor and in the efficacy of loss of IRS-1 or EGFR inhibition in the small intesitne. The combined effects of heterozygous deletion of IRS-1 and inhibition of EGFR were the same between male and female mice in the small intestine, so we predicted a similar observation in the colon. However, in the colon male mice on average were more likely to develop colon tumors

that female mice. While 89% of female IRS-1$^{+/+}$/*Apc*$^{Min/+}$ developed colon tumors, 100% of male IRS-1$^{+/+}$/*Apc*$^{Min/+}$ developed at least one colon tumor (Figure 4.2B). Even when given EGFR inhibitor or when IRS-1 was partially deleted, tumor incidence was still higher in male mice than in female mice (Figure 4.2B). This is consistent with human studies that have established an increased risk of colorectal cancer in men.[245,246,247,248] A recent meta-analysis of 17 studies from 18 different populations established a pooled risk estimate of colorectal cancer risk in men compared to women of 1.83 (95% confidence interval, 1.69 -1.97).[246] A female survival advantage has been found across different solid tumors such as melanoma and colorectal cancer.[248] Our studies support evidence for such an advantage. Several hypothesis have been suggested as to why females show a lower risk of developing colorectal cancer, including dietary intake and physical activity.[249,250] However, our studies controlled for any lifestyle effects, suggesting that differences may be inherent linked to the physiology of the different sexes. Our previous studies have not shown a sex-specific difference with regards to the IRS-1-mediated effects on intestinal growth.[59] There is some evidence that post-menopausal women on estrogen and progesterone hormone replacement therapy have an overall reduced risk of colorectal cancer suggesting that estrogen and/or progesterone may have a protective role in the development of colorectal cancer.[251,252,253] The importance of our study is that it suggests that combined inhibition of both EGFR and IGF-IR/IRS-1 signaling pathways may be more effective against colon cancer in females than males.

Another observation was that while inhibition of either pathway reduced tumor load in the small intestine and in the colon, the combined effect of heterozygous deletion of IRS-1 and EGFR inhibition was more pronounced in the colon compared to the small

intestine. IRS-1 mediates the anti-apoptotic effects of IGF-I in an organ-specific manner. Complete IRS-1 deficiency dramatically reduces IGF-I-induced increase in muscle growth, yet only partially inhibits IGF-I-induced small intestine growth.[59] In other studies, homozygous deletion of IRS-1 reduced mucosal mass of the colon and ablated IGF-I-induced increases in mucosal and muscularis weight.[60] These studies suggest that IRS-1 has more pronounced effects to mediate the anti-apoptotic effects in the colon and suggests a possible mechanism as to why heterozygous deletion of IRS-1 combined with EGFR inhibition has more pronounced effects to additively reduce tumor development in the colon. It must be noted our results reflect the effects of heterozygous deletion of IRS-1 and may not be the case with homozygous deletion of IRS-1. IRS-$1^{-/-}$ mice are born at a reduced frequency and no IRS-$1^{-/-}$/$Apc^{Min/+}$ mice were born during this study.[59] However, we predict that complete disruption of IRS-1 combined with EGFR inhibition would additively or synergistically reduce tumor development in $Apc^{Min/+}$ mice.

Together these results demonstrate that combined heterozygous deletion of IRS-1 and pharmacological inhibition of EGFR in $Apc^{Min/+}$ mice have additive effects to reduce tumorigenesis in the colon of female mice and not male mice. Although combined inhibition of both IRS-1 and EGFR in the small intestine did not show additive effects to reduce tumor development, these results are promising and pointing to interactions between loss of IRS-1 and inhibition of the EGFR to more effectively decrease tumor development even in the small intestine of $Apc^{Min/+}$ mice. These studies support a role for IRS-1 in mediating IGF-IR signaling in EGFR resistance. In addition, the results also support further analysis into gender-specific effects of pharmacological treatments against colorectal cancer.

Table 4.1. Inhibition of IRS-1 or EGFR does not affect final body weight.

Genotype	Treatment	sex	Body Weight
IRS-$1^{+/+}$/$Apc^{Min/+}$	Vehicle	F	17.4±0.7
IRS-$1^{+/-}$/$Apc^{Min/+}$	Vehicle	F	17.3±0.9
IRS-$1^{+/+}$/$Apc^{Min/+}$	AG1478	F	18.9±0.5
IRS-$1^{+/-}$/$Apc^{Min/+}$	AG1478	F	16.5±0.1
IRS-$1^{+/+}$/$Apc^{Min/+}$	Vehicle	M	20.5±1.2
IRS-$1^{+/-}$/$Apc^{Min/+}$	Vehicle	M	23.5±0.9
IRS-$1^{+/+}$/$Apc^{Min/+}$	AG1478	M	22.2±1.5
IRS-$1^{+/-}$/$Apc^{Min/+}$	AG1478	M	22.9±0.9

Table 4.2. Percent reduction in tumor number relative to vehicle-treated IRS-1$^{+/+}$/ *Apc*$^{Min/+}$ mice.

		SMALL INTESTINE			COLON		
Genotype	**Treatment**	**All**	**Male**	**Female**	**All**	**Male**	**Female**
IRS-1$^{+/+}$/Apc$^{Min/+}$	Vehicle	0.0±14.4	0.0±15.9	0.0±20.8	0.0±16.3	0.0±19.4	0.0±22.9
IRS-1$^{+/-}$/Apc$^{Min/+}$	Vehicle	34.8±8.8	48.8±8.9	22.1±17.7	59.4±9.7[a]	62.4±10.0	68.4±15.8
IRS-1$^{+/+}$/Apc$^{Min/+}$	AG1478	45.0±9.9[a]	36.8±24.1	49.3±9.0	37.5±18.2	15.4±23.1	52.6±22.3
IRS-1$^{+/-}$/Apc$^{Min/+}$	AG1478	65.5±4.8[a]	65.3±5.9[a]	66.9±7.2[a]	67.9±11.0[a]	50.0±15.3	91.4±5.8[a]

a = $p < 0.05$ versus IRS-1$^{+/+}$/*Apc*$^{Min/+}$ on vehicle diet

Table 4.3. Heterozygous deletion of IRS-1 or EGFR inhibition reduces tumor load in the small intestine and the combination does not additively reduce tumor load.

A. Whole Population (Males & Females)				SMALL INTESTINE		
Genotype	**Treatment**	**n**	**sex**	**Number**	**Size**	**Load**
IRS-$1^{+/+}$/$Apc^{Min/+}$	Vehicle	13	MF	84.3±12.2	0.82±0.05	71.4±10.9
IRS-$1^{+/-}$/$Apc^{Min/+}$	Vehicle	15	MF	54.9±7.4[a]	0.84±0.04	46.3±6.9[a]
IRS-$1^{+/+}$/$Apc^{Min/+}$	AG1478	13	MF	46.4±8.3[a]	0.62±0.04[a,b]	29.8±6.5[a]
IRS-$1^{+/-}$/$Apc^{Min/+}$	AG1478	19	MF	29.1±4.0[a,b]	0.70±0.03[a,b]	20.4±3.1[a,b]
B. Separate Sexes				**SMALL INTESTINE**		
Genotype	**Treatment**	**n**	**sex**	**Number**	**Size**	**Load**
IRS-$1^{+/+}$/$Apc^{Min/+}$	Vehicle	9	F	80.0±16.6	0.74±0.03	62.0±14.0
IRS-$1^{+/-}$/$Apc^{Min/+}$	Vehicle	6	F	62.3±14.1	0.80±0.07	51.7±13.6[a]
IRS-$1^{+/+}$/$Apc^{Min/+}$	AG1478	9	F	40.6±7.2[a]	0.58±0.04[a,b]	23.5±4.4
IRS-$1^{+/-}$/$Apc^{Min/+}$	AG1478	11	F	26.5±5.7[a,b]	0.63±0.03[a]	16.0±3.3[a,b]
IRS-$1^{+/+}$/$Apc^{Min/+}$	Vehicle	4	M	94.0±14.9	1.02±0.08	92.5±11.6
IRS-$1^{+/-}$/$Apc^{Min/+}$	Vehicle	9	M	50.0±8.4[a]	0.86±0.05	42.8±7.4[a]
IRS-$1^{+/+}$/$Apc^{Min/+}$	AG1478	4	M	59.5±22.6	0.69±0.05[a,b]	44.0±18.1[a]
IRS-$1^{+/-}$/$Apc^{Min/+}$	AG1478	8	M	32.6±5.6[a]	0.79±0.03[a]	26.6±5.4[a]

a = $p<0.05$ vs. IRS-$1^{+/+}$/$Apc^{Min/+}$ on vehicle diet
b = $p<0.05$ vs. IRS-$1^{+/-}$/$Apc^{Min/+}$ on vehicle diet

Table 4.4. Combined heterozygous deletion of IRS-1 and EGFR inhibition more effectively reduces tumor number, size, and load in the colon of female mice.

A. Whole Population (Males & Females)				COLON		
Genotype	Treatment	n	sex	Number	Size	Load
IRS-$1^{+/+}$/$Apc^{Min/+}$	Vehicle	13	MF	2.5±0.4	1.3±0.2	3.8±0.8
IRS-$1^{+/-}$/$Apc^{Min/+}$	Vehicle	15	MF	1.0±0.2[a]	1.0±0.3	1.7±0.5[a]
IRS-$1^{+/+}$/$Apc^{Min/+}$	AG1478	13	MF	1.5±0.4	0.7±0.2	1.8±0.6[a]
IRS-$1^{+/-}$/$Apc^{Min/+}$	AG1478	19	MF	0.8±0.3[a]	0.4±0.1[a,b]	1.0±0.5[a]
B. Separate Sexes				**COLON**		
Genotype	Treatment	n	sex	Number	Size	Load
IRS-$1^{+/+}$/$Apc^{Min/+}$	Vehicle	9	F	2.1±0.5	1.1±0.2	2.9±0.8
IRS-$1^{+/-}$/$Apc^{Min/+}$	Vehicle	6	F	0.7±0.3[a]	0.6±0.4	0.9±0.6[a]
IRS-$1^{+/+}$/$Apc^{Min/+}$	AG1478	9	F	1.0±0.5	0.6±0.3	1.3±0.7[a]
IRS-$1^{+/-}$/$Apc^{Min/+}$	AG1478	11	F	0.2±0.1[a]	0.1±0.1[a]	0.1±0.1[a]
IRS-$1^{+/+}$/$Apc^{Min/+}$	Vehicle	4	M	3.3±0.6	1.8±0.1	5.9±1.5
IRS-$1^{+/-}$/$Apc^{Min/+}$	Vehicle	9	M	1.2±0.3[a]	1.3±0.3	2.2±0.8
IRS-$1^{+/+}$/$Apc^{Min/+}$	AG1478	4	M	2.8±0.8	1.0±0.3	3.0±1.5[a]
IRS-$1^{+/-}$/$Apc^{Min/+}$	AG1478	8	M	1.6±0.5[a]	1.0±0.3	2.2±1.0[a]

a = $p<0.05$ vs. IRS-$1^{+/+}$/*Apc*$^{Min/+}$ on vehicle diet
b = $p<0.05$ vs. IRS-$1^{+/-}$/*Apc*$^{Min/+}$ on vehicle diet

Table 4.5. Percent reduction in tumor size relative to vehicle-treated IRS-1$^{+/+}$/*Apc*^{Min} mice

		SMALL INTESTINE			COLON		
Genotype	**Treatment**	**All**	**Male**	**Female**	**All**	**Male**	**Female**
IRS-1$^{+/+}$/Apc$^{Min/+}$	Vehicle	0.0±6.1	0.0±8.3	0.0±4.7	0.0±12.5	0.0±2.6	0.0±18.1
IRS-1$^{+/-}$/Apc$^{Min/+}$	Vehicle	2.1±5.0	15.1±5.1	9.3±9.6	21.3±18.0	29.9±17.6	36.7±30.8
IRS-1$^{+/+}$/Apc$^{Min/+}$	AG1478	25.2±4.4[a,b]	31.8±5.1[a]	21.1±5.9[b]	47.6±15.4	46.4±16.9	48.5±22.3
IRS-1$^{+/-}$/Apc$^{Min/+}$	AG1478	15.3±3.4[a]	22.6±3.2[a]	14.3±4.6	66.6±10.9[a]	49.4±14.0	91.1±6.0[a]

a = $p<0.05$ vs. IRS-1$^{+/+}$/*Apc*$^{Min/+}$ on vehicle diet
b = $p<0.05$ vs. IRS-1$^{+/-}$/*Apc*$^{Min/+}$ on vehicle diet

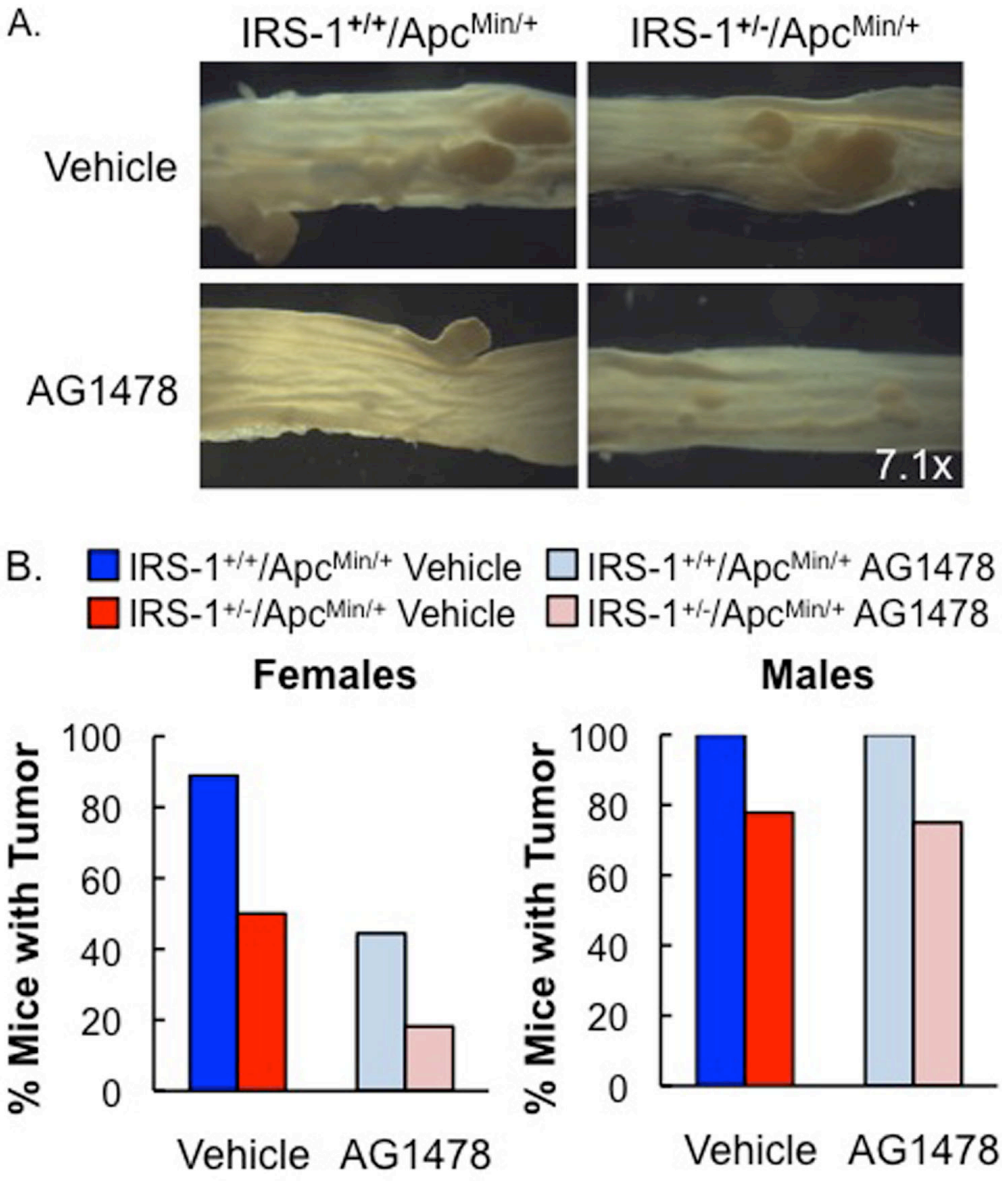

Figure 4.1 Combined heterozygous deletion of IRS-1 and inhibition of EGFR additively reduces tumor incidence in female mice *Apc*$^{Min/+}$ mice, but not male *Apc*$^{Min/+}$ mice. A. Bright field images of representative colons from female IRS-1$^{+/+}$/*Apc*$^{Min/+}$ and IRS-1$^{+/-}$/*Apc*$^{Min/+}$ mice given either diet containing the EGFR inhibitor AG1478 or vehicle diet for 12 weeks. Note the lack of tumors that develop when both IRS-1 and EGFR are inhibited. B. Histograms showing tumor incidence (% mice that developed one or more tumors) in female (left) or male (right) IRS-1$^{+/+}$/*Apc*$^{Min/+}$ and IRS-1$^{+/-}$/*Apc*$^{Min/+}$ mice given either vehicle diet or diet containing the EGFR inhibitor AG1478.

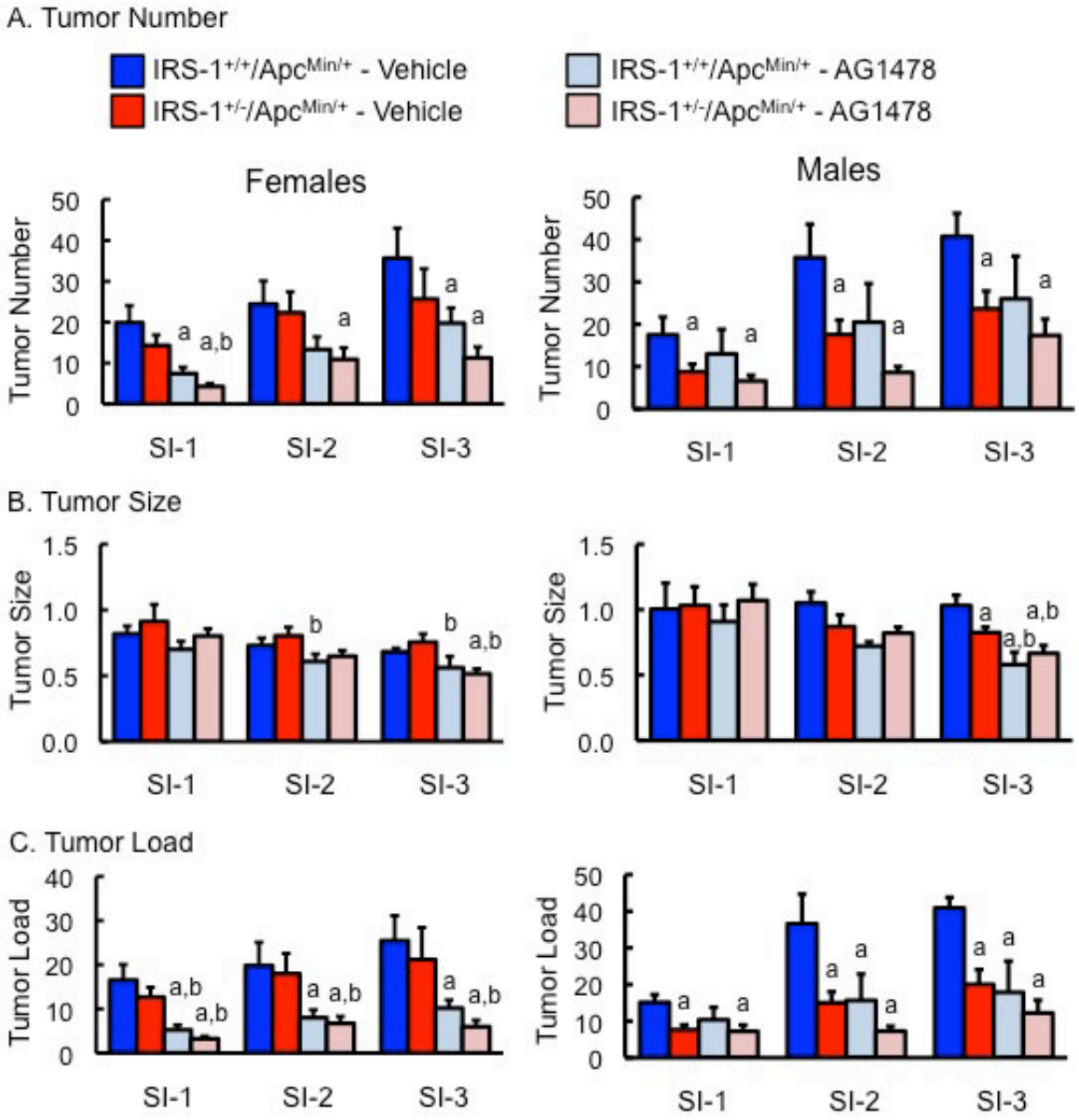

Figure 4.2. Combined inhibition of IRS-1 and EGFR does not have additive effects to reduce tumor number, size, or load in the small intestine. Mean ± S.E.M for A. tumor number, B. tumor size, and C. tumor load (number x size) for each tumor region in female (left) or male (right) IRS-$1^{+/+}$/*Apc*$^{Min/+}$ and IRS-$1^{+/-}$/*Apc*$^{Min/+}$ mice given either diet containing the EGFR inhibitor AG1478 or vehicle diet for 12 weeks. n≥4, SI-1 is the most proximal region and contains the duodenum and SI-3 is the most distal region and contains the ileum. a = p<0.05 vs IRS-$1^{+/+}$/*Apc*$^{Min/+}$ given vehicle diet; b = p<0.05 vs IRS-$1^{+/-}$/*Apc*$^{Min/+}$ given vehicle diet

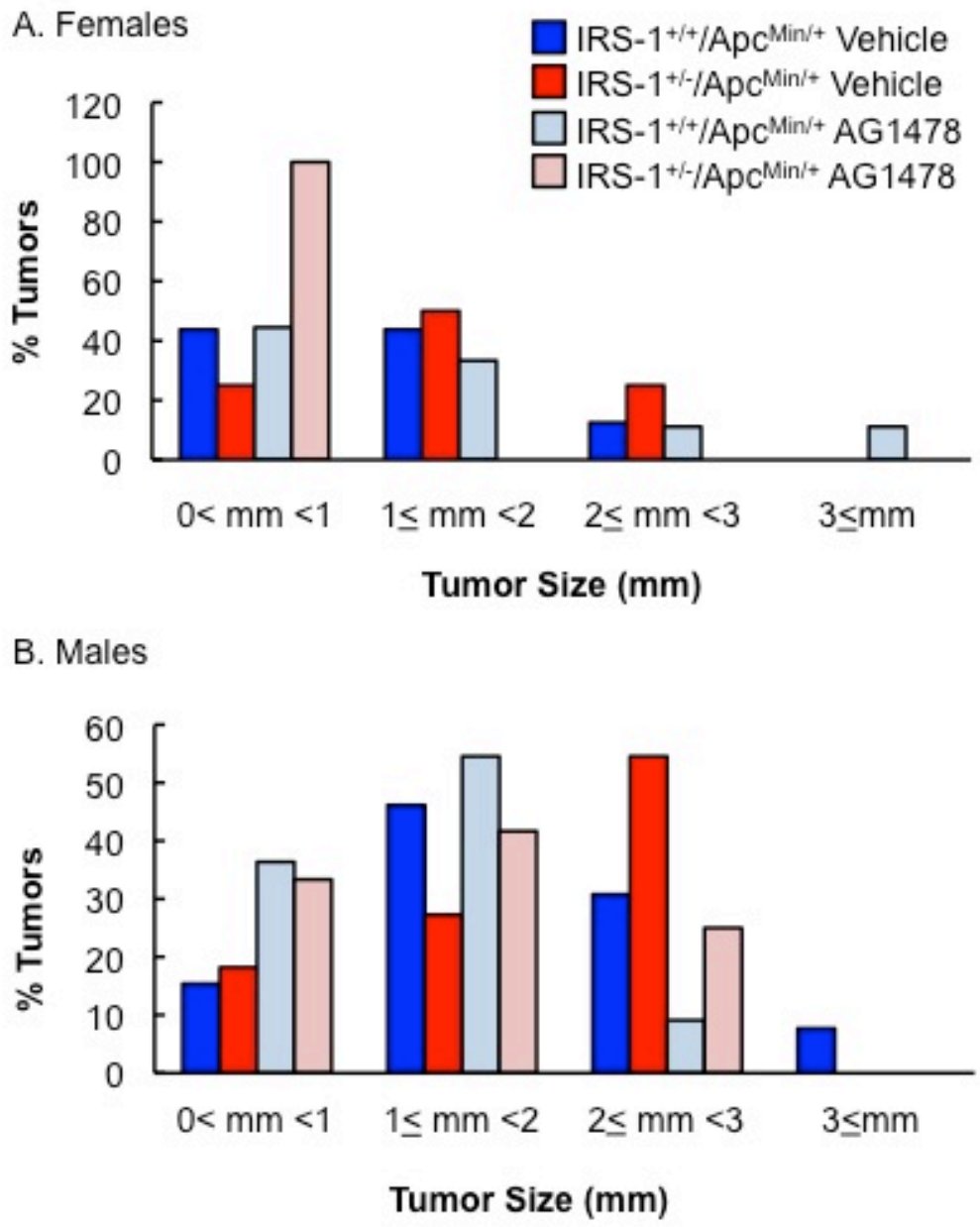

Figure 4.3 Female IRS-1$^{+/-}$/*Apc*$^{Min/+}$ mice given diet AG1478 develop small tumors. A. Histogram showing the percentage of tumors of a particular size compared to the overall tumor number in female mice. Note the lack of medium ($1 \leq$ mm < 2) and large ($2 \leq$ mm < 3, $3 \leq$ mm) tumors in female IRS-1$^{+/-}$/*Apc*$^{Min/+}$ mice given the EGFR inhibitor. B. Histogram showing % of tumors of a particular size in male mice. Note that while IRS-1$^{+/+}$/*Apc*$^{Min/+}$ and IRS-1$^{+/-}$/*Apc*$^{Min/+}$ mice given the EGFR inhibitor have an increase in small and medium sized tumors, there was no effect with combined inhibition of IRS-1 and EGFR in male mice.

CHAPTER 5

DISCUSSION AND FUTURE DIRECTIONS

A. SOCS are negative regulators and biomarkers of cancer in the gastrointestinal tract.(Adapted from [80])

Results in chapter II show that heterozygous or homozygous loss of SOCS2 increases tumor number, size, and load in the small intestine and colon of $Apc^{Min/+}$ mice and increases tumor incidence in the colon. Together with evidence that partial loss of SOCS2 in GH-Tg mice promotes dysplastic growth, these studies provide strong evidence that loss of SOCS2 promotes the growth of pre-cancerous lesions in the intestine. Thus, in addition to evidence that SOCS2 genes are silenced by promoter methylation in cancers of other organs (Table 1.1) and evidence for a role of other SOCS in gastrointestinal cancers, especially SOCS1 and SOCS3, a central role emerges for SOCS proteins as intrinsic negative regulators of tumor development in the gastrointestinal tract. Furthermore, SOCS proteins may have distinct roles to suppress development of different tumor types in the intestine. This regional- and context-specific role of the SOCS proteins may allow for their use as biomarkers of GI cancer or different subtypes.

SOCS1 and SOCS3 play a particular role in inflammation-associated tumorigenesis in several types of GI cancer. Available evidence suggests that SOCS1 and SOCS3 acts as suppressors of inflammation-associated cancer by decreasing proliferation, promoting apoptosis, and, in certain cases, reducing activation of NFκB and STATs.[113, 114] SOCS1 or SOCS3 silencing appears to drive tumorigenesis in context of inflammation, though some studies suggest an overlap between pathways linked to sporadic as well as inflammation-associated cancers. For example, in a study of tissue from patients with sporadic rather than IBD-associated CRC, there was a significant

correlation between nuclear accumulation of STAT3 and β-catenin, and this was associated with poor patient survival.[254] Likewise, in spontaneous stomach cancer, STAT3 positivity in tumors correlated with poorer prognosis, and this was unrelated to *Helicobacter pylori* infection or gastritis.[255] In esophageal squamous cell carcinoma, STAT3 is enhanced in tumor cells, and in vitro studies showed that transfected β-catenin induced transcription and expression of STAT3, and that dominant-negative TCF4 reversed this effect.[256] These studies all suggest that STAT3 plays a role in sporadic as well as inflammation-associated tumorigenesis; therefore, SOCS proteins are likely regulators of both tumor types. However, few studies have examined the interplay between SOCS proteins and sporadic cancer pathways such as Wnt/β-catenin.

The most striking direct evidence of SOCS2 as a tumor suppressor thus far lies in our studies of intestinal tumorigenesis. Studies showing increased small intestine and colon tumor load and incidence in SOCS2$^{-/-}$/*Apc*$^{Min/+}$ mice illustrate an intrinsic tumor suppressor role of SOCS2 in the absence of detectable inflammation, and our published studies show that the absence of SOCS2 has no effect of tumor formation in the AOM/DSS model.[116,117] Studies described in chapter II also show that loss of SOCS2 promotes increases in serine-phosphorylation of STAT3 as opposed to tyrosine-phosphorylation, which SOCS3 is known to inhibit.[113] This is exciting as it suggests that differential phosphorylation of STAT3 may be one mechanism that explains the preferential effects of SOCS1 and SOCS3 to limit inflammation-associated tumors, while SOCS2 limits spontaneous tumorigenesis in the intestine.

One common theme of among GI cancers is that SOCS proteins are silenced by aberrant hyper-methylation in tumor tissue.[104,106,257,258,259,260,261,262,263,264,265,266,267,268,269,270]

Recent studies show that IL-6 regulates the transcription factor Fli-1, which is required for expression and activity of DNA methyltransferase enzyme (dnmt-1).[184] Additional studies showed that IL-6 directly contributes to aberrant methylation of the tumor suppressor p53.[184] To date, the mechanisms regulating SOCS methylation are unknown. IL-6-mediated hyper-methylation of SOCS could be an as-yet-undefined mechanism of tumorigenesis in the GI tract, at least in the setting of inflammation.

The prevalence of SOCS hyper-methylation in GI cancers highlights the importance of developing therapies to reverse the epigenetic silencing of tumor suppressor genes. In cholangiocarcinoma cell lines that exhibit SOCS3 promoter methylation, treatment with the demethylating agent 5-aza-2'-deoxycytidine (DAC) reversed SOCS3 silencing and decreased STAT3 activation, providing exciting pre-clinical data on the prospect of using DAC to restore SOCS expression in cancer cells.[267] In addition to epigenetic therapies, small molecule SOCS agonists may be useful therapies to restore SOCS action and prevent or treat cancer. JAK inhibitors are currently being tested for a variety of myelo-proliferative disorders, but may have broader implications for other cancers where aberrant cytokine or STAT signaling occurs, such as those GI cancers where one or more SOCS proteins are silenced.[271] SOCS-based therapies may be attractive as combinational therapies with more traditional strategies such as conventional chemotherapy, radiation therapy, or even biologic therapies. The rationale underlying this suggestion is that SOCS are endogenous tumor suppressors and may have fewer side effects. Thus, it would be of interest in the future to test whether SOCS overexpression or SOCS mimetics synergize with or reduce the necessary dose of traditional therapies in ablating or inhibiting tumor cell growth in vivo or in vitro. While

some SOCS mimetics based on SOCS peptides have been reported, a remaining obstacle is to ensure permeability into cells.

B. Serine Phosphorylation of STAT3 and cancer

Constitutive activation of STAT3 is associated with poor prognosis in colorectal cancer, protection from apoptosis, and is commonly thought to occur through tyrosine phosphorylation of STAT3.[254, 272, 273,274,275] In chapter II, we demonstrated that homozygous deletion of SOCS2 in $Apc^{Min/+}$ mice increases serine phosphorylation of STAT3 in tumor and non-tumor intestinal tissue by both western blot and immunohistochemistry, but tyrosine phosphorylation of STAT3 was unaffected. These studies support an emerging hypothesis that serine phosphorylation of STAT3 has a role in cellular transformation and growth and regulates tumorigenic growth.

Upon cytokine or growth factor activation, STAT3 is phosphorylated at tyrosine residue 705 and/or at serine residue 727. Phosphorylation at Y705 has been shown to be required for dimerization and nuclear translocation of STAT3.[173,188] The role of phosphorylation at S727 is context- and cell-specific.[194,276,193,277] In some studies, serine phosphorylation is not required for DNA binding, but has been shown to be required for maximal transcriptional activity.[276,278] Others studies have suggested that STAT3 negatively regulates tyrosine phosphorylation of STAT3 through both ERK-dependent and –independent mechanisms.[193,192,277] Importantly, serine phosphorylation has demonstrated growth effects independent of tyrosine phosphorylation, including increased anchorage-independent growth in cancer cell lines and tumor formation in

immune compromised mice.[190] The role of serine phosphorylation in normal growth was demonstrated in mice with an inactivating serine to alanine mutation at residue 727 of STAT3 (SA/SA).[191] When combined with heterozygous loss of STAT3 (SA/-), these mice had a 10-15% reduction in birth weight, reduced survival, and had a 40-50% reduction in post-natal body and organ growth one week after birth.[191] These mice had reduced levels of plasma IGF-I in 8-day old littermates, suggesting that mechanisms of reduced growth occur through reduced circulating IGF-I.[191] Though SA/SA mice appeared grossly normal, there was reduced transcriptional activation in response to IL-6 and OSM, suggesting that in vivo serine-phosphorylation of STAT is necessary for maximal transcriptional activation of these cytokines.[191] Thus, one direction our laboratory would like to take in the future is to examine the S727A STAT3 mutant and test whether these animals show diminished risk of tumors. Use of constructs expressing this STAT isoform could also test directly whether serine phosphorylation of STAT is necessary for tumor promoting roles of SOCS2 silencing.

In congruence with its suggested role in growth, serine phosphorylation of STAT3 has recently been detected in vivo in mammary gland, prostate tumors, and in chronic myloid leukemia and in vitro in H-Ras(V12) transformed cells, bladder, lung, and medullary thyroid cancer cell lines.[189,174,190,172,279] Serine-phosphorylation was required for H-Ras-, N-Ras-, or K-Ras-induced tumor growth in soft agar, where STAT3 was shown to contribute to Ras-mediated oncogenic transformation.[279] Of particular importance, serine phosphorylation of STAT3 has recently been associated with tumor-initiating stem cells (TISCs) or cancer stem cells (CSC) in glioblastoma, a frequent and aggressive cancer.[280] This subpopulation of cells retains stem-cell characteristics and is

highly resistant to chemotherapeutics and ionizing radiation.[281,280] STAT3 is constitutively phosphorylated at S727 in these cells and inhibition of STAT3 reduces their proliferation and sensitizes them to chemotherapy.[280] In addition, recent studies to derive rabbit embryonic stem cells found that LIF, which was required for maintenance of these stem cells, promoted preferential phosphorylation at serine 727 of STAT3.[280] In chapter II, serine-phosphorylation of STAT3 was localized to the crypts in normal small intestine and was particularly evident in small cells intercalated between paneth cells, a region known to contain both intestinal stem cells or crypt based columnar cells.[282] Results of these studies in stem-like cells, in addition to a demonstrated role in normal and neoplastic growth, suggest that serine phosphorylation of STAT3 may influence growth by affecting the local stem cell populations.

C. STATS and stem cells

As previously mentioned, activated STAT3 is able to promote neoplastic growth in the presence of activating oncogenic mutations, in both sporadic and inflammation-associated colorectal cancer. Among the STAT family, STAT3 is the only STAT that when deleted in whole animals is embryonic lethal, suggesting a central role for this STAT in embryonic development.[283] In addition, studies in chapter II suggest that serine phosphorylated STAT3 is localized to the lower half of the crypt, where intestinal stem cells (ISCs) are thought to reside. A series of studies in the *Drosophila* midgut has recently highlighted the role of the JAK-STAT pathway in homeostasis and maintenance of the intestinal stem cell population.[284]

The *Drosophila* midgut is quickly becoming an excellent model to study behaviors of ISCs since it parallels stem cell differentiation in humans.[285,286] A population of self-renewing ISCs is maintained in the base membrane along with non-dividing, undifferentiated ISC daughter cells, called enteroblasts (EBs), that can differentiate into one of two lineages through Delta ligand-Notch receptor signaling, absorptive enterocytes (ECs) or secretory enteroendocrine cells (EEs).[287] Intestinal damage to due microbial infection, induced apoptosis, or JNK-mediated stress signaling induces the expression of three cytokine-like ligands called *unpaired* (*Upd1-3*) in the surrounding muscle cells to influence nearby enterocytes.[287,285,288] *Unpaired* binds to *domeless*, an IL-6-like receptor, that phosphorylates and activates a JAK homologue, *Hopscotch* (*Hop*), which in turn activates *STAT92E*, the sole STAT homologue in *Drosophila*.[287,284] *STAT92E* induces the expression of *domeless* and a negative regulator of *Upd* signaling, *socs36E.*[287]

The *Upd/Hop/STAT* pathway is implicated in maintenance and differentiation of ISCs.[284] Overexpression of *Upd* or *Hop* increases enterocytes mitosis in both terminally differentiated cells and ISCs.[287] Mutations in *STAT92E* inhibited the expression of differentiation markers and reduced the size of enterocytes nuclei.[287] JAK-STAT signaling functions cooperatively with Delta-Notch signaling to promote terminal differentiation and lineage decision, where $Notch^{High}/Hop^{Low}$ cells are driven towards an absorptive EC fate and $Notch^{Low}/Hop^{High}$ cells are driven towards a secretory EE cell fate.[285] As such, activated JAK-STAT signaling is not normally found in terminally differentiated enterocytes and loss of JAK-STAT signaling attenuates ISC differentiation.[289,285] Together, these studies support a role dual role for JAK-STAT

signaling in regulating the proliferation and differentiation of ISC and ISC daughter cells, though JAK-STAT signaling does not seem to have a role in self-renewal.[287,289,286] Dr. Scott Magness, who will be a mentor for my post-doctoral research, recently developed techniques that allow the isolation and in vitro culture of ISCs capable of self-renewal and differentiation into enterocyte lineages.[290,291] Using these techniques, future studies could test the ability of JAK-STAT signaling in mouse ISCs to influence maintenance and differentiation, in particular, whether serine or tyrosine phosphorylation has a role in promoting ISC growth and/or differentiation.

D. Using gene expression microarray to identify signaling pathways.

Our studies in chapter II demonstrated that increased tumorigenesis in SOCS2$^{-/-}$/*Apc*$^{Min/+}$ mice is associated with increased local IGF-I expression, serine-phosphorylation, and AP-1 DNA binding. However, we cannot rule out that other unknown mechanisms are promoted in response to loss of SOCS2 and whether these pathways are related to specific growth factors, such as IGF-I or EGF. Our studies in chapter III in IEC-6 cells showed that combined treatment of IGF-I and EGF additively increased nuclear β-catenin and TCF transcriptional activity. However, whether IGF-I and EGF have additive effects to promote transcription of β-catenin-dependent or independent genes is unknown. Ongoing studies to investigate the effects of SOCS2 deletion or EGF/IGF-I on expression of specific genes and pathways are using microarray technology to elucidate activation or repression of various genes and networks. Studies in Dr. David Threadgill's lab have highlighted the use of GeneSpring and Ingenuity

software to identify pathways related to cell signaling and survival.[292] Such studies are already currently underway in our lab. A post-doc, Dr. Shengli Ding and graduate student, Amanda Mah, have already performed microarray analysis on RNA from pooled tumors and matching non-tumor tissue collected from SOCS2$^{+/+}$/*Apc*$^{Min/+}$, SOCS2$^{-/-}$/*Apc*$^{Min/+}$, SOCS2$^{+/+}$, and SOCS2$^{-/-}$ mice. Data analysis is in progress. Future studies will investigate gene expression from tumors isolated in young mice, when tumors first present, to examine signaling expression when adenomas are just beginning to form as a result of loss of SOCS2 expression. We predict that genes that regulate AP-1 activation or expression will be altered, as well as STAT-regulated genes.

E. Use of combined targeted therapies in colorectal cancer.

In chapter III, we demonstrated that combined IGF-I and EGF treatment synergistically increases proliferation, nuclear β-catenin, and TCF transcriptional activation. We also showed in chapter IV, that inhibition of the EGFR combined with heterozygous IRS-1 deletion additively reduces tumor development in the colon of female *Apc*$^{Min/+}$ mice and may interact to decrease small bowel tumors. These results suggest that IGF-I and EGF interact to promote a key-tumor promoting pathway and enhance proliferation and that combined inhibition of both these pathways may be a promising treatment against colorectal cancer.

Studies in NSCLC and breast cancer support this hypothesis, where aberrant activation of the IGF-I pathway occurs in cells that are resistant to targeted EGFR inhibitors.[153, 226] In some cases, this is thought to occur though activation IRS-1.[154,221]

Though small molecule and antibody inhibitors have been developed that specifically target IGF-IR, we chose to use a model of IRS-1 inactivation on the *Apc*$^{Min/+}$ background.[145] Inactivation of IRS-1 is thought to inhibit the growth effects of all IGF ligands and we have previously demonstrated that partial loss of IRS-1 significantly reduces tumor load in *Apc*$^{Min/+}$mice.[61] In addition, at the time of the study, specific IGF-IR inhibitors had not been tested in the *Apc*$^{Min/+}$ mouse model. Recently, antibodies against IGF-I (KM3168) and IGF-II (KM1468) given by intraperitoneal (i.p.) injection to *Apc*$^{Min/+}$ mice led to reduced polyp number when given alone and additive effects when combined.[293] These inhibitors show promise in testing the utility of combined targeted therapies in mouse models, such as the *Apc*$^{Min/+}$ mouse.

Other inhibitors for have surfaced for use in combination with EGFR inhibitors, most notably VEGF. Targeted therapeutics have been developed that inhibit both the EGFR and VEGFR and reduced tumor growth orthopically in the nude mice.[294] This has also been demonstrated in a similar manner for NSCLC and salivary adenoid carcinoma cells.[295,296,297] Though, the efficacy of these treatments has yet not been evaluated in patients with colorectal cancer, these studies support our general hypothesis that combined inhibition of tumor-promoting pathways may be more effective in reducing tumor development than single-agent therapies.

F. Effect of underlying genetic mutations on efficacy of target therapeutics

Our studies in chapter IV showed an additive effect to reduce tumor incidence and tumor size with combined heterozygous deletion of IRS-1 and EGFR inhibition (Figure

4.1, Figure 4.3). While these studies support the use of combined targeted therapeutics to reduce tumor development, one cannot overlook the patient populations that will most benefit from this type of treatment. Recent lessons from studies of using the targeted EGFR inhibitors, cetuximab and panitumumab, in metastatic colorectal cancer patients demonstrate that underlying genetic mutations can dramatically affect the efficacy of such treatments to reduce colorectal cancer. Though preclinical studies demonstrated a remarkable effect of these inhibitors to reduce tumor development, only 10-20% of colorectal cancer patients responded to this therapy.[298,220] An initial retrospective analysis revealed that colorectal cancer patients who have activating *KRAS* mutations do not benefit from cetuximab or panitumumab treatment.[299,300] Activating *KRAS* mutations have since been defined as a negative predictor of clinical outcome of cetuximab-based treatments.[228] This breakthrough has led to the changes in FDA guidelines for these specific inhibitors, specifying that cetuximab and panitumumab should not be given to patients with *KRAS* mutations.[301] A recent study demonstrated longer disease-free survival in patients with wild-type (WT) *KRAS*.[229] Since, other mutations such as *BRAF* and *PTEN* mutations have also been defined as negative predictors of cetuximab clinical outcome.[228] These studies suggest that combined inhibition of EGFR and IGF-IR would be most effective in patients that do not harbor these mutations, since IGF-IR is also known to activate RAS/MAPK and PI3K/PTEN pathways.

Even with identification of such mutations, even the best responders to targeted EGFR develop resistance to cetuximab and panitumumab in 12 to 19 months.[228] These secondary resistance mechanisms are still unknown and up-regulation of the IGF-IR pathway could represent one such mechanism. Our studies demonstrated that when IRS-1

signaling was inactivated, thus impairing signaling through the IGF family, treatment with the EGFR inhibitor was more effective than inhibition of either IRS-1 or EGFR. This suggests that IGF-I pathways are still active in tumors that survive EGFR inhibition. Indeed, studies by Ming Yu in the Threadgill lab have shown that tumors that survive in *Apc*$^{Min/+}$ mice despite an EGFR inactivating mutation (EGFR$^{wav\text{-}2}$), have elevated IGFBP5 and IGF-IR expression (communication with Dr. Ming Yu and David Threadgill). We propose that IGF-IR targeted therapies may be useful in overcoming the secondary resistance seen in responders to EGFR inhibitor treatment. The animal models and strategies used in this thesis may be used to address the role of IGF-IR signaling in resistance to EGFR inhibitors.

G. Novel tumor imaging techniques to identify micro-adenomas and therapeutic mechanisms

Our studies in Chapter IV showed that female IRS-1$^{+/-}$/*Apc*$^{Min/+}$ mice treated with an EGFR inhibitor show additive reductions in tumor incidence greater than inhibition of IRS-1 or EGFR alone. The lack of obvious tumors in these mice was striking and suggests that combined inhibition of these pathways disrupts early stages of tumor development such as the initiation and establishment of adenomas. Genetic and pharmacological disruption of the EGFR in *Apc*$^{Min/+}$ has been shown to inhibit the establishment of adenomas.[134] What is not known is whether combined inhibition of IRS-1 and EGFR pathways promotes changes in the formation of aberrant crypt foci, the earliest indicator of neoplastic dysplasia, and microadenomas.[302] These features are near

impossible to detect by bright-field microscopy and are currently only detected through laborious histological detection. In addition, only histological detection can truly identify adenomas from similar, but benign, hyperplastic and lymphoid polyps.

Current studies in our lab have focused on developing enhanced tumor imaging techniques that would allow identification of adenomas, from benign polyps and areas of inflammation and dysplasia. Molecular probes have been developed that are activated by biomarkers up-regulated in adenomas, such as cathepsin B.[303,304] These probes are administered intravenously and are undetectable unless cleaved by locally activated enzymes causing to fluoresce at near-infared fluorescence and have been shown to be effective in detecting tumors in *Apc*$^{Min/+}$ mice.[303,304] Recent studies in our laboratory combined the use of these probes with capsule endoscopy, a capsule designed to be swallowed by a patient and record images as it goes through the digestive tract.[305] These studies demonstrated the cathepsin B probe, Prosense680^{TM}, was able to detect adenomas in both *Apc*$^{Min/+}$ mice and models of colits-induced colon cancer, but did not detect benign lesions.[305] More probes have been developed and these are currently being tested by a post-doc in the lab, Dr. Shengli Ding. Recently, a probe Annexin-750, can detect areas of apoptosis and would be useful in our model of combined IRS-1 and EGFR inhibition to determine if the mechanisms of tumor inhibition involve apoptosis.[306] Once tested, the ability of these probes detect microadenomas will be evaluated in AOM-treated and early-age *Apc*$^{Min/+}$ mice to test if these probes can detect early stage adenomas, which are not normally visible by bright-field microscopy.

H. Gender effects on colorectal cancer risk and potential mechanisms

We observed in chapter II and chapter IV that male $Apc^{Min/+}$ mice on the C57BL/J6 background were more likely to develop colon tumors than female $Apc^{Min/+}$ mice. This was quantified in Figure 4.1B where male IRS-$1^{+/+}$/$Apc^{Min/+}$ mice on vehicle or EGFR inhibitor diet had a higher tumor incidence compared to female IRS-$1^{+/+}$/$Apc^{Min/+}$ mice on the same diets. Female mice also had dramatic reductions in tumor load and incidence in response to combined heterozygous deletion of IRS-1 and inhibition of EGFR.(Table 4.4, Table 4.5, Figure 4.1, Figure 4.3) These observations support previous studies in humans, demonstrating an increased risk of colorectal cancer in men across multiple race/ethnic groups.[245,246,248] A meta-analysis of 17 independent studies found a pooled increased risk of 1.83 for advanced neoplasia in men and positive association between gender and advanced cancer.[246] Men also show a stronger association between increased BMI and colorectal cancer risk.[249,247] A female survival advantage has been demonstrated in melanoma, lung, esophageal, and pancreatic cancers as well as in soft tissue sarcoma.[248] Environmental and genetic factors play a role in the increased risk of colorectal cancer in men. These effects are most likely not due to preventative screening behaviors as men are more often screened for colorectal cancer.[307] Men have been reported to consume more alcohol and more high-fat meat, and eat less dietary fiber, than women, which may contribute to increased risk.[250,249] However, one cannot overlook the genetic differences inherent between the sexes.

Several studies suggest female sex steroids may have a role in reducing risk of colorectal cancer in women.[308,251,252,253] The Women's Health Initiative (WHI) recently showed a reduced risk of colorectal cancer risk with use of estrogen and progesterone hormone replacement therapy (HRT) in post-menopausal women.[308] In the Cancer Prevention Study cohort, estrogen-only HRT was associated with reduced risk of colorectal cancer and a strong inverse relationship between long-term estrogen use and colorectal cancer risk.[308] In another study, use of oral HRT was associated with a 63% relative reduction in the risk of colorectal cancer in post-menopausal women.[251] While mechanisms as to how estrogen and/or progesterone treatment reduces colorectal cancer risk are still unknown, one possible mechanism lies in estrogen action on growth hormone. A few studies demonstrate that estrogen inhibits the metabolic actions of GH by reducing JAK2 tyrosine phosphorylation and GH-induced STAT reporter activity.[309,310] Long-term estrogen was found to increase SOCS2 and SOCS3 mRNA in the liver through estrogen receptor α.[311] Since our studies in Chapter II demonstrated that heterozygous and homozygous deletion of SOCS2 in $Apc^{Min/+}$ mice reduced intestinal tumorigenesis, future studies could investigate whether estrogen mediates a protective effect against colorectal cancer through SOCS2. This is just one mechanism whereby sex-hormones augment tumor-promoting pathways and studies in this thesis highlight the need for gender-specific evaluations of the cancer therapies.

I. Summary and Working Model

These studies show that SOCS2 and EGF have opposite effects to influence IGF-IR signaling. In the working model shown in Figure 5.1, GH activates the GH receptor and thus induces local IGF-I expression to have paracrine/autocrine effects in the intestine to increase proliferation and survival of intestinal epithelial cells and promote intestinal tumorigenesis. SOCS2 normally inhibits GH-induced local expression of IGF-I and IGF-IR activation as well as inhibits serine phosphorylation of STAT3. In the absence of SOCS2, IGF-I expression is increased and, thus, increases activation of the IGF-IR. IGF-I-induced receptor activation, as well as signaling from additional cytokine and/or growth factors, promote the phosphorylation of STAT3 at serine residue 727 without affecting tyrosine phosphorylation. Serine phosphorylation has been shown to promote the activation of AP-1 transcription factor binding to DNA to promote gene transcription that enhances intestinal tumorigenesis. EGF has opposite effects on IGF-IR signaling and, instead, acts to promote enhanced IGF-I-induced DNA synthesis and proliferation by enhancing total IGF-IR and promoting synergistic activation of β-catenin/TCF-mediated gene transcription. Though the exact mechanism of synergistic β-catenin activation remains unknown, we suspect further expression analysis will reveal activation of PI-3K or MAPK-driven pathways. Our studies in chapter IV show that not only do combined IRS-1 and EGFR inhibition synergistically reduce tumor development, but that this occurs in a gender- and region-specific manner, in the colon of female $Apc^{Min/+}$ mice. Combined, these studies suggest new pathways through which growth factors regulate tumor development and highlight novel avenues of cancer therapeutics,

such as SOCS protein mimetics and use of combined therapeutics to overcome resistance to targeted inhibitors.

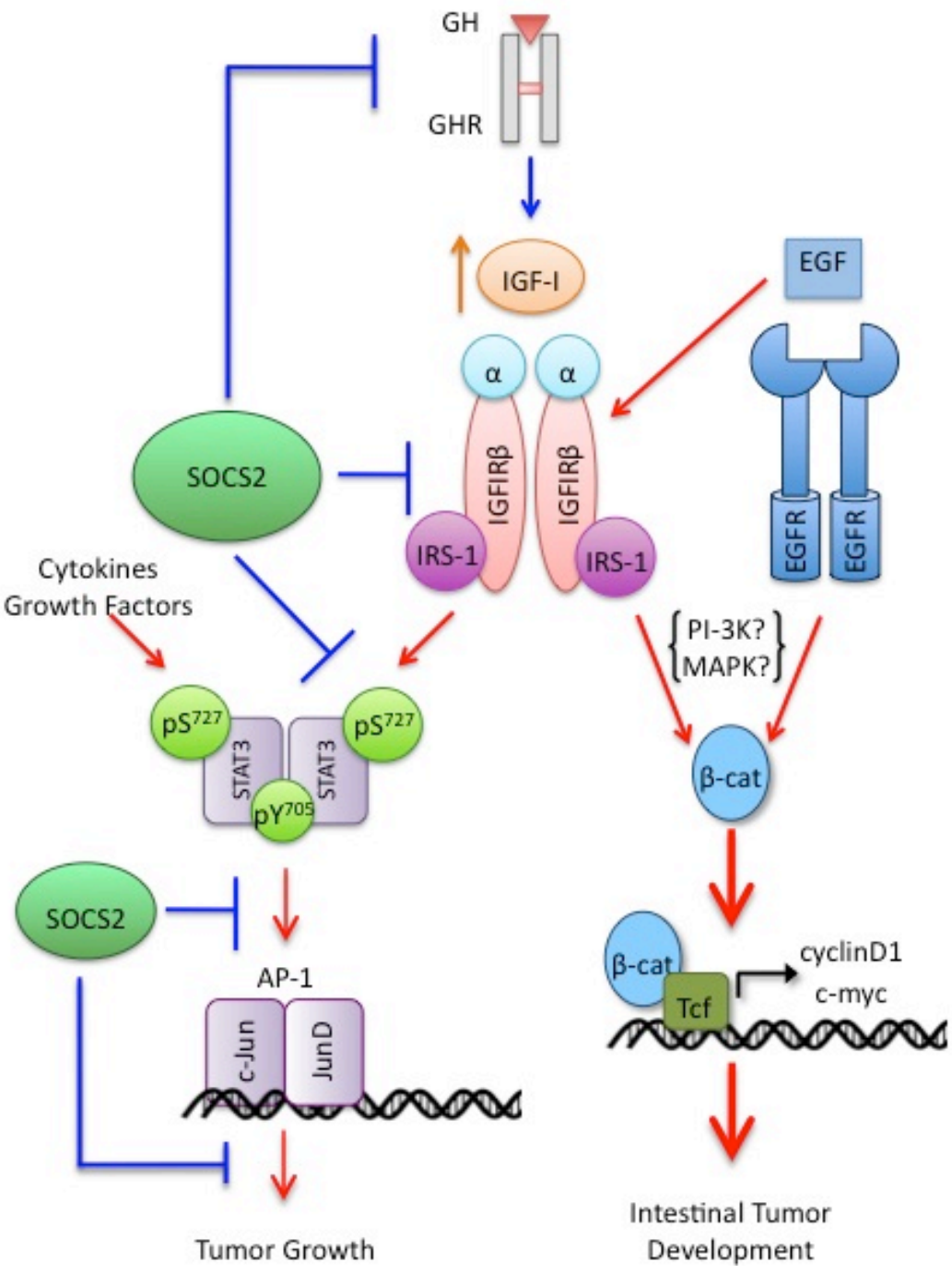

Figure 5.1. A Hypothetical model of SOCS2 and EGF action of IGF-IR signaling in intestinal cancer. Future directions will be aimed at identifying alterations in signaling pathways and networks as a result of loss of SOCS2 in *Apc*$^{Min/+}$ mice and EGF and IGF-I interactions in IEC-6 cells using microarray

CHAPTER VI

APPENDIX

A. LIST OF PUBLICATIONS

The list below provides citations of the publications to which Victoria A. Newton has contributed. Percent contribution by Victoria A. Newton is noted in parenthesis.

1. Newton VA, Ramocki NM, Scull BP, Simmons JG, McNaughton K, and Lund PK. Suppressor of cytokine signaling-2 gene disruption promotes *Apc*(*Min/*+) tumorigenesis and activator protein-1 activation. *Am J Pathol* 2010 May;176(5):2320-2332. (60%)

CHAPTER VII

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