OBESITY, HYPERINSULINEMIA, THE INSULIN AND INSULIN-LIKE GROWTH FACTOR 1 RECEPTORS, AND RISK OF COLORECTAL CANCER

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A dissertation submitted to the faculty at the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Program of Cell and Molecular Physiology in the School of Medicine.

Chapel Hill
2015

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ABSTRACT

Maria Agostina Santoro: Obesity, hyperinsulinemia, the insulin and insulin-like growth factor 1 receptors, and risk of colorectal cancer
(Under the direction of P. Kay Lund)

Insulin resistance and hyperinsulinemia associated with obesity or type 2 diabetes are strongly associated with increased risk of colorectal cancer (CRC). Elevated plasma insulin can increase the levels of "free" insulin-like growth factor 1 (IGF1) in the circulation. Both insulin and IGF1 can bind and activate the insulin receptor (IR) or the related IGF1 receptor (IGF1R). IGF1R is traditionally viewed as a major mediator of growth and anti-apoptosis and has been linked to cancer. IR is expressed as two isoforms, IR-A and IR-B. IR-A promotes growth of fetal and possibly cancer cells, while IR-B mediates the metabolic actions of insulin and promotes differentiation in some tissues. However, the specific roles of IGF1R, IR-A, and IR-B in colon physiology and tumorigenesis are unclear. This dissertation combined translational and pre-clinical approaches to explore the roles of IGF1R and IR in colorectal adenoma risk, tumorigenesis, and reduced apoptosis of genetically damaged colonocytes during obesity and hyperinsulinemia. Our studies showed that increased IR-A:IR-B ratio due to decreased IR-B mRNA predicted colorectal adenomas in patients with elevated plasma insulin. In a mouse model of inflammation-induced CRC, genetic deletion of IR in colon epithelial cells (CECs) enhanced tumor number *in vivo* and tumor cell growth *in vitro* and this was associated with enhanced IGF1-induced AKT activation. Obesity/hyperinsulinemia resulted in reduced apoptosis of CECs in normal colon after radiation-induced DNA damage. Surprisingly, loss of IGF1R in CECs had no effects on apoptosis, but loss of IR dramatically increased apoptosis of genetically-damaged CECs. However, IR loss did not prevent the anti-apoptotic effects of obesity/hyperinsulinemia. Overall, this dissertation provides novel evidence that maintained IR expression and function may protect against early stage colon tumorigenesis.

Since IR-B expression is reduced in colon tumors in mice and normal mucosa of hyperinsulinemic patients with adenomas, we propose that in the colon, IR-B normally attenuates the proliferative, anti-apoptotic, or tumorigenic actions of IGF1R or IR-A. Our studies suggest that therapeutic strategies to increase or maintain IR-B expression may improve prevention of CRC, particularly when IR-B function is impaired as occurs during insulin resistance associated with obesity or type 2 diabetes.

To my wonderful parents and sister who supported my decision of moving overseas to pursue my dreams and have accompanied me every step of the way, and to my boyfriend who is always there for me and has supported me daily throughout the excitements and challenges of graduate school.

ACKNOWLEDGEMENTS

First, I would like to thank my dissertation mentor Dr. Kay Lund for giving me the opportunity to engage in the type of basic and translational research I had always wanted to pursue since before my PhD. She was always there for me when I needed guidance yet allowed me to become an independent scientist. Apart from being a brilliant scientist, she is an exceptional writer and has helped my scientific writing improve tremendously. Her advice as a mentor and as a friend has been essential to this work, my formation as a scientist, and my wonderful experience as a doctoral student in her laboratory.

Second, I want to give special thanks to the current and former members of the Lund Lab. Dr. Laurianne Van Landeghem has been a fantastic bench mentor who taught me most of the techniques I know currently and helped me get started on some of my research projects. Working closely with her has made a great impact on the scientist I am today. Drs. Sarah Andres and Amanda Mah have not only been outstanding labmates and collaborators but also wonderful friends. We grew together as scientists and I have enjoyed working and discussing my data with them. Eric Blue has been of indispensable technical assistance in all mouse studies, and I could not have done these experiments without him. Drs. Emily Moorefield, Shengli Ding, and Jim Simmons have provided helpful intellectual advice as well as training. Adeola Keku helped with mouse genotyping and Josh Robbs was always there for us to help with computer and administrative issues. I also want to acknowledge my summer undergraduate students Marienid Flores-Colón, Grisselle Burgos-Santana, and Christian Agosto-Burgos who made contributions to my projects and whose excitement for science and scientific success were very rewarding.

I would also like to thank my collaborators Drs. Tope Keku, Robert Sandler, and Joe Galanko from the Department of Medicine and the UNC Center for Gastrointestinal Biology and Disease (CGIBD) for providing mentoring, clinical exposure, and invaluable contributions to the human project; the UNC Intestinal Stem Cell Group, particularly Drs. Scott Magness, Christian Dekaney, and Susan Henning for

sharing equipment and providing scientific input during lab meetings; Kirk McNaughton and Ashley Ezzell at the Histology Research Core Facility in the Department of Cell Biology and Physiology for their histology services and training in immunohistochemistry; Carolyn Suitt at the CGIBD Histology Core for assistance in paraffin embedding of tissues; Drs. Andrea Azcarate-Peril and Belen Cadenas at the CGIBD Microbiome and qRT-PCR Core for providing laboratory space and training on equipment during the human research project; Nikki McCoy for assistance with apoptosis assays in human samples; Qing Shi from the UNC Department of Nutrition for assistance with plasma insulin measurements; Carlton Anderson and Dr. Scott Magness at the CGIBD Advanced Analytics Core for their assistance with ELISA and high throughput PCR experiments; and the CGIBD Biostatistics and Data Management Core for providing statistical assistance. Also, I want thank the Department of Cell Biology and Physiology and the Program in Cell and Molecular Physiology, especially Dr. Carol Otey for her guidance and professional advice throughout my graduate studies; Dr. Kathleen Caron, Adriana Tavernise, and Janice Warfford for helping me stay on top of graduate requirements and timelines; Tonya Murrell and Vicki Morgan for administrative assistance; and Dr. Ann Stuart for her helping me with oral presentations during the Presentation Class. I am also grateful for the grad-into-med training I received from the UNC Program in Translational Medicine and the career development support provided during my final year by the UNC Royster Society of Fellows. Last but not least, I want to thank Drs. Carol Otey, Robert Sandler, John Rawls, and Scott Magness for providing valuable guidance during committee meetings.

PREFACE

Chapter 2 of this dissertation was published in the October issue of *Cancer Epidemiology, Biomarkers & Prevention,* 2014. Author contributions were: M.A. Santoro designed and performed experiments, analyzed and interpreted the data, and wrote the manuscript; S.F. Andres developed methodology and reviewed manuscript; J.A. Galanko performed the statistical analyses; R.S. Sandler designed study and reviewed manuscript; T.O. Keku designed study, contributed to acquisition of patient samples and interpretation of data, and reviewed the manuscript; P.K. Lund designed and supervised study, developed methodology, wrote the manuscript, and contributed to acquisition, analysis and interpretation of data.

Chapter 4 of this work has been finalized and is in preparation for submission. Contributions of authors were as follows: M.A. Santoro designed and conducted all experiments, analyzed and interpreted data, and wrote the manuscript; S.F. generated the intestinal epithelial IR knockout model and provided intellectual input; E.R. Blue assisted in all radiation procedures and mouse dissections, L. Van Landeghem designed study, generated the intestinal epithelial IGF1R knockout model, and provided valuable intellectual guidance; P.K. Lund supervised study, designed all experiments, interpreted data, and wrote the manuscript.

This dissertation work was supported by grants from the National Institutes of Health: RO1 DK40247 (P.K. Lund), RO1 AG041198 (P.K. Lund), P30 DK034987 (UNC Center for Gastrointestinal Biology and Disease), R01 CA044684 (Diet and Health Study V), and RO1 CA136887 (T.O. Keku). Additional support was received from the Lovick P. Corn Dissertation Fellowship, UNC Royster Society of Fellows, and the UNC-HHMI Fellowship in Translational Medicine (M.A. Santoro).

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LIST OF ABBREVIATIONS AND SYMBOLS

=	Equal to
±	Plus-minus
≥	Greater than or equal to
ACF	Aberrant crypt foci
Actb	β-actin gene
AKT	Protein kinase B
Aldh	Aldehyde dehydrogenase 1
AMPK	Adenosine monophosphate -activated protein kinase
ANOVA	Analysis of variance
AOM	Azoxymethane
APAF-1	Apoptotic protease activating factor 1
APC	Adenomatous polyposis coli
ATM	Ataxia telangiectasia mutated
ATP	Adenosine triphosphate
BAD	Bcl-2-associated death promoter
BAX	Bcl-2-associated X protein
BCL-2	B-cell lymphoma 2
BCL-XL	B-cell lymphoma-extra large
BMI	Body mass index
Bmp	Bone morphogenetic protein
BRAF	Raf murine sarcoma viral oncogene homolog B
CBC	Crypt base columnar
CD133	Cluster of differentiation 133

CD44	Cluster of differentiation 44
Cdkn1b	Cyclin-dependent kinase Inhibitor 1B
CDX2	Caudal-related homeobox protein 2
CEC	Colon epithelial cell
CESC	Colonic epithelial stem cell
CI	Confidence interval
CIMP	CpG island methylator phenotype
COX-2	Cyclooxygenase-2
CRC	Colorectal cancer
CR-CTEC	Conditionally-reprogrammed colon tumor epithelial cells
CSC	Cancer stem cell
CTL	Control
CUGBP1	CUG RNA binding protein 1
db/db	Mice with homozygous mutation in the leptin receptor gene
DHS V	Diet and Health Study V
DIABLO	Direct inhibitor of apoptosis-binding protein with low pI
DM	Myotionic distrophy
DMPK	Myotonic distrophy protein kinase
DNA	Deoxyribonucleic acid
DR4/5	Death receptor 4/5
DSB	Double-stranded break
DSS	Dextran sodium sulfate
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EdU	5-Ethynyl-2´-deoxyuridine

EGF	Epidermal growth factor
EGFP	Enhanced green fluorescent protein
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immuno assay
EpCAM	Epithelial cell adhesion molecule
ER	Endoplasmic reticulum
ERK	Extracellular-signal-regulated kinase
FAP	Familial adenomatous polyposis
FasL	Fas ligand
FasR	Fas receptor
FITC	Fluorescein isothiocyanate
fl/fl	Floxed/floxed. Homozygous for LoxP modifications
GF	Germ-free
GH	Growth hormone
GLP-2	Glucagon-like peptide 2
GLUT	Glucose transporter
Gy	Gray, unit of radiation
H&E	Hematoxylin and eosin
HFD	High fat diet
HMBS	Hydroxymethylbilane synthase
HOMA	Homeostatic model assessment
HR	Hybrid receptor
i.p.	Intraperitoneal
IAP	Inhibitor of apoptosis protein complex
IBD	Inflammatory bowel disease

IEC	Intestinal epithelial cell
IGF1	Insulin-like growth factor 1
IGF1R	Insulin-like growth factor 1 receptor
IGF2	Insulin-like growth factor 2
IGF2R	Insulin-like growth factor 2 receptor
IGFBP	Insulin-like growth factor binding protein
Ihh	Indian hedgehog
IL-12	Interleukin-12
IL-18	Interleukin-18
IL-6	Interleukin-6
INSR	Insulin receptor gene
IR	Insulin receptor
IR-A	Insulin receptor isoform A
IR-B	Insulin receptor isoform B
IRS-1	Insulin receptor substrate 1
IRS-2	Insulin receptor substrate 2
ISC	Intestinal stem cell
KO	Knockout
KRAS	Kirsten Rat Sarcoma Viral Oncogene Homolog
Lgr5	G-protein-coupled receptor 5
LKB1	Liver kinase B1
LRC	Label-retaining cell
Lrig1	Leucine-rich repeats and immunoglobulin-like domains 1
MBNL	Muscleblind protein
MIN	Multiple intestinal neoplasia

MMR	Mismatch repair
mRNA	Messenger ribonucleic acid
MSI	Microsatellite instability
Msi-1	Musashi-1
mTOR	Mammalian target of rapamycin
Myc	V-myc avian myelocytomatosis viral oncogene homolog
NEMO	NF-κB essential modulator
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NOXA	PMA-induced protein 1
ob/ob	Mice with homozygous mutation in the leptin gene
OR	odds ratios
Ovt/Ob	Overweight/obese
p21	Cyclin-dependent kinase inhibitor 1
PBS	Phosphate-buffered saline
Perp	P53 apoptosis effector related to PMP-22
PFA	Paraformaldehyde
pH2AX	Phosphorylated histone H2AX
pH3	Phosphorylate histone H3
PI3K	Phosphoinositide 3-kinase
PUMA	P53 upregulated modulator of apoptosis
qRT-PCR	Quantitative real-time polymerase chain reaction
RNA	ribonucleic acid
ROS	Reactive oxygen species
SCFA	Short-chain fatty acid
SEM	Standard error of the mean

Shc	Src homology 2 domain containing proteins
shRNA	Small hairpin RNA
Smac	Second mitochondria-derived activator of caspase
SMAD2/4	Mothers against decapentaplegic homolog 2/4
Sox9	Sex determining region Y-box 9
SRSF	Serine/arginine-rich splicing factor
STAT3	Signal transducer and activator of transcription 3
TBS	Tris-buffered saline
TdT	Terminal deoxynucleotidyl transferase
TNFR2	Tumor necrosis factor receptor 2
TNF-α	Tumor necrosis factor-α
TRAIL	TNF-related apoptosis-inducing ligand
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
UC	Ulcerative colitis
VC	Villin-Cre
WHO	World Health Organization
WHR	Waist-to-hip ratio
WT	wild-type
α	Alpha
β	Beta
Δ/Δ	Delta/delta. Homozygous for gene deletion
κ	Kappa
μ	Micro-

CHAPTER 1: INTRODUCTION TO COLON PHYSIOLOGY, COLORECTAL CANCER, THE INSULIN/IGF SYSTEM, OBESITY, AND APOPTOSIS

The colonic epithelium

Structure and function

In humans, the colon consists of ascending, transverse (proximal colon), and descending (distal colon) segments and the rectum [1]. Absorption of fat, carbohydrates, and proteins occurs primarily in the small intestine and remaining luminal contents flow into the proximal colon which reabsorbs fluids and electrolytes [1-3]. The proximal colon is also the primary site for absorption of short-chain fatty acids (SCFAs) synthesized by bacterial fermentation of carbohydrates that were not absorbed in the small intestine [1,4]. The distal colon also produces SCFAs but at much lower levels due the decreased carbohydrate availability [4]. The main function of the distal colon is to desiccate stool and store it until propelled into the rectum for expulsion [1].

The colon has a tubular structure with an inner space called lumen. The wall of the colon is composed of four main layers: mucosa (which is the closest to the lumen), submucosa, muscle layer or muscularis propria, and serosa (Figure 1.1A). The latter constitutes the outer layer of the intestine but is difficult to visualize by histology. The mucosa consists of an epithelial layer known as the **colonic epithelium**, an underlying stromal connective tissue termed **lamina propria**, and the **muscularis mucosa**, a thin layer of smooth muscle cells (Figure 1.1A) [1]. The colonic epithelium consists of a single layer of columnar epithelial cells, which form invaginations or "crypts" that extend down towards the muscularis mucosa. Current views indicate that the base of the crypts harbors stem and progenitors cells, which divide as they migrate up the crypt giving rise to three main terminally differentiated cell lineages: enteroendocrine cells, goblet cells, and colonocytes (Figure 1.1B) [5]. Colonocytes and goblet cells are the

most abundant cell types, constituting ~25% and ~75%, respectively, of the total cells per crypt [6]. Enteroendocrine cells secrete various peptide hormones and goblet cells secrete mucus [5]. Colonocytes are primarily absorptive but secrete chloride ions during diarrhea, which is accompanied by secretion of potassium ions by goblet cells leading to water release into the lumen [7]. Once differentiated cells reach the surface epithelium, they undergo detachment-mediated cell death (anoikis) and are shed into the lumen [5].

Colonic epithelial stem cells and their niche

Renewal of the human colonic epithelium occurs every 3-8 days [8]. Maintenance of the colon epithelial integrity and constant renewal requires highly active proliferation, which is driven by a small population of colonic epithelial stem cells (CESCs) located at the base of the crypts (Figure 1.1B). Current views indicate that a stem cell gives rise to two daughter cells in a process called asymmetric division, in order to renew itself and give rise to a transient amplifying progenitor cell that will continue to divide before they terminally differentiate [9]. Therefore, stem cells are characterized by their ability to self-renew, give rise to all other differentiated cell types (multipotency), and live for very long periods of time (longevity) [9]. In 1974, the existence of two intestinal stem cell (ISC) populations was proposed based on studies in the small intestine: crypt base columnar (CBC) cells which actively divide and reside at the very base of the crypt and a slowly-cycling, label-retaining cell (LRC) population located immediately above CBCs at approximately cell position 4 from the crypt base, termed "+4" [10,11]. In the late 2000s, a number of putative markers for CBCs and +4 stem cells have been identified in the mouse small intestine, the first being the leucine-rich-repeat-containing G-protein-coupled receptor 5 (Lgr5) [12]. However, the specificity of these markers appears to be complex, as studies showed that CBCs can express proposed markers of +4 and vice versa [13]. Stem cells in the colon have been significantly less well characterized than in the small intestine. Proposed CESC markers include Lgr5 [12], Musashi-1 (Msi-1) [14], aldehyde dehydrogenase 1 (Aldh) [15], leucine-rich repeats and immunoglobulin-like domains 1 (Lrig1) [16], and high levels of sex determining region Y-box 9 (Sox9) [17].

Surrounding the colonic crypts in the lamina propria are pericryptal myofibroblasts (Figure 1.1B), which are considered to be a key component of the stem cell niche [18]. These mesenchymal myofibroblasts are thought to contribute to differentiation by secreting bone morphogenetic protein (Bmp) signals that are induced by Indian hedgehog (Ihh) signals from colonocytes (Figure 1.1B) [19]. Myofibroblasts are also thought to provide CESCs with Wnt signals that are important for proliferation but this is based on the fact that mouse colon crypts need exogenous Wnt to grow in culture [20,21]. Notch ligands produced by epithelial cells at the crypt base are also involved in maintaining the balance between proliferation and differentiation in the colon (Figure 1.1B) [22,23]. This has been directly shown by a recent mouse study which indicated that a subpopulation of crypt goblet cells marked by cKit is regulated by Notch and secretes epidermal growth factor (EGF) to support Lgr5+ stem cells [22].

Colorectal cancer (CRC)

Colorectal cancer (CRC) is the third most common cancer in men and the second in women worldwide [24]. In the United States, CRC is the second leading cause of cancer death despite the decline in incidence and mortality over the last 30 years [16,17]. Risk factors include being a male, family history, inflammatory bowel disease, diabetes, obesity, and physical inactivity [17]. Smokers and individuals who have one or more alcoholic drinks per day are at increased risk for CRC [25,26]. A number of epidemiological studies have linked consumption of red and processed meats to CRC risk, but these associations remain weak and unclear [27] . CRC risk in the context of obesity and diabetes will be reviewed in more detail in later sections. The majority of CRCs are sporadic, but some can result from inherited germline mutations. The most common of these is a mutation in the adenomatous polyposis coli (*APC*) gene, which encodes a tumor suppressor protein that normally inhibits Wnt/β-catenin signaling [28]. Patients with germline *APC* mutation develop familial adenomatous polyposis (FAP), a pre-cancerous disease that usually progresses to CRC. Other genes and pathways linked to CRC are reviewed below.

Models of CRC initiation and progression

Aberrant crypt foci (ACF) are thought to be the earliest precursor of CRC, usually resulting from a mutation that causes inactivation *APC* [29]. Mutated *APC* leads to genetic instability which favors the occurrence of mutations in other genes such as the oncogene *KRAS* and the tumor suppressor complex *SMAD2/4* [30,31], causing the formation of an adenoma. RAS protein is downstream of the growth-promoting epidermal growth factor receptor (EGFR), and monoclonal antibodies against EGFR are currently being used in the clinic to treat advanced CRC [32]. However, this therapy was shown to be ineffective in patients whose tumors carry mutated *KRAS* [33]. Progression from adenoma to malignant adenocarcinoma is associated with loss-of-function mutations in other genes including the tumor suppressor p53 [34]. This model of genetic alterations during the progression from normal colonic mucosa to adenocarcinoma is illustrated in Figure 1.2 and was originally proposed by Fearon and Vogelstein in 1990 [35]. However, it is now known that CRCs are much more heterogeneous and significant efforts are being made to define CRC subtypes based on the involvement of multiple pathways that lead to genomic instability [36-40]. These include microsatellite instability (MSI), epigenetic modifications in genes involved in mismatch repair (MMR), CpG island methylator phenotype (CIMP), and mutations in the oncogenes *KRAS* and *BRAF* [38-40]. These studies showed that MMR-proficient (unaltered MMR genes) tumors with mutations in *KRAS* or *BRAF* genes are associated with poorer survival outcome than MMR-proficient tumors without *KRAS* and *BRAF* mutations [40]. In all these mechanisms of colorectal carcinogenesis, the common denominator is the accumulation of DNA damage that leads to mutations and allows the cell to acquire cancerous potential.

Cancer stem cells

Tumors are heterogeneous lesions containing cells of different phenotypes and genotypes. It was originally thought that any cell within a tumor is able to initiate and sustain growth of new tumors. The cancer stem cell (CSC) theory, on the other hand, proposes that only a small subset of cells within a tumor has the proliferative capability of tumor formation and propagation [41]. Evidence for the existence of CSCs

was initially documented in leukemia [42,43]. Presence of CSCs was later shown in solid tumors of the breast and the brain [44,45]. These studies involved transplantation of human tumor cells in immunodeficient mice and the observation that only a small fraction of these cells was able to grow tumors or yield tumors when surgically transplanted [44,45]. The growth characteristics of these cells resembled that of stem cells. CD133 has been shown to mark CSCs in the brain given that CD133-positive cancer cells had the ability to originate tumors, while CD133-negative cells did not [45]. In 2007, two studies provided evidence that CD133+ was a putative marker of cancer stem cells in the colon [46,47]. In the same year, another study reported that a subpopulation of cells from primary CRC tissue which expressed high levels of epithelial cell adhesion molecule (EpCAM) and was positive for CD44 was able to initiate tumors in immunodeficient mice [48].

More recently, the development of stem cell reporter models have permitted further identification of tumor-initiating stem cells in the intestine. Clevers' group used an Lgr5-EGFP stem cell reporter mouse crossed with a conditional *Apc* knockout (KO) mouse to provide evidence that Lgr5-positive stem cells represent a tumor-initiating population [49]. This study revealed that a subset of adenoma cells expressing Lgr5 was able to form adenomas and give rise to multiple cell types as well as additional Lgr5-positive cells [49]. In line with this evidence, other reports showed that human colon carcinomas were enriched for a stem-like cell population that expressed Lgr5 [50,51]. Furthermore, a recent study by Powell et al. demonstrated that loss of one *Apc* allele in colonic progenitors expressing the stem cell marker Lrig1 led to formation of distal adenomas in mice [52].

Additional evidence for the involvement of the stem cell niche in colorectal tumorigenesis is supported by studies linking regulators of crypt cell proliferation and differentiation such as Wnt, Notch, and BMP to CRC [21,23]. Constitutive Wnt activation leads to accumulation of nuclear β-catenin, which activates transcription of mediators of cell proliferation such as cMyc and CyclinD1 [53,54]. Notch is highly expressed in human CRC cell lines and in mouse colon tumors and has been linked to metastasis, transepithelial migration, and tumor neovascularization [23]. Interestingly, two recent studies showed that

BMP signaling, which normally inhibits proliferation to promote differentiation, acquires pro-tumorigenic and pro-invasive roles when SMAD4 expression is lost in CRC cells [55,56].

Nevertheless, the cancer stem cell theory has been a topic of controversy due to the high degree of heterogeneity among patients and variation in laboratory assays, which hindered validation of cancer stem cell markers [57,58]. In fact, a recent study showed that activation of intestinal NFκB, a transcription factor involved in cell survival and inflammation, causes differentiated cells to acquire a stem-like phenotype and tumorigenic properties [59]. This "dedifferentiation" resulted from enhanced Wnt/β-catenin signaling induced by NFκB [59]. This evidence does not disprove the CSC model but rather supports the concept of "bidirectional interconversion" between stem cells and non-stem cells that can initiate tumors [57,59].

Inflammation and CRC

An important contributor to colorectal carcinogenesis is the presence of chronic inflammation. This has been established by numerous studies showing increased CRC risk in patients with inflammatory bowel disease (IBD) and accelerated tumor development in mouse models of CRC where mucosal inflammation is induced [60].

During chronic inflammation, constant production of reactive oxygen species (ROS) can be mutagenic and lead to DNA damage favoring carcinogenesis [61]. In humans, elevated levels of pro-inflammatory interleukin-12 (IL-12) in normal rectal mucosa were associated with presence of colorectal adenomas [62]. Cytokines or inflammatory mediators implicated in growth of colorectal tumors, metastasis, and poor prognosis are signal transducer and activator of transcription 3 (STAT3), cyclooxygenase-2 (COX-2), tumor necrosis factor-α (TNF-α), NFκB, and the downstream interleukin-6 (IL-6) [59,63,64].

Obesity and high fat diet (HFD) have been strongly linked to inflammation. It is well established that adipocytes are a main source of pro-inflammatory cytokines such as TNF-α and IL-6 during obesity [65]. However, there is increasing evidence that obesity-associated inflammation occurs in the intestine of mice and humans [66]. For example, mice fed a HFD showed increased expression of TNF-α mRNA and

NFκB activation relative to mice fed a low fat diet [67]. Particularly, TNF-α mRNA levels strongly and positively correlated with body weight gain, fat mass, and plasma glucose. Elevated intestinal TNF-α preceded weight gain and adverse metabolic consequences of obesity such as elevated plasma insulin [67]. Similar results were obtained by a more recent study, where diet-induced obesity led to increased mRNA expression of TNF-α and interleukin-18 (IL-18) in the mouse colon [68].

Current views support a role for the microbiota in inducing intestinal inflammation during obesity. Obesity induces changes in the gut microbial composition and promotes activation of the pro-inflammatory toll-like receptor 4 (TLR4) [69,70]. In germ-free mice, HFD feeding does not induce obesity and does not increase intestinal TNF-α [67,71]. Colonization of germ-free NFκB-EGFP reporter mice with fecal slurries from HFD-fed mice activated the reporter, demonstrating that fecal contents, which include microbiota, were sufficient to induce inflammation [67]. Furthermore, a recent study using mice with mutated *K-ras* showed that fecal transfer from HFD-fed donors with small intestinal tumors to healthy recipients fed a standard diet was sufficient to induce tumors in their small intestine [72]. This effect was blocked by antibiotics, indicating that gut microbes play a key role in promoting obesity-associated cancer [72].

Based on the links between obesity, inflammation, and colorectal tumorigenesis, a topic of interest to this dissertation is how signaling pathways that are altered during obesity play a role in CRC risk in the context of obesity-associated inflammation.

Mouse models of CRC

Both genetic and chemically-induced models of colorectal carcinogenesis are typically used in rodents. The **Apc$^{Min/+}$** mouse model carries a heterozygous mutation on the *Apc* gene and relates to FAP in humans. This genetic mouse model was discovered in 1990 by forward genetics, where ethyl-nitrosoure-induced mutagenesis led to numerous intestinal adenomas, and this mutation was named multiple intestinal neoplasia (MIN) [73]. Two years later, it was found that the MIN phenotype was caused by a nonsense mutation in one allele of the *Apc* gene, which resulted in a truncated protein [74]. The

$Apc^{Min/+}$ mouse represents a good animal model to study adenomas, since somatic mutations on the *APC* gene usually occur in human colorectal adenomas and CRC. However, a limitation of this mouse model is that it develops many adenomas in the small intestine and relatively few adenomas in the colon. This contrasts with human FAP, where polyposis occurs in the colon. There are also chemically-induced models of CRC (Figure 1.3). Administration of **azoxymethane (AOM)** in rodents offers one better system to study non-hereditary, sporadic CRC [75]. AOM is a chemical agent which, when given to animals via 4-6 weekly intraperitoneal injections, can induce colon tumors (Figure 1.3 A). AOM travels through the bloodstream to the liver, where it gets hydroxylated and secreted into the bile for delivery in the intestine, where it gets further metabolized by the microbiota [75]. The activated metabolite causes base mismatches in DNA which promotes formation of colorectal tumors, particularly on the mid to distal part of the colon [75]. Unlike $Apc^{Min/+}$ mice, AOM-treated animals develop tumors specifically in the colon and rectum, providing an advantage in terms of similarity to human disease. However, AOM-induced tumor formation can take as long as 5 months and susceptibility to AOM doses and number of injections required differ across different mouse strains. In our hands, the AOM model as applied to mice on the C57BL/6 background has proved problematic. Mice either do not develop tumors or develop very few tumors, making the model difficult to use if attempting to define interventions or genetic modifications that reduce tumorigenesis. Doses and numbers of AOM injections that yield tumors in C57BL/6 mice have resulted in liver toxicity and often unacceptable death rates. This has been particularly true in animals fed HFD, which was tested due to our interest in evaluating the role of obesity in colon tumorigenesis. Combined treatment with AOM and dextran sodium sulfate (DSS), a polysaccharide known to induce mucosal damage and inflammation in the colon, was shown to dramatically accelerate tumor development so that colon tumors are reliably observed at 2.5 months after AOM administration [75]. This model consists of a single AOM injection and 3 DSS treatments (5-7 days long), each alternated with a 2-week recovery period (Figure 1.3 B). Like in the AOM model, tumors are seen primarily in the mid to distal colon and rectum, and rarely in the proximal colon. The AOM-DSS model was initially developed to model tumorigenesis in chronic inflammation as occurs in patients with ulcerative colitis

(UC) [76]. However, because of its reliability, this model is widely used and is used in this dissertation to test the effects of loss of the insulin receptor (IR) on tumor development.

The insulin/IGF system

Ligands and receptors

The insulin/insulin-like growth factor (IGF) system comprises three ligands, insulin, IGF1, and IGF2, and two receptors, insulin receptor (IR) and IGF1 receptor (IGF1R) (Figure 1.4 A). IGF1R is expressed at high levels in most, if not all, tissues in the body, while IR expression is most predominant in skeletal muscle, liver, and adipose tissues in adults [77]. Traditional views consider IGF1R as a key mediator of the trophic and pro-tumorigenic actions of IGFs and IR as a mediator of the metabolic actions of insulin [78-81]. Although it will not be further discussed here, a receptor specific for IGF2 (the IGF2R or mannose-6 phosphate receptor) also exists and it is thought to serve as a "sink" to clear IGF2 and attenuate its signaling [82]. IR and IGF1R belong to the family of receptor tyrosine kinases, which are located at the cell membrane. These receptors consist of two extracellular or "α" subunits, which represent the ligand-binding domain, and two intracellular or "β" subunits, which have tyrosine kinase activity and auto-phosphorylate each other upon activation by ligand binding. In humans, the gene encoding IR (*INSR*) is located on chromosome 19 and the *IGF1R* gene in chromosome 15. Both genes derive from a common ancestor gene and their proteins share a high degree of structural homology: 64-67% in the extracellular subunit and 84% in the intracellular tyrosine kinase subunit [83]. As a result, insulin and IGF1 have the ability to bind both IR and IGF1R. IR has higher affinity for insulin than IGFs and IGF1R has higher affinity for IGFs than insulin. Therefore, at normal physiological concentrations, each ligand activates its "preferred" receptor [84]. However, elevated concentrations of insulin can bind the IGF1R and elevated levels of IGFs or "free" IGFs (not bound to IGF binding proteins) can activate IR.

During evolution of mammals, the IR gene acquired a 36-nucleotide exon and the ability to skip this exon by alternative pre-mRNA splicing [85,86]. In 1985, the human IR cDNA was cloned by two different research groups and they each predicted the size of the protein to be 1,382 and 1,370 amino

acids [87,88]. Four years later, it was found that this 12-amino acid difference corresponded to exon 11, which was present or absent depending on the tissue and the developmental stage [89]. These two IR isoforms resulting from alternative splicing were termed IR-A and IR-B (Figure 1.4 B). IR-A lacks exon 11, is highly expressed in the fetus and in cancer cells, and is an isoform that binds IGF2 as well as insulin with high affinity [90-92]. IR-B, which is only present in mammals, includes exon 11, binds primarily to insulin, and its highest expression has been reported in insulin target tissues such as liver, muscle, and adipose tissue [93-95]. IR-B has therefore been associated with a major role in mediating the metabolic actions of insulin, and more recent evidence, including evidence from our laboratory, has linked IR-B to differentiation of some tissues [86,96,97].

Hybrid receptors and ligand specificity

The high structural homology of IGF1R and IR can lead to the formation of hybrid receptors (HRs), which gives the insulin/IGF system an extra level of complexity [98]. These HRs form when one hemireceptor (an α and a β subunit) of IGF1R heterodimerizes with a hemireceptor of IR. Both IR-A and IR-B can heterodimerize with IGF1R [99], making five possible combinations of receptors (Figure 1.5A). Significant efforts have been made to investigate the ligand binding affinities of these receptors *in vitro* and the data are summarized in Figure 1.5A [99-103]. IGF1R and the hybrids HR-A and HR-B bind primarily to the IGFs but can bind to insulin at elevated concentrations. IR-A and IR-B have a similar binding affinity for their main ligand insulin, but IR-A binds much more strongly to the IGFs, especially IGF2, than does IR-B.

The function and signaling of HRs remain unclear, but their expression has been found to be elevated in cancer [77]. The formation of HR-A or HR-B in a particular tissue depends on the abundance of IR-A and IR-B. As a result, during fetal development and carcinogenesis where IR-A is highly expressed, HR-A formation may allow insulin to crosstalk with IGF1R signaling [77,99]. However, in normal differentiated cells where IR-B is more highly expressed, HR-B may attenuate IGF1 signaling through IGF1R to limit proliferative effects [77,99]. Furthermore, signaling through HRs is thought to be dictated by

the type of HR that predominates. *In vitro* studies using a variety of cell lines showed that in cells expressing predominantly HR-A, IGFs promoted cell proliferation and migration more potently than in cells expressing primarily HR-B [99]. Brierley et al. used a CRC cell line to demonstrate that knockdown of IR-A promoted formation and signaling through IGF1R homodimers, thus enhancing cell viability [104]. Therefore, heterodimerization of IR and IGF1R may provide a mechanism to attenuate IGF1R receptor.

Major downstream mediators

Insulin and IGF action can be mediated by receptor-induced activation of the insulin receptor substrates 1 and 2 (IRS-1 and IRS-2) or the Src homology 2 domain containing (Shc) proteins, which are immediately downstream of IGF1R and IR (Figure 1.5B). Tyrosine phosphorylation of IRS-1/2 can activate phosphoinositide 3-kinase (PI3K), which leads to phosphorylation of AKT and subsequent activation of molecules involved in glucose and lipid metabolism. Signaling through AKT can also favor cell differentiation [96,105] as well as cell growth and survival, for example, via inhibition of the pro-apoptotic BAD [106]. Shc proteins lead to activation of the RAS/MAPK pathway to promote proliferative and anti-apoptotic signals [103,107].

IRS-1 knockout mice are about 50% smaller than their wild-type (WT) littermates and become mildly insulin resistant as they age [108,109]. Loss of IRS-1 did not prevent insulin-induced phosphorylation of PI3K in liver and muscle and this residual insulin signaling was attributed to IRS-2 action [108,109]. IRS-2 knockout mice, on the other hand, are normal in size but develop peripheral insulin resistance and pancreatic β-cell dysfunction, consistent with a diabetic phenotype [110]. Studies on fibroblasts isolated from IRS-1 knockout mice showed that IRS-1 deletion significantly decreased IGF1-induced proliferation and PI3K signaling [111] . Transfection of IRS-2 into these cells rescued activation of PI3K but had minimal effects on proliferation [111]. In the mouse intestinal epithelium, IRS-1 was shown to be required for the anti-apoptotic actions of IGF1 and disruption of the IRS-1 gene dose-dependently increased apoptosis and reduced tumorigenesis [112,113]. On the other hand, IRS-2 was shown to be induced by caudal-related homeobox protein 2 (CDX2) to promote differentiation in normal and tumor cells derived from human

colon [114]. Together, these *in vivo* and *in vitro* data suggest that IRS-1 plays a larger role in mediating the mitogenic and anti-apoptotic effects of IGF1 while IRS-2 primarily mediates metabolic and differentiation signals. Overall, these studies lead to the concept that IR-B may signal preferentially through IRS-2 to mediate metabolic effects and differentiation, whereas IGF1R and IR-A may signal primarily via IRS-1 to promote cell proliferation, reduced apoptosis, and tumorigenesis (Figure 1.5 B).

Impact of the insulin/IGF system on growth

IGF1 is produced at highest levels in hepatocytes, but it is also expressed in several other tissues in the body, including the gut mesenchyme [115,116]. IGF1 plays an important role in mediating the trophic actions of growth hormone (GH) to promote growth and development of various organs and is clinically used to treat children with growth failure due to genetic defects in the GH receptor [117]. Mice deficient for IGF1R exhibit severe growth retardation and die shortly after birth [118]. IGF2 is expressed in the fetus to regulate proliferation and apoptosis during embryonic development, while in human adults it is expressed mainly in the liver and exerts anti-apoptotic and proliferative actions through IR-A [82,90]. In normal cells, the *IGF2* gene is maternally imprinted and therefore the paternal allele is only expressed, and loss of imprinting has been found in many tumors [82].

It is well established that IGF1 is a potent mediator of intestinal growth [119]. IGF1 is produced in the mesenchyme and acts in a paracrine manner to induce mucosal growth and adaptation to surgical resection of the bowel [116,120,121]. Local synthesis of IGF1 is stimulated by glucagon-like peptide 2 (GLP-2), a gastrointestinal hormone that acts on GLP2 receptor in intestinal mesenchymal cells to stimulate IGF1 secretion[121]. GLP-2 exerts enterotrophic effects exclusively via IGF1 and a GLP-2 analog has been recently approved for treatment of short bowel syndrome [122-125]. Mouse studies on the mechanisms of GLP-2 action to improve gut barrier function showed that signaling through IGF1R in intestinal epithelial cells was essential and this was associated with IGF1R-induced modulation of tight junction proteins [126].

IGF1 produced in the liver and released into the circulation constitutes another source of IGF1 that acts on the intestinal epithelium. In mice, circulating IGF1 increases intestinal mass and crypt cell

proliferation and survival during normal conditions, and infusion of IGF1 promotes growth of small intestinal epithelium during lack of luminal nutrients as occurs with total parenteral nutrition [127,128]. Furthermore, exogenous IGF1 has been recently shown to enhance epithelial regeneration by expanding ISC following high-dose radiation [129]. Studies performed in rats indicated that IGF1 treatment enhanced colonic mucosal growth and function and promoted healing after colonic anastomoses [130,131].

Insulin is secreted into the bloodstream by the β-cells of the pancreatic islets after ingestion of a meal to regulate carbohydrate, lipid, or protein metabolism via IR [132]. Mice deficient for IR are normal at birth but die of diabetic ketoacidosis 2-3 days after birth [80,133]. In addition to the traditional actions of IR on metabolism, a role for IR in growth and development emerged from work by Dr. Efstratiadis' group in 1993 [118,134]. In these studies, the researchers observed that growth retardation was more dramatic in double mutant mice lacking IGF1 and IGF2 ligands or IGF2 and IGF1R than the respective single knockouts. Furthermore, in mice with intact IGF2 and null mutations in both IGF1 and IGF1R, residual growth was observed. These studies suggested the existence of some unknown receptor capable of mediating IGF2 signaling [118,134]. In 1997, this unknown receptor was identified as IR and later found to be IR-A [90,135].

Some studies have suggested that insulin can induce growth of the intestinal epithelium. In rat models of short bowel syndrome and mucosal damage, oral insulin treatment led to increases in overall small intestinal mass [136,137]. These growth effects induced by insulin were associated with increased proliferation in the crypts and decreased apoptosis in the villi [138]. It is important to note that despite these few studies, the impact of insulin in intestine has been under-investigated relative to studies of IGF1. Given the similarities in IGF1R and IR structure and the ability of IGFs and insulin to activate both receptors, the studies in this dissertation took the approach of genetic deletion of IGF1R or IR in mouse intestinal epithelium to better define their roles.

IGF1R overexpression has been found in a number of tumors including colorectal adenocarcinomas and metastases [139-141]. Furthermore, increased IGF1R signaling in tumor cells has been linked to resistance to chemotherapy and radiation treatments [142,143]. IR-A is overexpressed relative to IR-

B in tumors of the breast, thyroid, ovary, prostate, and colon [90,107]. IR-A:IR-B mRNA ratio is increased in aggressive human colorectal cancer cell lines and in colon tumors of $Apc^{Min/+}$ mice [97]. This reflected primarily loss of IR-B rather than increased IR-A since total levels of IR mRNA were actually reduced in tumors relative to normal tissue [97]. The ability of IR-A to mediate IGF1 and IGF2 action has presented a difficulty in the efficacy of anti-cancer drugs designed to block IGF1R. IGF1R inhibitors have been tested as anti-cancer therapies but in some tumors IR was able to confer resistance and support survival or growth despite IGF1R inhibition [144-147]. This compensatory response of IR to IGF1R-targeted therapies led to development of dual IGF1R/IR inhibitors [148-151]. However, the concern with blocking IR is the potential for decreased insulin sensitivity and adverse metabolic consequences. Therefore, it is expected that therapies targeted to IGF1R and IR-A combined with insulin sensitizing agents would provide the most beneficial strategy [151]. Defining risk factors for development of pre-cancerous adenomas and improved screening and prevention represent desirable goals to reduce CRC. One chapter of this dissertation undertook an epidemiologic study to assess whether levels of expressed IGF1R, IR, or IR-A:IR-B mRNAs were associated with colorectal adenoma risk.

Role of IGF binding proteins in regulating IGF action

Normally, circulating IGFs are about 95% bound to IGF binding proteins (IGFBPs) [152]. When IGFs are present in the unbound form, they become "free" to bind their receptors and initiate signaling. IGFBPs therefore limit "bioavailable" IGF for binding to IGF1R or IR. There are six IGFBPs that bind IGFs with high affinity. **IGFBP-1** is synthesized by the liver and its production is known to be strongly suppressed by elevated plasma insulin. Human studies showed that IGFBP-1 is found at low levels in the plasma of obese, hyperinsulinemic individuals [153,154]. Suppressed IGFBP-1 correlates with increased free IGF1 in serum, which was shown to be 50-70% higher in obese than in non-obese subjects [154]. This increase in bioavailable IGF1 is associated with the adverse effects of obesity and hyperinsulinemia on cancer risk, including pre-cancerous colon adenomas [155]. **IGFBP-2** binds primarily to IGF2 and plays a role in regulating growth of a number of tissues during embryonic development [156,157]. **IGFBP-3** is the most

abundant in the circulation and is expressed in virtually all tissues in the body. Its major role is to modulate levels of free IGFs that signal through IGF1R to regulate cell proliferation, differentiation, and apoptosis [158,159]. Although numerous studies investigated the roles of IGFBP-3 in cancer and metabolism, results have been inconsistent and whether IGFBP-3 promotes or protects against tumorigenesis and metabolic disorders remains unclear [160,161]. In the intestine of rodents, IGFBP-3 mRNA is expressed mainly in the lamina propria and is decreased after small bowel resection, potentially facilitating the ability of IGF1 to promote mucosal growth [162,163]. In human colon, IGFBP-3 protein expression is decreased in adenomas and adenocarcinomas, and low IGFBP-3 mRNA levels in normal mucosa have been associated with increased risk of colorectal adenomas [164,165]. Consistent with this evidence, *in vitro* work has shown that IGFBP-3 is a transcriptional target of the tumor suppressor p53 and may promote apoptosis independent of IGF1 [166,167]. **IGFBP-4** is expressed in several tissues including the intestine. A recent report using IGFBP4-KO mice concluded that circulating IGFBP-4 inhibits basal intestinal growth but is required to promote the trophic actions of GLP-2 on the intestinal epithelium [168]. Another study using CRC cell lines overexpressing SOX9 and mice deficient for SOX9, an ISC marker [169,170], showed that IGFBP-4 mediates anti-proliferative actions of SOX9 on CRC cells and IEC [171]. **IGFBP-5** is also expressed in most tissues and, in the intestine, its expression is high in the muscularis layer and in mesenchymal cells of the lamina propria [163]. Unlike IGFBP-3, local IGFBP-5 expression is thought to potentiate the trophic actions of IGF1 on the small intestine [116,172]. In line with this concept, some evidence links IGFBP-5 to tumorigenesis in a number of cell types [173]. **IGFBP6** has a much higher binding affinity for IGF2 than IGF1 and may inhibit proliferative or anti-apoptotic actions of IGF2 through IGF1R [174,175]. A large body of data recently reviewed by Bach et al., 2013, indicate that IGFBP-6 is a potential inhibitor of cancer, as its expression is reduced by β-catenin and increased by p53 in tumors [175]. Additionally, studies have linked IGFBP-6 to decreased tumor growth and metastasis in a number of cancers, including colon cancer [175].

Obesity, hyperinsulinemia, and colorectal cancer

The World Health Organization (WHO) defines overweight and obesity as "abnormal or excessive fat accumulation that presents a risk to health". Obesity is currently at epidemic levels, affecting 13% of the world population and 35% of the US population [135,176]. Body mass index (BMI) is widely used to define obesity and is calculated using the formula (weight in kg)/(height in meters)^2. Normal or lean BMI ranges between 19.5 and 24.9 kg/m^2, overweight corresponds to a BMI between 25.0 and 29.9 kg/m^2, and obese individuals have a BMI equal to or greater than 30.0 kg/m^2. Waist-to-hip ratio (WHR) is another measure of obesity that takes into account abdominal fat. WHR equal to or above 91 and 99.5 cm in women and men, respectively, indicates abdominal or "central" obesity, which is one component of metabolic syndrome along with dyslipidemia, hypertension, and hyperglycemia [177].

Insulin is produced by the β-cells of the islets of Langerhans in the pancreas and released into the circulation in response to a rise in blood glucose, aminoacids, and secretion of intestinal hormones after ingestion of a meal [132]. Some of the effects of insulin include 1) glucose uptake in muscle and adipose, 2) decreased hepatic gluconeogenesis, 3) glycogen synthesis in muscle and liver, and 4) lipogenesis in liver and adipose tissue (Figure 1.6A) [132,178]. To induce glucose uptake, insulin binding to IR recruits glucose transporters (GLUTs) to the cell membrane to facilitate glucose transport into the cell [132]. Reduced blood glucose then signals to pancreatic β-cells to inhibit insulin production. **Insulin resistance** is a condition where peripheral tissues have a reduced ability to respond to circulating insulin at physiological levels and therefore glucose uptake is impaired (Figure 1.6B). The molecular basis of insulin resistance is not completely understood. Some proposed mechanisms include lipotoxicity, inflammation, hyperglycemia, mitochondrial dysfunction, and endoplasmic reticulum (ER) stress [179]. All these mechanisms, which are reviewed in detail in Boucher et al., 2014, lead to phosphorylation of IR, IRS-1/2, or AKT at Ser/Thr residues that inhibit their kinase activity and therefore impair insulin signaling [179]. As a result of insulin resistance, blood glucose levels increase (**hyperglycemia**) and the pancreas therefore secretes more insulin to maintain normal glycemic levels and glucose metabolism [180]. This compensatory response to insulin resistance leads to elevated plasma insulin, known as **hyperinsulinemia** (Figure 1.6B). Some

obese patients have hyperinsulinemia but whether this is a cause or a consequence of obesity remains unclear [153,181]. In mouse models of diet induced obesity, hyperinsulinemia typically develops after increases in fat mass, indicating a role for obesity or functional consequences of obesity in driving insulin resistance and hyperinsulinemia [67]. Insulin resistance and the inability to uptake glucose result in hepatic glycogen breakdown and conversion into glucose, which increases glucose output and further exacerbates hyperglycemia (Figure 1.6B) [132,178]. Lipolysis occurs in adipose tissue, which leads to lipid accumulation in muscle [178]. When increased insulin production is not sufficient to overcome insulin resistance and maintain normal glucose levels, fasting hyperglycemia and hyperinsulinemia occur, marking the onset of **type 2 diabetes**. At later stages in the progression of the disease, pancreatic β-cells become exhausted and dysfunctional, resulting in partial or complete insulin deficiency (Figure 1.6C) [132]. Therefore, obesity, hyperinsulinemia, and type 2 diabetes are strongly linked and can lead to long-term complications such as metabolic syndrome, cardiovascular disease, and cancer.

Evidence for the link between obesity and CRC

Obesity and type 2 diabetes have been widely associated with increased risk of multiple cancers, including CRC [182-185]. Hyperinsulinemia and insulin resistance have been linked to increased risk of colorectal adenomas and cancer [155,186-188]. Interestingly, those patients with elevated plasma insulin and adenomas had significanlty reduced apoptosis in their normal rectal mucosa, suggesting a potential mechanism by which insulin may promote formation of pre-cancerous lesions [155,186]. In colorectal cancer patients treated with chemotherapy and EGFR inhibitors, elevated blood glucose and high BMI predicted accelerated disease progression [189]. Additionally, rectal cancer patients with type 2 diabetes showed a lack of response to chemoradiotherapy [190]. Obesity has been associated with increased recurrence and mortality following CRC treatment, as obese and morbidly obese patients with colon cancer appear to have increased recurrence and poorer survival after chemoradiotherapy [191,192]. In contrast, some epidemiological studies suggested that weight loss decreases CRC risk [193,194].

In the last years, there has been growing evidence supporting the concept that cancer risk associatd with type 2 diabetes may be influenced by anti-diabetic treatments. Human studies have shown a positive relationship between insulin therpies and cancer [195,196]. Insulin analogs such as insulin glargine have also been associated with increased cancer risk, but results have been inconsistent [196-198]. In contrast, use of biguanides such as metformin has been suggested to decrease cancer incidence in a number of organs via increased activation of adenosine monophosphate -activated protein kinase (AMPK) [180,196,199,200]. AMPK is activated when energy levels in the cell are low and therefore stimulates catabolic pathways to produce energy [200,201]. Thus, metformin-induced AMPK activation leads to increased glucose uptake and glycolysis and decreased hepatic gluconeogenesis, which attenuate hyperglycemia and hyperinsulinemia [200,201]. Interestingly, AMPK activation is mediated by the tumor suppressor liver kinase B1 (LKB1), which is deficient in patients with Peutz-Jeghers syndrome, a hereditary polyposis disease that increases susceptibility to CRC [202]. The signaling pathways downstream of AMPK that are involved in the anti-tumor effects of metformin are reviewed in Perncova and Korbonits, 2014 [200]. These mechanistic studies were performed mainly in cell lines and mice with the limitation that the doses of metformin used were much higher than those clinically used in humans.

Together, elevated levels of plasma insulin associated with metabolic disease or anti-diabetic therapies represent a risk factor for colorectal carcinogenesis, poor CRC treatment efficacy, or increased moratlity after CRC treatment (Figure 1.7).

Mediators and mechanisms of colorectal cancer risk during obesity

There are many proposed mechanisms of increased intestinal cancer risk associated with obesity and are summarized in Figure 1.8. The insulin/IGF1 pathway is likely to play a role in carcinogenesis given that obese individuals tend to have elevated plasma insulin and free IGF1 as well as decreased IGFBP1, which allows more free IGF1 in the circulation [153,181]. Numerous human studies have found associations between IGF1 and IGF1R overexpression and CRC [139-141,203,204]. IGF1 promotes activation of the oncogenic RAS/MAPK pathway, and constant exposure of tissues to IGF1 can therefore enhance

proliferation and tumor growth. *In vitro* studies reported that inhibition of IRS-1 decreased proliferation of colorectal cancer cells [205]. Furthermore, stabilization of β-catenin and phosphorylation of IRS-1 were induced by IGF1treatment, and Wnt/β-catenin signaling is an important transcriptional regulator of the *IRS1* gene [206,207]. β-catenin stabilization results in nuclear translocation and activation of Wnt targets genes such as the stem cell marker *Lgr5* [208]. In fact, a human study reported that *LGR5* mRNA levels were increased in colorectal tumors relative to normal mucosa and this correlated with mRNA up-regulation of oncogenic *cMYC* [209]. Furthermore, patients with high tumor *LGR5* expression had decreased disease-free survival [209]. Together, these studies suggest that mediators downstream of IGF1R/IR promote tumorigenesis but whether IGF1R or IR mediates these effects in the colon has not been directly tested.

Hyperinsulinemia associated with obesity and diabetes is caused by hyperglycemia, as pancreatic β-cells attempt to lower blood glucose when glucose uptake is impaired. Hyperglycemia facilitates consumption of glucose by cancer cells, which obtain energy from glycolysis and lactate production in the cytosol rather than by oxidative phosphorylation in the mitochondria [210,211]. This metabolic switch in cancer cells is called the Warburg effect and is thought to provide cancer cells with metabolites that favor cell proliferation [211]. In line with this concept, hyperglycemia has been associated with increased cancer risk by positively influencing pathways that enhance proliferation, migration, and anti-apoptosis [212].

Adipokines are hormones that are produced mainly in adipose tissue and are also thought to contribute to the mechanisms of tumor growth during obesity [210]. Leptin is secreted by adipocytes during the fed state and acts on the hypothalamus to suppress appetite, but it has also been implicated in tumor cell growth in the mouse colon [213]. Proliferative effects of leptin on colon cancer cells appeared to be mediated by the signal transducer and activator of transcription 3 (STAT3) [213]. In humans, obesity is linked to leptin resistance at the level of the leptin receptor, but epidemiological studies linking serum leptin concentrations to cancer risk remain inconclusive [210]. On the other hand, adiponectin, which is another hormone released by adipose tissue, is found at low levels in the plasma of obese and diabetic patients [214]. In the mouse colon, adiponectin deficiency led to increased polyp number and colon cell proliferation only during diet-induced obesity, and administration of adiponectin inhibited colon tumor

growth in obese animals [215,216]. *In vitro* experiments in a mouse colon adenocarcinoma cell line showed that adiponectin exerts anti-proliferative actions via decreased STAT3 phosphorylation [217].

As mentioned earlier, chronic inflammation associated with obesity has been widely implicated in CRC development. Elevated plasma concentrations of the pro-inflammatory cytokine IL-6 have been found in patients with colorectal adenomas and cancer and were associated with high BMI and abdominal obesity [218,219]. Moreover, number and size of tumors were reduced in AOM-DSS treated mice lacking IL-6 [220]. In **Apc$^{Min/+}$** mice, HFD feeding led to increased expression of markers of inflammation such as IL-12, IL-6 and TNF-α in adipose and intestinal tumor tissue [221]. *In vitro* studies in CRC cells lines have shown that IL-6 and TNF-α act via STAT3 to promote expression of TNF receptor 2 (TNFR2) and proliferation [222]. In summary, a large body evidence exists to support the contributions of insulin/IGF1 signaling, hyperglycemia, adipokines, and inflammation to the mechanisms underlying increased CRC risk during obesity and insulin resistance (Figure 1.8).

Overview of apoptosis

Intrinsic apoptosis pathway

Apoptosis is a programmed cell death that the body uses to eliminate unwanted cells and is essential during tissue development, regeneration, and maintenance. In adult tissues, apoptosis generally occurs in order to remove cells with damaged and unrepaired DNA, which may otherwise accumulate mutations and acquire cancerous potential. Stimuli that trigger apoptosis include growth factor withdrawal and DNA damage caused by toxins, infection, or ionizing radiation [223]. The balance between apoptosis and survival is critical to tissue homeostasis, as excessive apoptosis can lead to degenerative diseases and insufficient apoptosis can lead to the development of cancer [223].

Depending upon whether the pro-apoptotic signals are intracellular or extracellular, apoptosis occurs via the *intrinsic* or *extrinsic* pathway, respectively, which are reviewed in detail by Ashkenazi, 2008 [223]. Both forms of apoptosis involve cysteinyl aspartate-specific proteases called **caspases**, which

are activated by proteolytic cleavage in a process known as the caspase cascade, leading to DNA fragmentation in the nucleus and execution of apoptosis [224].

The **intrinsic** pathway, also known as the *mitochondrial* pathway, is initiated by intracellular events such as DNA damage and is illustrated in Figure 1.10. Radiation, chemotherapy, UV light, and other types of cellular stresses cause double-stranded breaks (DSB) in DNA. DSBs are recognized by the kinase ataxia telangiectasia mutated (ATM) [225], which initiates the DNA damage response by activating p53 [226,227], a tumor suppressor that is critical to mediating death and survival signals. As a consequence, p53 translocates to the nucleus to initiate transcription of genes encoding pro-apoptotic proteins such as NOXA, PUMA, and PERP, as well as genes encoding mediators of cell cycle arrest such as p21 [228]. Pro-apoptotic targets of p53 inhibit anti-apoptotic BCL-2 and BCL-XL and activate pro-apoptotic BAX and BAK. BAX and BAK directly promote permeabilization of the mitochondrial membrane which causes the release of Cytochrome C and Smac/DIABLO from the mitochondrion into the cytosol [223]. Cytochrome C binds to apoptotic protease activating factor 1 (APAF-1) to recruit the initiator caspase-9 into the apoptosome complex. The apoptosome stimulates cleavage and activation of caspase-9, which in turn cleaves and activates effector caspases such as caspase-3. Smac/DIABLO further contributes to the caspase cascade by inactivating the inhibitor of apoptosis proteins (IAPs) [223]. Cleaved caspase-3 is a critical mediator of chromatin condensation, DNA fragmentation, and membrane blebbing, which result in the formation of apoptotic bodies that are engulfed by phagocytosis [229].

The **extrinsic** pathway is triggered by cytotoxic immune cells which release pro-apoptotic ligands that belong to the TNF superfamily such as TNF-related apoptosis-inducing ligand (TRAIL) and Fas ligand (FasL) [223]. TRAIL binds to death receptors 4 or 5 (DR4/5) and FasL signals through Fas receptor (FasR), which are located at the surface of the target cell. This promotes activation of initiator caspases 8 and 10 and subsequent activation of downstream effector caspases (Figure 1.9) [223]. The current studies have used cleaved caspase-3 as a major readout for radiation/genetic damage-induced apoptosis.

Apoptosis in the colonic crypts

Cell death in the colonic epithelium occurs at two main sites: 1) in the bottom half of the crypts by apoptosis in order to remove genetically damaged stem or progenitor cells that could initiate neoplastic lesions and 2) at the luminal surface where differentiated cells are detached and shed into the lumen by a process known as anoikis, which is related to apoptosis and part of normal intestinal epithelial renewal. Levels of spontaneous apoptosis in the intestinal crypts are low and much lower in the colon than in the small intestine [230]. The particularly low rates of apoptosis in the colon are attributed to decreased basal expression of p53 and increased BCL-2, and this may contribute to the higher incidence of tumors in the colon than in the small intestine [231,232]. Another difference between the two bowel regions is that while in the small intestine basal and induced apoptosis occurs primarily within the stem cell zone at the crypt base, in the colon apoptotic cells are present throughout the length of the crypt [230,233].

During homeostasis, the major mediator of apoptosis in colonic crypts is BCL-2, as *Bcl2* knockout mice exhibited increased levels of apoptosis relative to WT mice [232]. However, p53 and BAX appear to have little role in spontaneous apoptosis since mice deficient for these proteins showed similar apoptosis levels to those in WT animals in the basal state [234]. To better study the apoptotic response in the intestinal crypts, models of DNA damage induced by 1-6 Gy radiation have been widely used [113,233,235,236]. Studies in rodents showed that following radiation, there two large waves of apoptosis [237]. The first one occurs 3-6 hours after radiation and requires p53, as mice lacking p53 showed significantly reduced apoptosis at 3-4.5 hours post-radiation [231,238]. The second wave of apoptosis, also known as "mitotic catastrophe" [231], occurs 24 hours later and is thought to result from genetically damaged cells with unrepaired DNA that re-enter the cell cycle and attempt to undergo mitosis but die due to chromosomal aberrations. This later wave of apoptosis has been shown to be p53-independent [231]. BCL-2 was also reported to play an important role in regulating colon crypt cell apoptosis within the initial hours after DNA damage, whereas BAX was shown to have little impact on p53-dependent apoptosis [234]. Whether obesity, IGF1R, or IR affects apoptosis of genetically damaged colon epithelial cells has not been

explored and could provide mechanistic insight into early events that promote survival of aberrant cells that could initiate tumors.

Research hypotheses

Distinguishing the specific functions of the IGF1R *versus* the IR in situations of elevated insulin has been a long standing challenge due to the high degree of crosstalk between both receptors. Understanding the individual contributions of each receptor to CRC risk and initiation is critical to developing and improving strategies for CRC prevention, diagnosis, and treatment, especially in the current epidemic of obesity and insulin resistance. This work has used human biopsies and genetic mouse models to investigate the specific roles of IGF1R and IR in colon adenoma risk, tumorigenesis, and epithelial cell survival after DNA damage during hyperinsulinemia, obesity, or inflammation. The following hypotheses have been tested (Figure 1.10):

1. Increased *IGF1R* relative to *IR* mRNA or increased IR-A:IR-B ratio in normal mucosa predicts colorectal adenomas in humans, and this is associated with elevated plasma insulin.
2. IGF1R is the main mediator of colorectal tumorigenesis while IR exerts protective effects by attenuating IGF1R signaling. Therefore, IR loss favors formation of tumors.
3. Diet-induced obesity and hyperinsulinemia lead to decreased apoptosis of genetically damaged colon epithelial cells.
4. IGF1R is a critical mediator of the anti-apoptotic actions of obesity and hyperinsulinemia.

Figures

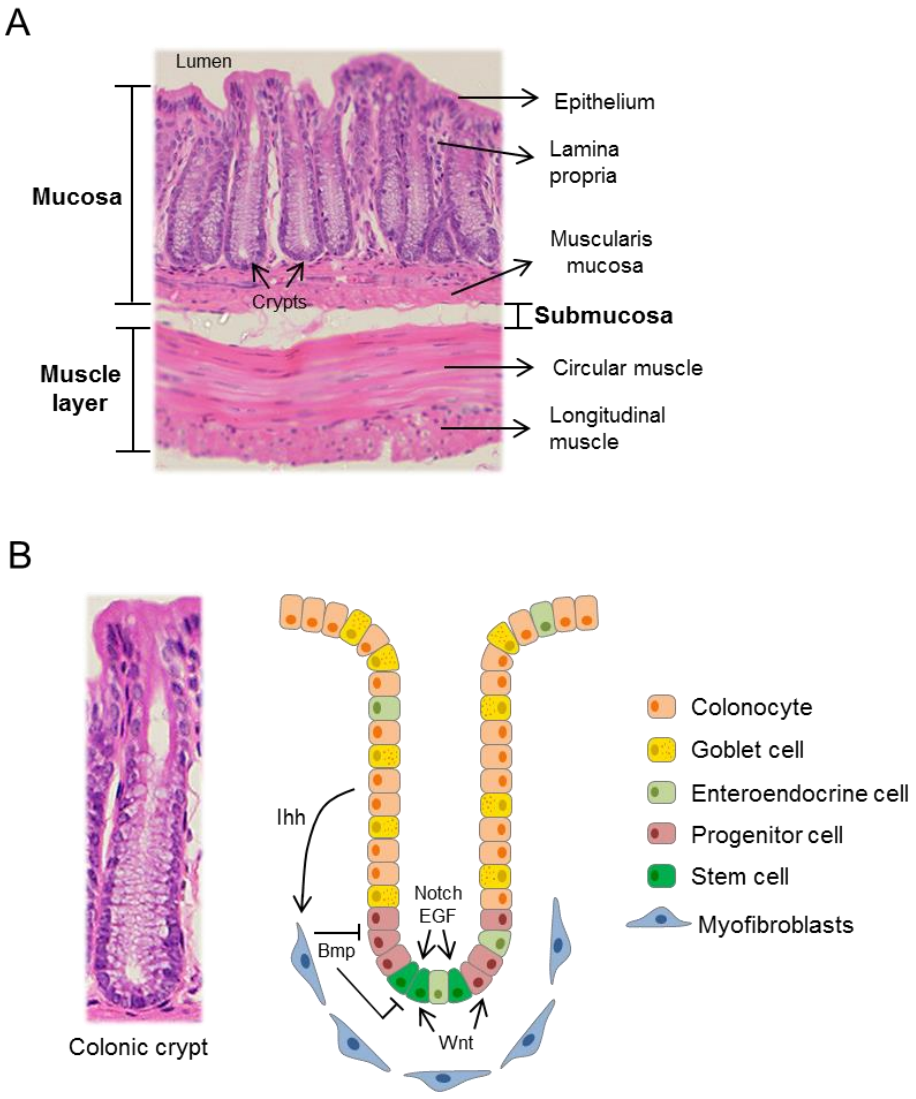

Figure 1.1: Tissue layers and crypt cell types in the mammalian large intestine.
(A) The colon epithelium consists of three main layers that can be visualized by histology. The **mucosa** consists of (i) a single layer of columnar epithelial cells that forms the crypts, (ii) stromal connective tissue surrounding the crypts (lamina propria) that contains immune cells important for defense, and (iii) an underlying layer of smooth muscle cells (muscularis mucosa). The **submucosa** is a thin layer of connective tissue containing small blood vessels. The **muscle layer**, or muscularis propria, is composed of circular and longitudinal smooth muscle tissues. Although not visible in this figure, two neural plexi exist: the submucosal plexus and the myenteric plexus between the two muscle layers of the muscularis propria. (B) Histological image (left) and schematic representation (right) of the colonic crypt. Stem cells residing at the base of the crypts give rise to progenitor cells which actively divide and migrate upwards as they differentiate into colonocytes, goblet cells, or enteroendocrine cells. Mesenchymal myofibroblasts surround the crypts and may provide Wnt ligands to regulate proliferation. Indian hedgehog (Ihh)

produced by differentiated cells stimulate myofibroblasts to secrete Bmp to attenuate proliferation and promote differentiation. Notch and EGF secreted by epithelial cells also help maintain stem cell function. (References: van Dop et al., 2009; Sato et al., 2011, Krausova et al., 2014; Rothenberg et al., 2012. Crypt diagram modified from Medema and Vermeulen, 2011).

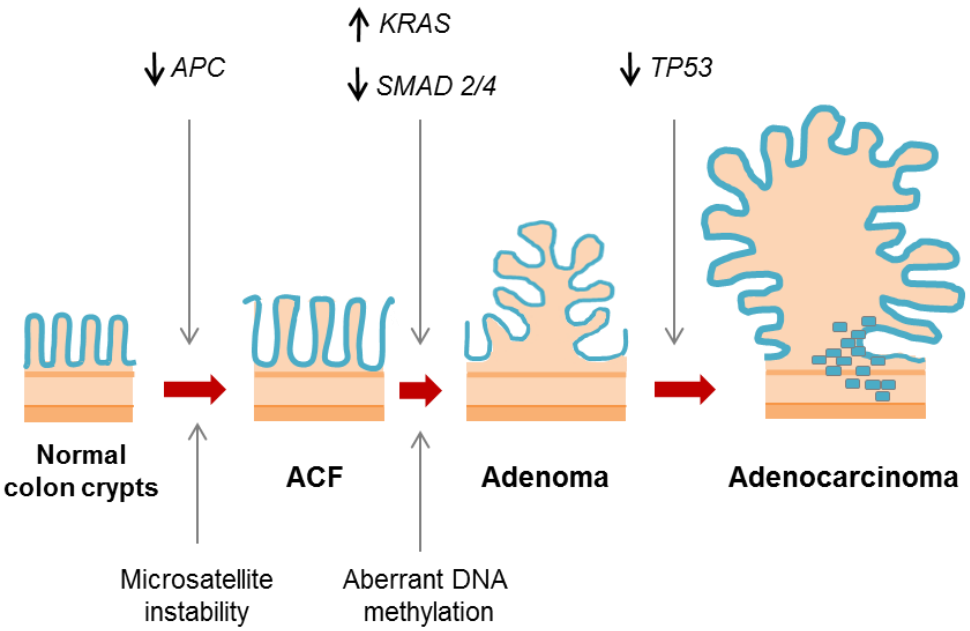

Figure 1.2: Progression from normal colon epithelium to adenocarcinoma.
A loss of function mutation in the *APC* gene and microsatellite instability can lead to the formation of aberrant crypt foci (ACF). Progression to adenoma can involve mutations that lead to overexpression of *KRAS* and loss of *SMAD2/4* as well as altered epigenetic modifications in DNA repair genes. Mutations on the gene encoding the tumor suppressor p53 cause progression to malignancy and development of invasive adenocarcinoma. (Diagram modified from Davies et al., 2005, and Markowitz and Bertagnolli, 2009).

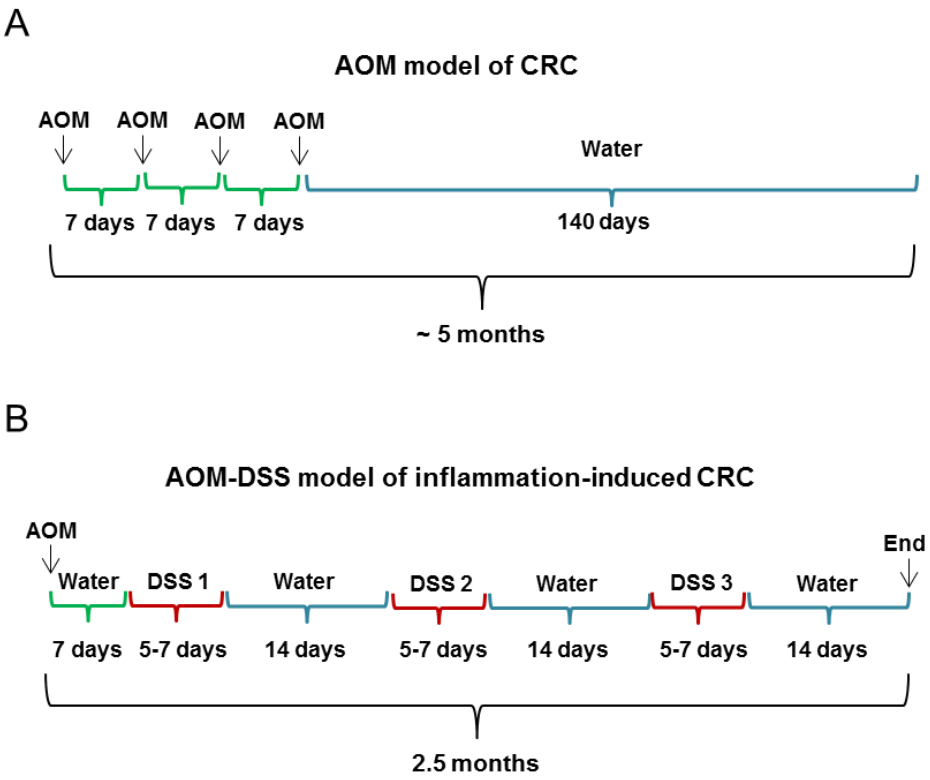

Figure 1.3: Chemically-induced models of colorectal cancer (CRC).
(A) The AOM model of CRC consists of 4-6 weekly injections with AOM with not further treatment. Animals tend to develop colorectal tumors approximately 20 weeks after the last AOM injection. (B) The AOM-DSS model of CRC involves a single intraperitoneal injection of AOM followed by three DSS treatments for 5-7 days. A recovery period consisting of water drinking occurs between each DSS cycle. This model allows numerous colorectal tumors to develop by 2-2.5 months as a result of chronic inflammation and mucosal damage induced by multiple exposures to DSS.

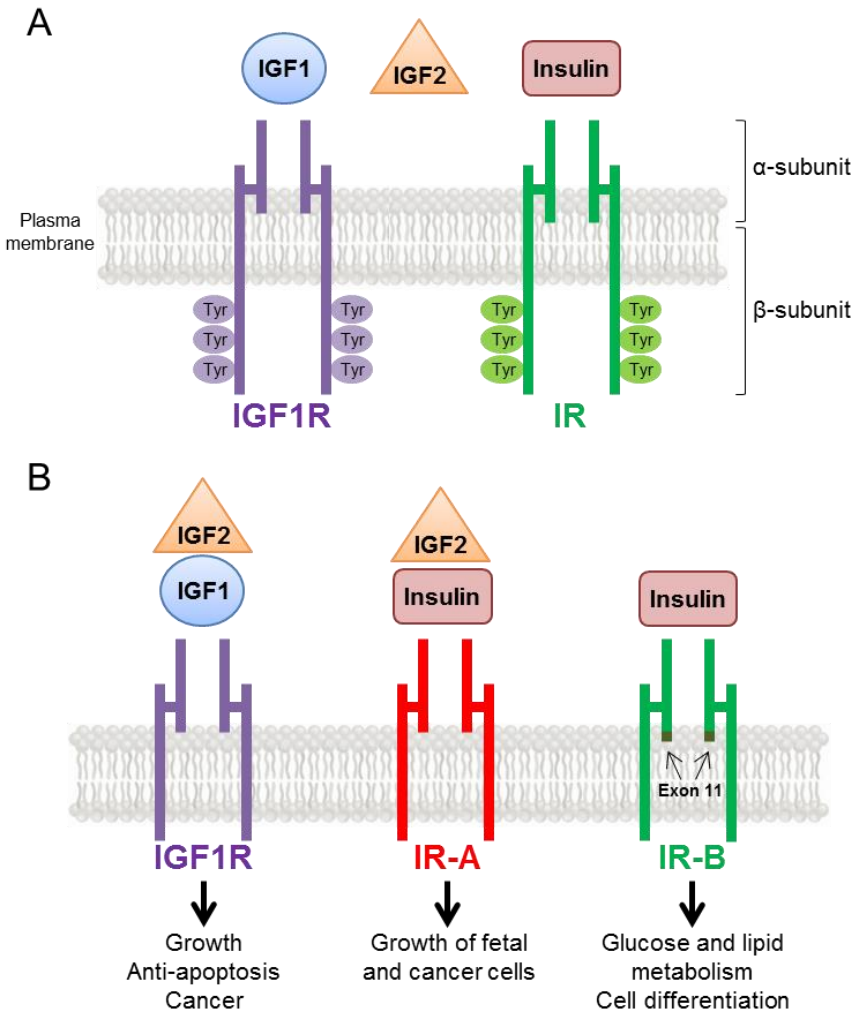

Figure 1.4: The insulin and IGF1 receptors.
(A) The insulin-like growth factor 1 receptor (IGF1R) and the insulin receptor (IR) belong to the receptor tyrosine kinase family. They are composed of two α-subunits (ligand binding domain) and two β-subunits (tyrosine kinase domain). Although they bind preferentially to their own ligand, both receptors can be activated by IGF1, IGF2, or insulin when present at high levels in the circulation. (B) Traditional views associate IGF1R with growth, anti-apoptotic, and tumorigenic actions of IGFs. IR is expressed as two isoforms resulting from alternative pre-mRNA splicing. IR-A lacks exon 11, binds strongly to insulin and IGF2, and is overexpressed in fetal and cancer cells. IR-B includes exon 11, binds primarily to insulin, mediates glucose and lipid metabolism in insulin-target tissues, and may play a role in cell differentiation.

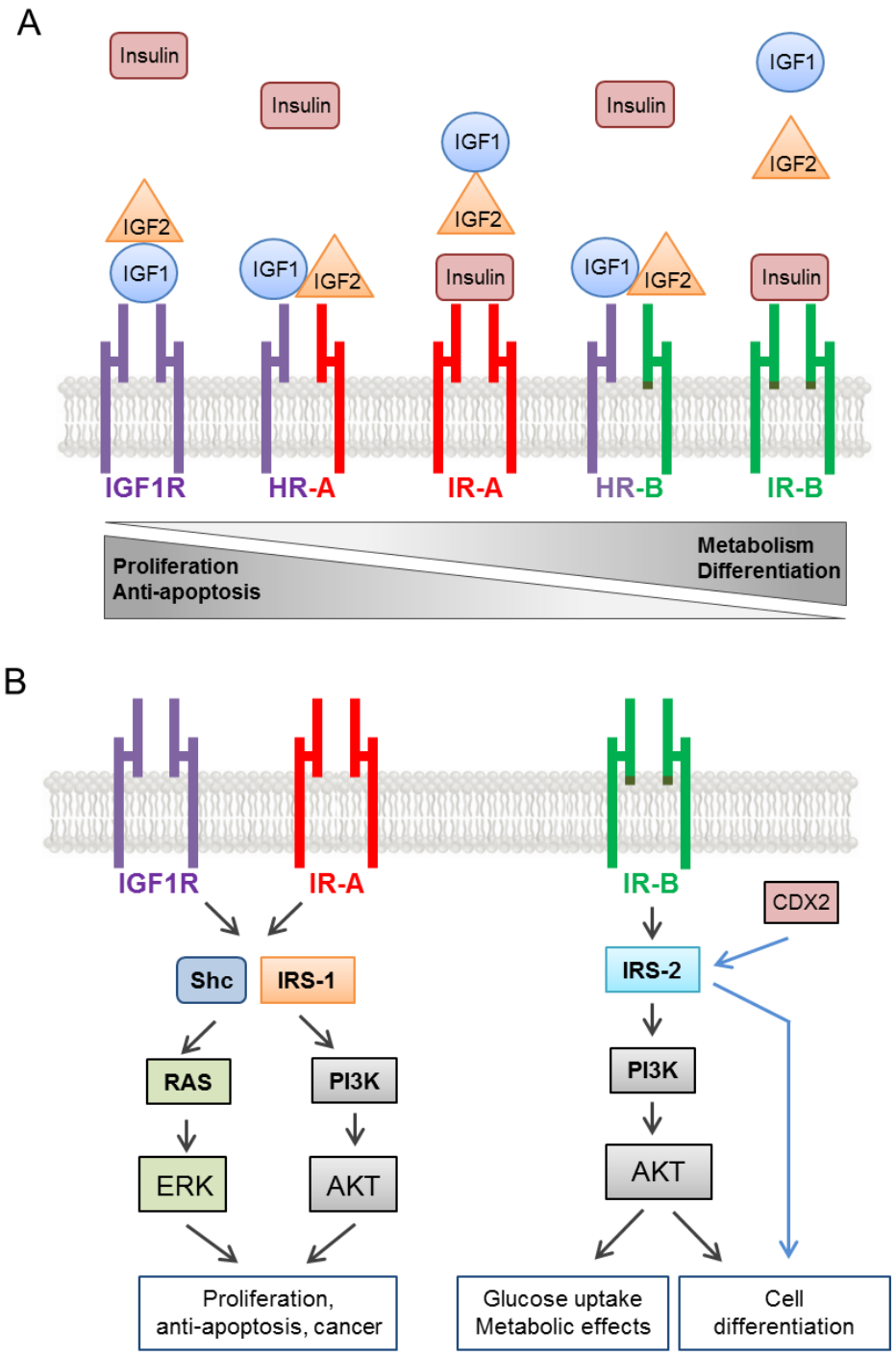

Figure 1.5: Ligand binding affinities and proposed downstream signaling pathways of IGF1R, IR-A, and IR-B.

(A) IGF1R and both hybrid IGF1R:IR-A (HR-A) and IGF1R:IR-B (HR-B) receptors bind to the IGFs with higher affinity than to insulin. Both IR isoforms bind preferentially to insulin, but IR-A has a high affinity for IGFs and IR-B does not. In the diagram, closer proximity of a ligand to a receptor indicates increased binding affinity. (B) Growing evidence suggests that IGF1R and IR-A share a common

signaling pathway in which Shc and IRS-1 proteins are phosphorylated following ligand binding. Shc activates the MAPK pathway via phosphorylation of RAS and ERK, while IRS-1 activates PI3K/AKT signaling, leading to cell growth, survival, and cancer. IR-B likely signals through IRS-2 to activate PI3K/AKT and mediate the metabolic effects of insulin in insulin-target tissues or promote cell differentiation. IRS-2 actions on differentiation are positively regulated by CDX2, as suggested by Modica et al., 2009. (Adapted and modified from Frasca et al., 2008, and Belfiore and Malaguarnera, 2011).

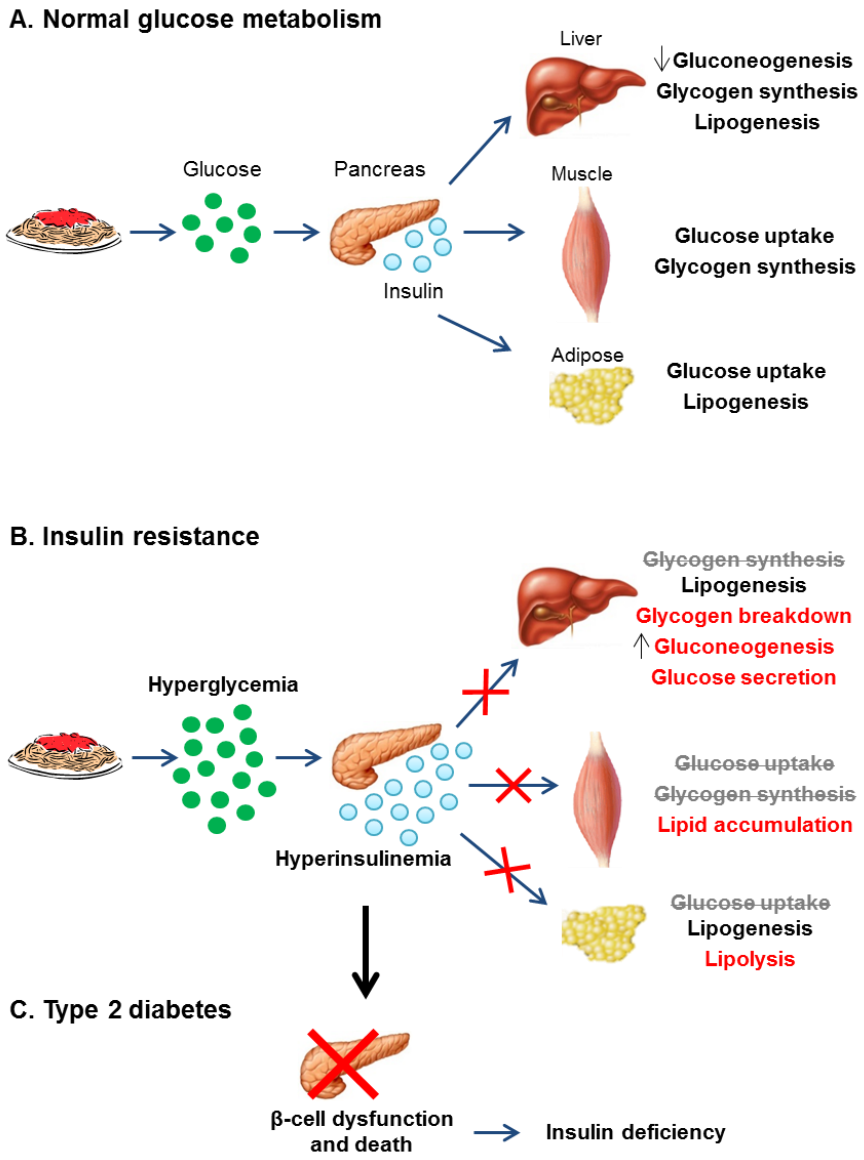

Figure 1.6: Glucose metabolism during healthy conditions and insulin resistance.
(A) After a carbohydrate-rich meal, glucose is sensed by the β-cells of the pancreatic islets which secrete insulin into the circulation to decrease hepatic gluconeogenesis and promote glucose uptake in skeletal muscle and adipose, glycogen synthesis in liver and muscle, and lipogenesis in liver and adipose tissue. As a result, blood glucose levels decline and the pancreas ceases to secrete insulin. (B) During insulin resistance, tissues are insensitive to circulating insulin. This causes hyperglycemia, which in turn causes the pancreas to secrete more insulin, leading to hyperinsulinemia. The inability of tissues to uptake glucose causes the liver to increase glycogen breakdown, gluconeogenesis and glucose secretion, which further increases blood glucose levels. Additionally, lipogenesis is increased in adipose tissue, leading to lipid accumulation in muscle. (C) When the pancreatic β-cells can no longer secrete enough insulin to maintain homeostatic glucose levels, they become dysfunctional and die, resulting in insulin deficiency and type 2 diabetes. (References: Samuel and Shulman 2012 and Lippincotts' Illustrated Reviews, Biochemistry. 3rd ed).

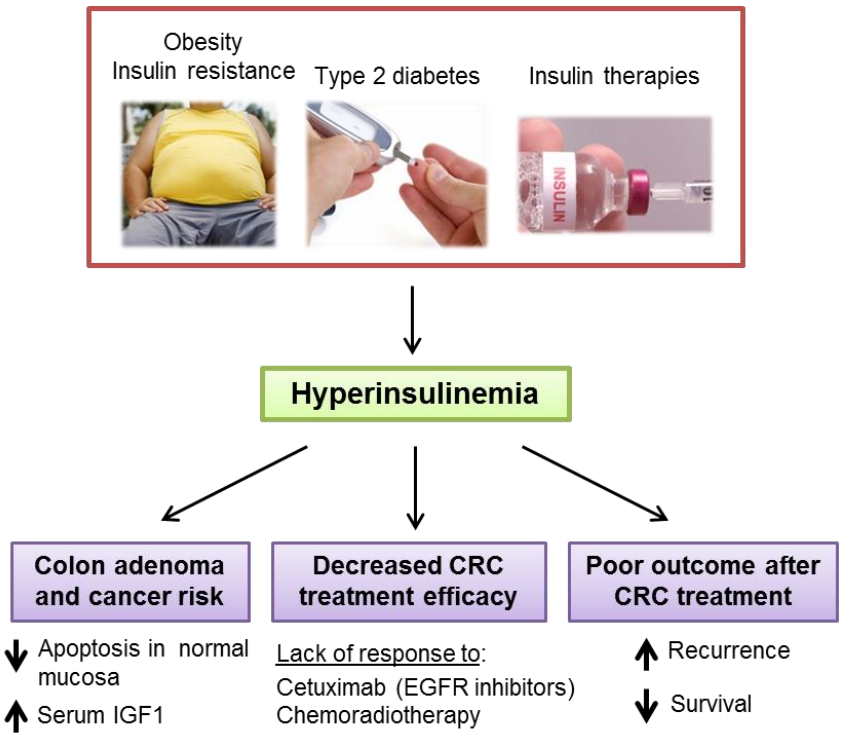

Figure 1.7: Adverse consequences of hyperinsulinemia on the colon.
Hyperinsulinemia caused by obesity, insulin resistance, type 2 diabetes, and/or insulin therapies can have implications in increased risk of colorectal adenomas or cancer, decreased response to treatments, and increased recurrence and mortality following treatment. (References: Keku et al., 2005; Vidal et al., 2012; Giovannucci, 2007; Tsai and Giovannucci, 2012; Pantano et al., 2013; Caudle et al., 2008; Dignam et al., 2006; Sinicrope et al., 2013).

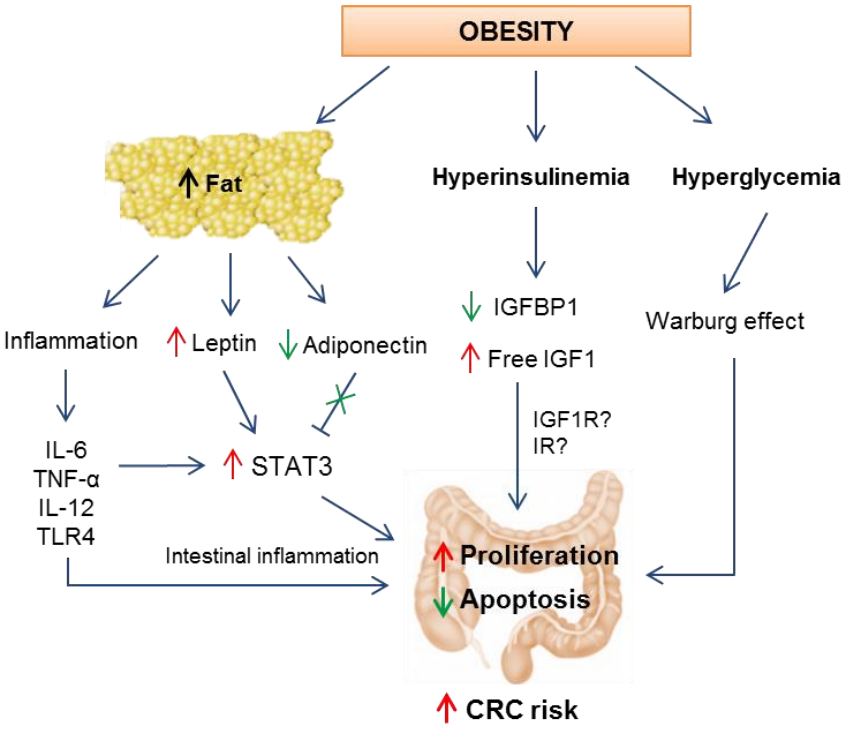

Figure 1.8: Suggested mechanisms of obesity-induced colorectal cancer risk.
Excess adiposity during obesity leads to enhanced production of inflammatory cytokines and leptin, which induce STAT3 activation to increase proliferation and reduce apoptosis in the colonic mucosa. Production of adiponectin, which normally inhibits STAT3, is reduced during obesity. Hyperinsulinemia decreases hepatic production of IGFBP1, allowing more free IGF1 in the circulation. IGF1, as well as insulin, can activate IGF1R or IR in colon epithelial cells to exert proliferative and anti-apoptotic actions. Finally, hyperglycemia associated with obesity or hyperinsulinemia promotes the switch from aerobic to anaerobic glycolysis, known as the "Warburg effect", which favors production of metabolites and nutrients that are utilized by tumor cells. (See section "*Mediators and mechanisms of colorectal cancer risk during obesity*" of this dissertation for references).

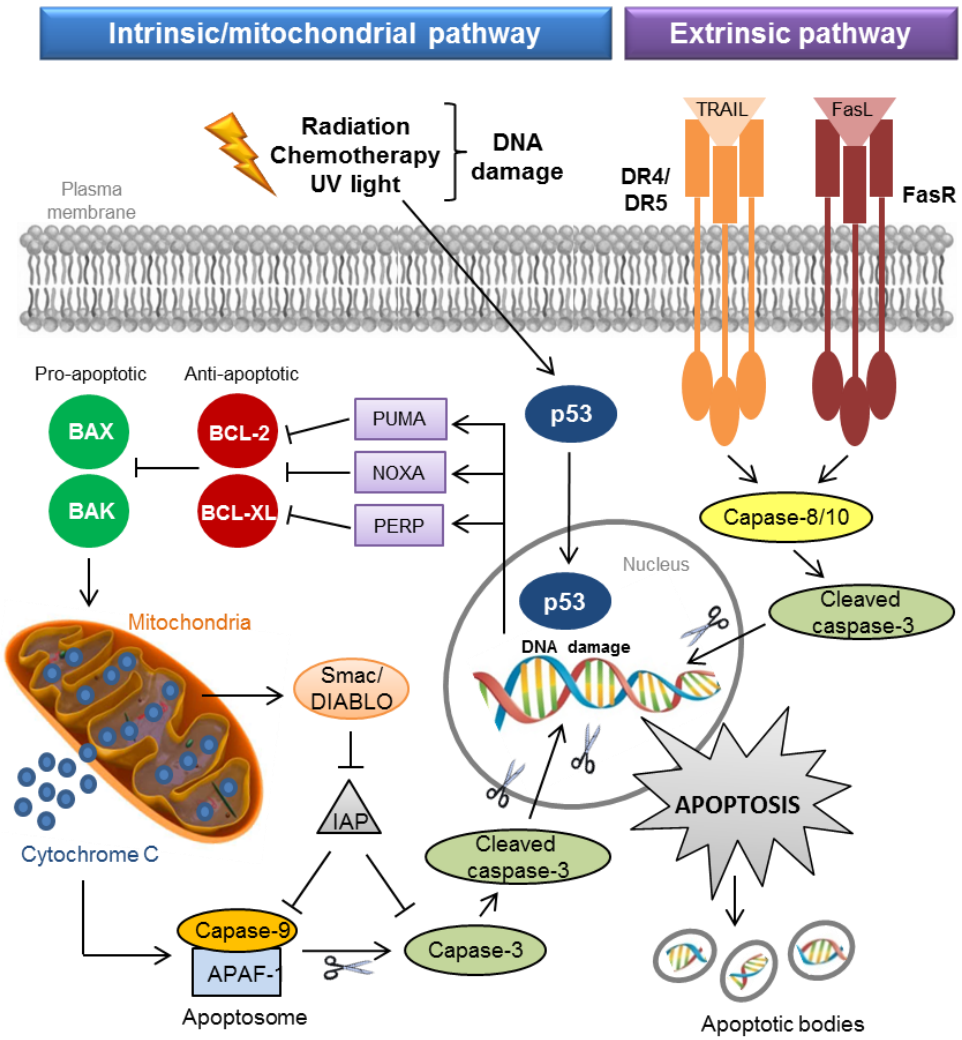

Figure 1.9: Apoptosis pathways.
Intrinsic pathway: DNA damage caused by cellular stressors such as radiation is sensed by p53, which translocates to the nucleus to induce transcription of apoptotic mediators PUMA, NOXA, and PERP. These mediators inhibit anti-apoptotic BCL-2 and BCL-XL, thereby allowing activation of pro-apoptotic BAX and BAK, which cause permeabilization of the mitochondrial membrane and efflux of Cytochrome C and Smac/DIABLO. Cytochrome C promotes formation of the apoptosome where the apoptotic protease activating factor 1 (APAF-1) cleaves and activates caspase-9, which in turn cleaves and activates caspase-3. Smac/DIABLO released by the mitochondrion negatively regulates the inhibitor of apoptosis protein (IAP) complex, further allowing caspase activation. *Extrinsic pathway*: Extracellular factors such as TNF-related apoptosis-inducing ligand (TRAIL) and Fas ligand (FasL) are secreted from cytotoxic immune cells. TRAIL binds to death receptor 4 or 5 (DR4/5) and FasL to Fas receptor (FasR) in order to activate caspases 8 and 10, which subsequently activate caspase-3. Cleaved caspase-3 moves to the nucleus to fragment DNA and execute apoptosis, which ultimately results in the formation of apoptotic bodies that are phagocytized by macrophages. (References: Attardi and DePinho, 2004, diagram modified from Ashkenazi, 2008).

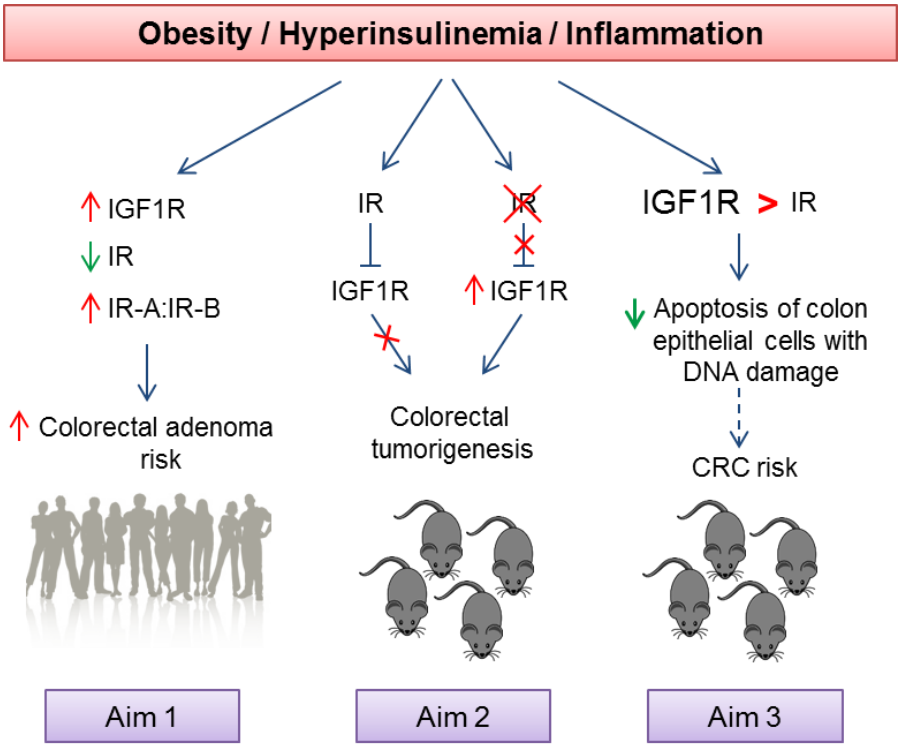

Figure 1.10: Research aims and hypotheses.
This dissertation tested the central hypothesis that IGF1R is an essential mediator of the adverse effects of obesity, hyperinsulinemia, and inflammation that favor risk of CRC. Aim 1 in Chapter 2 used normal rectal biopsies from human patients to examine whether increased *IGF1R* mRNA, decreased *IR* mRNA, or elevated IR-A:IR-B ratio predicted colorectal adenoma risk, especially during obesity or hyperinsulinemia. Aim 2 in Chapter 3 used a genetic mouse model of IR deletion in the intestinal epithelium to test the hypothesis that IR protects against inflammation-induced CRC and its loss leads to increased tumorigenesis by enhancing IGF1R action. Finally, Aim 3 in Chapter 4 evaluated the roles of IGF1R and IR in apoptosis of colon epithelial cells that underwent DNA damage, which is considered an early step in the initiation of neoplastic lesions. This final aim tested the hypothesis that obesity or hyperinsulinemia decreases apoptosis of genetically damaged colon epithelial cells and IGF1R is the main mediator of these anti-apoptotic effects in mice.

CHAPTER 2: REDUCED INSULIN-LIKE GROWTH FACTOR 1 RECEPTOR AND ALTERED INSULIN RECEPTOR ISOFORM mRNAs IN NORMAL MUCOSA PREDICT COLORECTAL ADENOMA RISK[1]

Introduction

Despite increased colonoscopy-based screening and improved treatment strategies [239], colorectal cancer (CRC) remains the second leading cause of cancer-related deaths in the United States [3]. Obesity, insulin resistance and type 2 diabetes are considered risk factors for CRC [187,240,241]. Previous work by our group has linked elevated plasma insulin and low apoptosis in normal rectal mucosa to increased adenoma risk [155,165,186]. Elevated plasma insulin (hyperinsulinemia) can increase levels of free insulin-like growth factor 1 (IGF1) in the circulation by inhibiting the production of IGF binding protein 1 (IGFBP1) [153,242]. In recent years, there has been increasing interest in targeting the insulin/IGF pathway for cancer treatment, as a large body of evidence links insulin/IGF1-mediated activation of insulin receptor (IR) or IGF1 receptor (IGF1R) to cancer of multiple organs [86,107,112,180,243,244]. Furthermore, a number of studies have shown that IGF1R confers resistance to radiation therapy and chemotherapy [245,246], and clinical evidence links IGF1R over-expression to colorectal tumor formation and progression [140,247]. Although IGF1R inhibitors showed a potential to reduce tumor growth [144,145], recent reports suggested that IR may permit tumors to resist IGF1R inhibition, which led to the development of dual IGF1R/IR inhibitors[146-149].

Considerable evidence has highlighted the potential significance of different IR isoforms in growth and cancer [90,92,107]. The *IR* gene yields two distinct IR isoforms due to alternate pre-mRNA splicing. IR-B is encoded by an mRNA that includes exon 11 and is the primary mediator of the metabolic actions of insulin [86,96]. IR-A is encoded by an mRNA that lacks exon 11, plays a role in fetal

[1] This chapter was published in the journal *Cancer Epidemiology, Biomarkers & Prevention*. The citation for this article is as follows: M.A. Santoro, S.F. Andres, J.A Galanko, R.S. Sandler, T.O. Keku, and P.K. Lund. Reduced Insulin-like Growth Factor I Receptor and Altered Insulin Receptor Isoform mRNAs in Normal Mucosa Predict Colorectal Adenoma Risk. *Cancer Epidemiol Biomarkers Prev*. 2014 Oct;23(10):2093-100.

growth and may mediate proliferative or anti-apoptotic actions of insulin or the IGFs [90,92]. Evidence that IR-A may be the predominant IR isoform in tumors or tumor cells [86,90-92,107], including colon tumors [97,104], has increased attention on this isoform as a possible mediator of cancer development or growth. However, the finding that IR-A knockdown increased viability of a colon cancer cell line via enhanced IGF1R activation indicates that when IGF1R signaling is intact, IR may limit IGF1R signaling [104]. Thus, the roles of IGF1R *vs.* IR in promoting colorectal tumorigenesis are not defined.

Little attention has been given to *IGF1R* and *IR* mRNA expression patterns during pre-neoplastic stages of malignancy, including whether *IGF1R* or *IR* levels in normal colorectal tissue differ between patients with adenomas and patients without adenomas. We hypothesized that elevated mRNA levels of *IGF1R vs. IR* or elevated IR-A:IR-B ratio in normal mucosa are associated with increased colorectal adenoma risk, elevated plasma insulin, and overweight/obese body mass index (BMI). To address this hypothesis, biopsies from normal rectal mucosa were obtained from adenoma or adenoma-free patients undergoing routine colonoscopy. Levels of mRNAs encoding *IGF1R*, *IR* and IR isoforms were quantified and the relationship between their expression, adenoma status, BMI and plasma insulin was evaluated.

Materials and methods

Participants

Participants were randomly selected from eligible subjects enrolled in the Diet and Health Study V (DHS V) who provided written informed consent and underwent routine colonoscopy at the University of North Carolina Hospitals, Chapel Hill, NC. The DHS V cohort has been described in previous studies [62,248-250]. For the present study, a subset of 100 cases and 98 controls were selected so that the two groups were matched based on age, gender and BMI. Patients were excluded from the study if they had cancer, colitis, 100 or more polyps (polyposis), prior resection of the colon, or history of colorectal adenomas. Colonoscopy was performed by certified gastroenterologists and all polyps were removed for pathological examination and were not available for research purposes. Adenomas were confirmed and defined according to standard pathological criteria. Subjects with one or more adenomas were classified as

"cases" and those without adenomas as "controls". The study was approved by the School of Medicine Institutional Review Board at the University of North Carolina, Chapel Hill.

Data collection

Methods for data collection were previously described [62,248-250]. Briefly, participants fasted overnight and body weight, height, and waist and hip circumference were measured at the time of colonoscopy. Within 3 months after colonoscopy, patients were interviewed by telephone to provide information about their lifestyle, diet and demographics. BMI between 18.5-24.9 kg/m^2 (lean) was defined as "normal" and BMI equal to or higher than 25 kg/m^2 (overweight/obese) was defined as "Ovt/Ob".

Biological specimens and laboratory assays

Prior to the endoscopic procedure, normal mucosal pinch biopsies were obtained 8-12 cm from the anal verge using standard disposable, fenestrated forceps. Sampling site was the same in all patients. Two biopsies were pooled for RNA extraction and immediately flash frozen in liquid nitrogen and later transferred to -80ºC. Another biopsy was fixed in 10% buffered formalin for histology and evaluation of apoptosis. Blood was collected via an intravenous catheter prior to administration of medication. Plasma was separated and insulin levels assayed for 95 controls and 79 cases by ELISA (Diagnostic Systems Laboratory, Webster, TX) as previously described [186]. Plasma insulin levels below or above the median were defined as "low" or "high", respectively.

Assays for apoptosis

Formalin fixed rectal biopsies were embedded in paraffin. Apoptosis was scored by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) using ApopTag Peroxidase In Situ Apoptosis Detection Kit (Millipore, Billerica, MA). This technology detects apoptotic cells by incorporating digoxigenin-conjugated nucleotides to the 3'OH termini of DNA fragments utilizing

terminal deoxynucleotidyl transferase (TdT). Briefly, samples were deparaffinized in 100%, 95% and 70% ethanol, digested in proteinase K and blocked in 2% hydrogen peroxide. TdT reaction was performed for 1.5 hours at 37ºC. Anti-digoxigenin conjugate was applied to the slides for 30 minutes, followed by a DAB (3, 3'-Diaminobenzidine) reaction for 1 minute. Samples were counterstained with hematoxylin and dehydrated with 95% and 100% ethanol and xylene. Slides were coverslipped using Eukitt mounting medium (Sigma-Aldrich, St. Louis, MO) and visualized with a bright-field microscope. Open crypts with good orientation were selected for scoring. The mean number of TdT-labeled apoptotic cells per crypt was calculated for each patient sample by investigators blinded to adenoma status. Due to the low number of samples available for apoptosis scoring (21 controls and 68 cases), it was only possible to compare apoptosis in cases *versus* controls without further stratification.

RNA Extraction, Reverse Transcription (RT), and PCR

RNA was extracted from biopsies using RNeasy Kit (Qiaen, Valencia, CA) and reverse transcribed with High Capacity cDNA Reverse Transcription Kit, including RNase inhibitor (Applied Biosystems, Carlsbad, CA) according to manufacturer's protocol. Quantitative real-time polymerase chain reaction (qRT-PCR) used the 7500 Real-Time PCR System (Applied Biosystems, Carlsbad, CA) to quantify *IGF1R* and *IR* mRNA levels. Hydroxymethylbilane synthase (HMBS), which we have found to be invariant across rectal biopsy mRNAs, was used as the housekeeping gene for normalization. The following TaqMan primer/probes (Applied Biosystems, Carlsbad, CA) were used: Hs00951562_m1 (*IGF1R*), Hs00961550_m1 (*IR*), and Hs00609297_m1 (*HMBS*). Pooled cDNA from colorectal cancer cell lines (Caco-2, SW480, Colo205) was run in all assays as a positive, internal control to account for inter-run variability. Samples were run in duplicate and water was run as a negative control. Reaction cycles consisted of initial denaturation at 95°C for 5 minutes, 45 cycles of 95°C denaturation for 15 seconds and 60°C annealing for 45 seconds. Data were analyzed using the Applied Biosystems 7500 software v2.0.1 and expression levels were calculated using the standard curve method. These values were then normalized to *HMBS* and to the internal control.

IR isoforms A and B were assessed by traditional, semi-quantitative PCR using 150 ng of cDNA template. Forward primer 5'-GAATGCTGCTCCTGTCCAAA -3' and reverse primer 5'-TCGTGGGCACGCTGGTCGAG -3' (Integrated DNA Technologies, Coralville, IA) were designed to flank exon 11, resulting in 250 bp (IR-B) and 214 bp (IR-A) amplified fragments. PCR protocol was modified from Brierley et al.[104] and consisted of initial denaturation at 92°C for 5 minutes followed by 40 cycles of 92°C denaturation for 30 seconds, 60°C annealing for 30 seconds and 72°C extension step for 30 seconds. Water and the internal control cDNA mentioned above were included in every assay. PCR products were run and visualized in a 2.5% agarose gel and band intensities were measured using Image J software (National Institutes of Health). Ratios of IR-A to IR-B were calculated for each patient sample and normalized to the internal control. All PCR assays were performed by an investigator blinded to case-control and BMI status, and samples were randomly organized by another investigator so that all the groups were represented in each assay run.

Statistical analysis

Means and standard errors were computed for continuous variables. Differences in continuous or categorical variables between adenoma cases and adenoma-free controls were compared by Student's t-test or Chi-square test, respectively. BMI was divided into "normal" and "Ovt/Ob" (overweight/obese) and plasma insulin levels into "low" (below the median) and "high" (above the median) subgroups as described above. For each receptor mRNA, the levels in controls were used to generate quartiles, and the lowest quartile was considered as reference. Logistic regression models were used to compute odds ratios (ORs) and 95% confidence intervals (CIs) to examine the association between mRNA quartiles (predictors) and adenoma status (response). We also calculated P-values for interactions between mRNA variables and BMI/insulin subgroups in a model testing for an association with case status. The relationship between plasma insulin and receptor mRNA levels was evaluated by Spearman's correlation coefficient. P-values less than 0.05 were considered statistically significant. All analyses were performed using SAS Version 9.3 (SAS Institute, Cary, NC).

Results

Patient samples in this study were selected so that cases and controls were matched on age, sex and BMI. Subject characteristics are summarized in Table 2.1. Race was not associated with control or case status, and no differences in WHR, reported calorie intake or reported physical activity were observed between cases and controls. Consistent with previous studies from our group [155,165], adenoma cases showed lower apoptosis ($P = 0.008$) and a trend towards increased plasma insulin ($P = 0.055$) relative to adenoma-free controls.

IGF1R and *IR* mRNA levels in normal rectal mucosa were quantified by qRT-PCR. IR-A and IR-B mRNAs were assessed by standard PCR, where amplification of both isoforms by identical primers allowed us to calculate the ratio of IR-A:IR-B amplicon in each patient sample. We first compared mean mRNA levels between controls and cases overall and after stratifying for BMI and plasma insulin (Table 2.2). Overall, cases had significantly lower *IGF1R* mRNA levels ($P = 0.0003$) than controls. This reduction in *IGF1R* mRNA was statistically significant in both normal ($P = 0.02$) and Ovt/Ob ($P = 0.01$) BMI subgroups and in subjects in the lower half of plasma insulin ($P = 0.007$). Since cases had slightly higher plasma insulin levels than controls, and elevated insulin can down-regulate IGF1R as a consequence of increased free IGF1 in the circulation [251,252], we asked if the lower *IGF1R* mRNA observed in cases could be associated with higher plasma insulin. Therefore, we examined *IGF1R* mRNA levels in controls *versus* cases after adjusting for plasma insulin. This analysis showed that even after controlling for insulin, cases still had lower *IGF1R* than controls ($P = 0.005$). Total *IR* mRNA levels did not differ between cases and controls in any subgroup categorized for BMI or plasma insulin. IR-A:IR-B ratio was 1.96 ± 0.04 in controls and 1.96 ± 0.03 in cases, demonstrating approximately 2 fold higher IR-A mRNA expression in human rectum compared with IR-B, but no significant difference in cases and controls as a whole or when stratified for BMI (Table 2.2). Interestingly, among patients with high plasma insulin, adenoma cases had small but significant increases in IR-A:IR-B ratios relative to controls ($P = 0.006$), which qualitatively reflected reduced IR-B mRNA (Figure 2.1).

To further evaluate the potential relationship between mRNA levels and colorectal adenoma risk, we studied the association between quartiles of *IGF1R*, *IR* and IR-A:IR-B mRNA expression and the odds of being a case, with the lowest quartile set as the reference (Table 2.3). Subjects in the highest two quartiles for *IGF1R* mRNA were significantly less likely to be cases. There were no significant associations between *IR* mRNA, IR-A:IR-B ratio and case status. To explore the association between receptor mRNA expression and adenoma risk in each subgroup, we used a logistic regression model to test for interactions between mRNA levels and BMI or plasma insulin status (Table 2.4). We found no interactions between BMI or plasma insulin and either *IGF1R* or *IR* mRNA levels. We did, however, observe a significant interaction between plasma insulin and IR-A:IR-B ratio ($P = 0.005$). With increasing IR-A:IR-B mRNA ratios, patients with high plasma insulin were more likely to have adenomas than were patients with low plasma insulin (Figure 2.2).

We next compared mRNA expression between subgroups in controls and cases separately. We found that in the control group, subjects with high plasma insulin had reduced mean *IGF1R*, *IR* and IR-A:IR-B mRNA levels ($P = 0.048$, $P = 0.02$, $P = 0.01$, respectively) relative to subjects with low plasma insulin. This association was not found in cases. Qualitative evaluation of the IR isoforms suggested that the reduced IR-A:IR-B ratio observed in controls with high insulin reflected higher IR-B (Figure 2.1). To further examine the possible effect of elevated insulin on gene expression, we calculated the correlation coefficients between plasma insulin and *IGF1R*, *IR* and IR-A:IR-B mRNA levels (Table 2.5). We indeed found significant negative correlations between plasma insulin and all three mRNA variables in controls, while in cases this relationship was significant only for *IGF1R* mRNA. In fact, in cases there was a non-significant trend for a positive correlation between IR-A:IR-B ratio and plasma insulin ($P = 0.06$).

Discussion

This case-control study provides novel evidence that, compared to adenoma-free controls, rectal biopsies from grossly normal mucosa of patients with adenomas are likely to have i) significantly lower levels of *IGF1R* mRNA, ii) unaltered *IR* mRNA, and iii) higher ratios of IR-A:IR-B isoforms in those

individuals with elevated plasma insulin. Consistent with our previous findings in three different patient groups [155,165,186], the presence of adenomas was associated with reduced apoptosis in normal appearing rectal mucosa and increased plasma insulin, although the latter was borderline significant in this smaller study population.

Identifying molecular biomarkers that predict early pre-cancerous lesions could significantly improve our understanding of factors that promote CRC risk, which could eventually contribute to better CRC prevention or screening. This study aimed to establish whether elevated mRNA expression of *IGF1R, IR* or relative expression of isoforms IR-A and IR-B in normal rectal mucosa predicts adenomas and whether this is influenced by BMI or plasma insulin levels. IGF1R signaling can be activated during elevated insulin and has been linked to reduced apoptosis and cancer progression in a number of organs, including the intestine [77,112,243]. Thus, we hypothesized that patients with adenomas would have up-regulated *IGF1R* mRNA expression in their normal rectal mucosa, particularly in those with high plasma insulin. Unexpectedly, we found that cases had significantly lower *IGF1R* mRNA levels than controls, and the odds of having colorectal adenomas diminished with increasing *IGF1R* mRNA expression. We considered whether elevated insulin could be linked to the reduced *IGF1R* mRNA in cases, since elevated insulin is known to down-regulate IGFBP1 resulting in higher levels of free circulating IGF1 that can down-regulate IGF1R [153,242,252]. However, the association between decreased *IGF1R* mRNA and presence of adenomas persisted even after adjusting for plasma insulin, suggesting that the reduced *IGF1R* mRNA observed in cases was not merely a result of elevated plasma insulin in this group. We next tested for interactions between mRNA levels and BMI or plasma insulin that may impact case status. We found a significant interaction between IR-A:IR-B ratio and plasma insulin, where increased IR-A:IR-B ratio was associated with increased colorectal adenoma risk in patients with high plasma insulin compared to those with low plasma insulin. This suggests that circulating insulin levels may play an important role in influencing tumor risk associated with high IR-A:IR-B expression, and that more attention should be given to the impact of hyperinsulinemia on relative tissue expression of these IR isoforms.

Insulin has long been known to down-regulate its own receptor by negative feedback to properly regulate glucose uptake in a number of tissues [253-257], and some evidence suggests that hyperinsulinemia and insulin resistance can impact isoform expression [258-261]. Insulin can also down-regulate IGF1R, potentially by increasing levels of free IGF1 in plasma [244,251,252]. Down-regulation of *IGF1R* transcript in situations of high insulin has been described in skeletal muscle of diabetic db/db mice, where reduced *Igf1r* mRNA relative to normoglycemic littermates was associated with increased *Igf1r* promoter methylation [262]. These numerous lines of evidence for negative feedback effects of elevated insulin are supported by the present study showing that in adenoma-free controls, levels of *IGF1R* and *IR* mRNAs, and IR-A:IR-B ratios each negatively and significantly correlated with plasma insulin. Qualitative analysis of IR isoforms suggested that reduced IR-A:IR-B ratio in controls with high plasma insulin appeared to be due primarily to increased IR-B. Patients with adenoma differed from controls in that only *IGF1R* mRNA levels significantly and negatively correlated with insulin, and for IR-A:IR-B mRNA ratios there was actually a trend for a positive correlation with insulin. This suggests a difference in the relationship between plasma insulin and *IR* mRNA levels or IR-A:IR-B mRNA ratios in cases *versus* controls that may be relevant to mechanisms underlying adenoma risk.

IR isoforms in humans have been studied primarily in breast and prostate cancers and it is well established that IR-A exerts proliferative actions and is overexpressed in tumor tissue [90,92,101,107]. However, little is known about the relative expression of IR isoforms in normal gastrointestinal organs including the colorectum. Our findings that mean levels of IR-A mRNA are about 2 fold higher than IR-B mRNA in the human rectal mucosa are relevant to normal and aberrant growth of colon epithelium. A predominance of IR-A might contribute to the relatively low levels of spontaneous colonocyte apoptosis[232] and increased susceptibility to insulin-mediated reductions in apoptosis. Our recent publication demonstrated a switch from predominance of IR-A in proliferative intestinal stem or progenitor cells to IR-B predominance in differentiated enterocytes [97]. Furthermore, IR-B expression was reduced in mouse pre-cancerous adenomas *versus* normal colon and was dramatically reduced in aggressive, poorly differentiated human CRC cell lines *versus* differentiated CRC cells [97]. Consistent with this finding, other studies have recently

shown that the relative mRNA levels of IR-A *versus* IR-B are elevated in both tumor and grossly normal adjacent tissue of human breast and prostate, compared to purely benign tissue [263,264]. However, whether IR isoform expression is altered in normal colorectum in the presence or absence of pre-malignant lesions had not to our knowledge been investigated previously. Our study suggests that among patients in the upper half of plasma insulin, those with adenomas had higher mean IR-A:IR-B ratio in their normal rectal mucosa compared to controls, which appeared to result from decreased IR-B and maintained IR-A as observed by qualitative examination. These data were supported by logistic regression analyses, which showed that increasing IR-A:IR-B ratios predicted adenomas in patients with elevated plasma insulin. A limitation of these findings is that they resulted from a subgroup comparison, in a relatively small number of patients. However, they do suggest that the relationship between plasma insulin and relative IR-A:IR-B expression in normal tissues should be further explored, as they may be relevant to improved understanding of the roles of hyperinsulinemia and impact of IR isoforms on colorectal tumorigenesis.

A limitation of this study is that alterations in receptor mRNA levels do not necessarily reflect changes in protein expression and phosphorylation, as increased activation of IGF1R and IR has been reported in cancer [265,266]. This is particularly difficult to address for IR-A due to the lack of available antibodies to permit immunohistochemistry or western immunoblot for this isoform. We chose to analyze RNA because sufficient RNA for evaluation of receptor levels is readily obtained from biopsies but we recognize the limitation with regard to predicting protein expression or activation. Another limitation of our study is the lack of access to actual adenomas as these are considered clinical specimens and were not available to us for research. Recent findings from our group using pre-clinical adenoma models provided evidence for increased IR-A:IR-B ratios in colon adenomas relative to normal colon mucosa in mice [97], but whether this is altered in humans and in the context of elevated plasma insulin needs further investigation. An additional limitation is that the differences in mean receptor mRNA expression across patient groups in this study are relatively small. Despite these limitations, the potential significance of our observations is highlighted by the growing interest in the role of the insulin/IGF pathway in cancer and IR/IGF1R inhibitors as potential therapies [145,148,149]. To date, IR and IR isoforms have been understudied

in the gastrointestinal tract, and our work suggests that further studies focusing on these receptors and relative IR-A and IR-B expression are needed to better understand their roles in initiation and pathophysiology of colorectal pre-cancerous lesions. Therefore, our previous and current work indicates that additional attention to the relative expression levels and biological roles of IR-A and IR-B is warranted.

Overall, this is to our knowledge the first study to show that the presence of colorectal adenomas is associated with decreased *IGF1R* mRNA and, during elevated plasma insulin, increased IR-A:IR-B mRNA ratio in normal rectal mucosa. Particularly, our data raise the important possibility that high IR-A:IR-B mRNA ratio may contribute to colorectal adenoma initiation during elevated plasma insulin. In addition, reduced *IGF1R* expression and increased relative expression of IR-A:IR-B in normal mucosa should be further investigated as potential predictive biomarkers of pre-malignant colorectal lesions.

Tables and Figures

Table 2.1: Descriptive characteristics of study participants.

Variable	Control	Case	*P**
Age (mean (se))	55.4 (0.7)	55.5 (0.7)	0.92
Race (n (%))			
White	76 (92)	79 (84)	0.17
Black	7 (8)	15 (16)	
Sex (n (%))			
Female	41 (48)	43 (45)	0.66
Male	44 (52)	53 (55)	
BMI (n (%))			
Normal	45 (48)	49 (49)	0.98
Overweight	29 (31)	29 (29)	
Obese	20 (21)	21 (21)	
Physical Activity in MET-minutes per week (mean (se))	2,981 (341)	2,485 (263)	0.25
Apoptosis (mean (se))	1.42 (0.12)	1.08 (0.06)	0.008†
Plasma insulin (mean μU/ml (se))	7.1 (0.8)	10.8 (1.7)	0.055
Calories (mean (se))	2,101 (88)	1,949 (79)	0.20
Waist / Hip ratio (mean (se))	0.908 (0.01)	0.915 (0.01)	0.54

BMI: body mass index
MET: metabolic equivalent of task
*Chi square for age, race and gender and student's t-test for remaining variables
†Significant at $P < 0.05$

Table 2.2: Mean expression of IGF1R, IR, and IR-A:IR-B mRNAs in controls *versus* cases overall and grouped by BMI and plasma insulin status.

Variable	Subgroup		n	Control mean (se)	n	Case mean (se)	*P**
IGF1R	Everyone		98	1.04 (0.03)	100	0.90 (0.02)	0.0003†
	BMI	Normal	45	1.06 (0.05)	49	0.91 (0.04)	0.02†
		Ovt/Ob	49	1.00 (0.04)	50	0.88 (0.03)	0.01†
	Insulin	Low	48	1.09 (0.05)	36	0.93 (0.03)	0.007†
		High	47	0.97 (0.04)	43	0.88 (0.03)	0.09
IR	Everyone		98	1.17 (0.03)	100	1.17 (0.06)	0.94
	BMI	Normal	45	1.14 (0.05)	49	1.18 (0.11)	0.78
		Ovt/Ob	49	1.17 (0.04)	50	1.15 (0.04)	0.82
	Insulin	Low	48	1.24 (0.05)	36	1.12 (0.05)	0.10
		High	47	1.08 (0.04)	43	1.12 (0.04)	0.51
IR-A:IR-B	Everyone		98	1.96 (0.04)	100	1.96 (0.03)	0.94
	BMI	Normal	45	2.01 (0.07)	49	1.96 (0.05)	0.52
		Ovt/Ob	49	1.91 (0.04)	50	1.95 (0.03)	0.48
	Insulin	Low	48	2.07 (0.07)	36	1.95 (0.05)	0.18
		High	47	1.85 (0.04)	43	2.01 (0.04)	0.006†

*Student's t-test
†Significant at $P < 0.05$

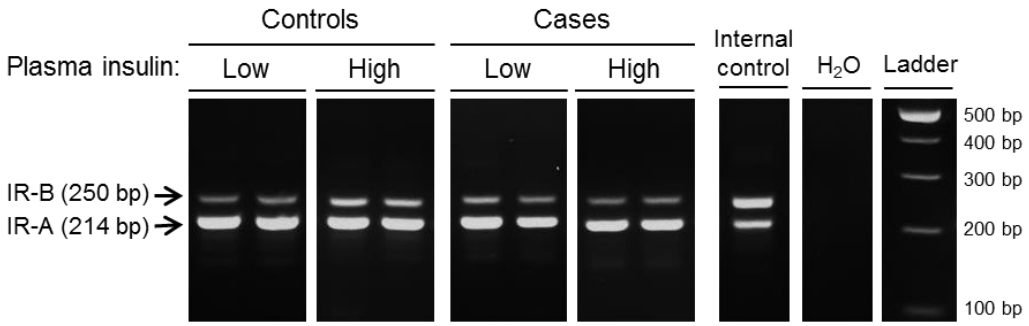

Figure 2.1: Representative gels showing IR-A and IR-B mRNAs in cases and controls with low and high plasma insulin.

Controls with high insulin have decreased IR-A:IR-B ratios compared to controls with low insulin, potentially due to increased IR-B and maintained IR-A. Among patients with high insulin, cases have higher IR-A:IR-B ratios than controls, and this appears to result from decreased IR-B and unaltered IR-A. Samples were run in groups of 19 per gel, and representative images were obtained from different originals or multiple fields from the same image.

Table 2.3: ORs and 95% CIs for the association between colorectal adenomas and IGF1R, IR, and IR-A:IR-B mRNA expression.

Variable	*n* (Control/Case)	OR* (95% CI)	*P**
IGF1R			
Q1	25/46	1.0 (Reference)	-
Q2	25/23	0.5 (0.2-1.1)	0.07
Q3	25/19	0.4 (0.2-0.9)	0.02†
Q4	23/12	0.3 (0.1-0.7)	0.004†
IR			
Q1	25/26	1.0 (Reference)	-
Q2	24/28	1.1 (0.5-2.4)	0.77
Q3	25/30	1.2 (0.5-2.5)	0.71
Q4	24/16	0.6 (0.3-1.5)	0.30
IR-A:IR-B			
Q1	25/29	1.0 (Reference)	-
Q2	24/20	0.7 (0.3-1.6)	0.42
Q3	25/23	0.8 (0.4-1.7)	0.56
Q4	24/28	1.0 (0.5-2.2)	0.99

*Odds of being a case
†Significant at $P < 0.05$

Table 2.4: Association between colorectal adenomas and IGF1R, IR, and IR-A:IR-B mRNA expression influenced by BMI and plasma insulin.

Variable	*P* for interaction	
	BMI (Normal *vs.* Ovt/Ob)	Insulin (Low *vs.* High)
IGF1R	0.78	0.47
IR	0.74	0.11
IR-A:IR-B	0.34	0.005[†]

[†]Significant at $P < 0.05$

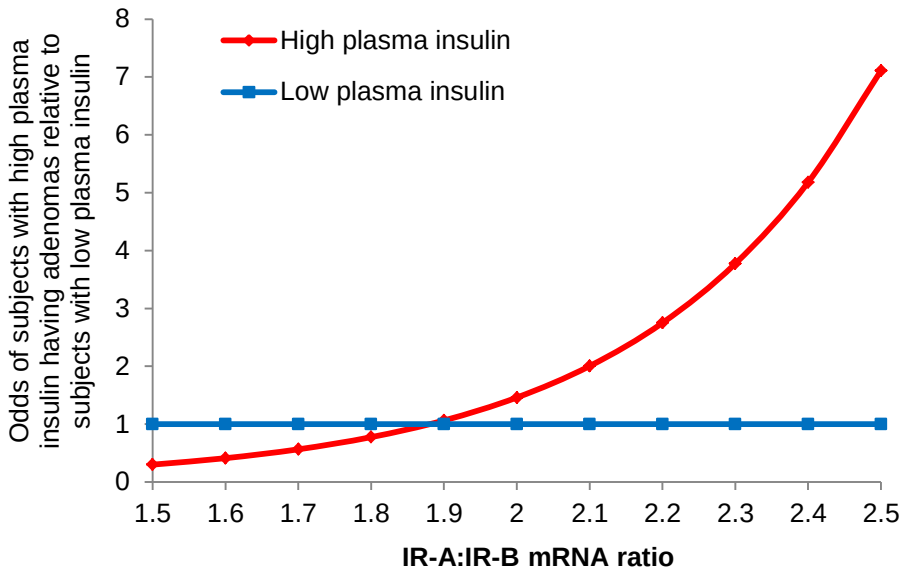

Figure 2.2: Increased IR-A:IR-B ratios predicts adenomas in patients with high plasma insulin. As IR-A:IR-B ratios increase, patients with high plasma insulin are more likely to have colorectal adenomas than patients with low plasma insulin.

Table 2.5: Correlation between plasma insulin and IGF1R, IR, and IR-A:IR-B mRNA expression.

Variable	Plasma insulin			
	Controls (n=95)		Cases (n=79)	
	r*	*P*	r*	*P*
IGF1R	-0.21	0.045†	-0.30	0.01†
IR	-0.26	0.01†	-0.14	0.21
IR-A:IR-B	-0.31	0.002†	0.21	0.06

*Spearman's correlation coefficient
†Significant at $P < 0.05$

CHAPTER 3: IMPACT OF INTESTINAL EPITHELIAL INSULIN RECEPTOR LOSS ON AOM-DSS INDUCED COLON TUMORIGENESIS

Introduction

Colorectal cancer (CRC) is the second most common cause of cancer death in the United States[146]. A growing body of evidence links increased risk of colorectal carcinogenesis to chronic inflammation resulting from inflammatory bowel disease (IBD) and to obesity and insulin resistance [60,210]. The insulin and insulin-like growth factor (IGF) signaling pathway has been implicated in risk of CRC [243], but a major unanswered question is whether the insulin receptor (IR) or the related IGF1 receptor (IGF1R) mediates this risk. IR exists as IR-B and IR-A isoforms. IR-B is considered the primary mediator of the metabolic actions of insulin, although growing evidence in mammary epithelium and our recent findings in colon epithelial cells or cancer cell lines indicate that IR-B induces cell differentiation [96,97]. IR-A binds IGFs as well as insulin and shares proliferative and anti-apoptotic roles with IGF1R [90,91,107]. IGF1R mediates anti-apoptotic and trophic actions of IGF1 or IGF2 and has been implicated in cancer of many organs, including the colon [78,118,140,141,145,265]. Due to reports of elevated IR-A expression in cancer cells and findings that IR-A promotes tumor progression when IGF1R is pharmacologically inhibited or deleted, IR has been viewed as a culprit in promoting cancer growth [90,97,146,147,263,264,267]. Opposing this evidence is a study showing that knockdown of IR-A in SW480 colon cancer cells increased viability and enhanced activation of the related oncogenic IGF1R [104]. This raises the intriguing possibility that IR may limit oncogenic IGF1R signaling.

Insulin resistance during hyperinsulinemia, obesity, or type 2 diabetes involves impaired insulin signaling through IR-B in insulin-target organs such as liver, skeletal muscle, and adipose tissue, which show higher expression of IR-B relative to IR-A [93,107]. In multiple human cohorts, we and others have linked hyperinsulinemia to increased risk of colorectal adenomas and cancer [155,186,187,268,269]. Whether this

risk involves insulin acting on IGF1R or IR-A when IR-B signaling is impaired, as occurs in insulin resistance, is unknown. The goal of the current study was to evaluate the impact of loss of IR function in the intestinal epithelium on colon tumorigenesis. We hypothesized that intact IR signaling normally attenuates the tumor-promoting actions of IGF1R, and IR loss would therefore favor tumor formation. Here, we used a mouse model of disrupted insulin signaling in all intestinal epithelial cells (IEC) of small intestine and colon via villin-Cre (VC) recombinase-mediated deletion of the IR gene (VC-IR$^{\Delta/\Delta}$) [270]. These mice and littermate controls with loxP-modified but intact IR (WT-IR$^{fl/fl}$) were subjected to azoxymethane (AOM) and dextran sodium sulfate (DSS) treatment which lead to inflammation-induced colon cancer. In this preliminary study performed in two independent cohorts of WT-IR$^{fl/fl}$ and VC-IR$^{\Delta/\Delta}$ mice, we found that deletion of IR in colon epithelial cells (CECs) dramatically increases tumor number *in vivo* and enhances growth of tumor-derived CECs in a matrigel culture system. This novel evidence that loss of IR promotes colon tumorigenesis indicates that maintained IR signaling may be essential for CRC prevention.

Materials and methods

Animals

Mice with loxP sites flanking exon 4 of the *Insr* gene (IR$^{fl/fl}$) were provided and described by Dr. Ronald Kahn's group [271]. Mice with IEC-specific IR deletion were generated by crossing IR$^{fl/fl}$ mice with transgenic mice expressing Cre recombinase under the control of the villin promoter (The Jackson Laboratory, Bar Harbor, ME) [272]. All mice were on a C57BL/6 background and co-housed littermate pairs with intact but floxed IR (WT-IR$^{fl/fl}$) or villin-Cre (VC)-mediate disruption of IR (VC-IR$^{\Delta/\Delta}$) were used in this study. Genotyping on tail DNA was performed using published protocols [273]. All mice were housed in a pathogen-free animal facility at the University of North Carolina (Chapel Hill, NC) and given food and water *ad libitum*. All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of North Carolina.

AOM-DSS treatment, tissue harvest, and histology

The experimental design is illustrated in Figure 3.1 and was adapted from previously published protocols, which reproducibly induce colon tumors in C57BL6 mice [75,274]. Male and female WT-IR$^{fl/fl}$ and VC-IR$^{\Delta/\Delta}$ mice were given a single intraperitoneal (i.p.) injection of AOM (10 mg/kg body weight, MRI Chemical Carcinogen Repository). A week later, a subset of mice were subjected to 3 cycles of 2% DSS (TbD Consultancy AB) in water for 5 days followed by a 14-day recovery period where mice were given water. At the end of the 3rd recovery period, all animals were euthanized with isoflurane (Baxter) and cervical dislocation. Colons were removed and flushed with cold 1x phosphate-buffered saline (PBS) pH 7.4 and opened longitudinally. The number of tumors was quantified by two researchers blinded to animal genotype. Individual tumors from the middle portion of the distal colon were collected for RNA extraction or culture experiments. The remaining regions of the distal colon were fixed in 10% zinc formalin overnight for histology. Fixed colon tissue was embedded in paraffin and longitudinal sections were stained with hematoxylin and eosin (H&E). Histological sections were visualized with an Axio Imager.A2 bright field microscope (Zeiss) and a ProgRes CF Scan camera (Jenoptik).

Conditional reprogramming of colon tumor epithelial cells (CR-CTEC)

Protocols for preparation and maintenance of CR-CTEC were adapted from Liu et al., 2012 [64]. Dispersed CECs obtained from WT-IR$^{fl/fl}$ and VC-IR$^{\Delta/\Delta}$ tumors were plated on mitotically inactive 3T3-J2 feeder cells in F-medium containing 10 μM Y27532 and Sato growth factors (Noggin, EGF, and R-Spondin) [275]. Sato additives were withdrawn 1 day after plating or passage and CR-CTEC were maintained in regular F medium thereafter.

3D matrigel culture

Feeders were removed from the wells using TrypLE Express Enzyme (Life Technologies) and discarded. CR-CTEC were removed by a second incubation in TrypLE Express, washed in F medium, and resuspended in 50 μl of Matrigel Matrix Growth Factor Reduced (BD Biosciences) at a density of

5,000 cells per well of a 24-well plate. The same plating and maintenance conditions described above were used. Plates were imaged using a using an inverted bright field microscope with an incubation chamber (Olympus IX83). Cells started to form spheroids/tumorspheres on day 2 after plating. Diameter was measured on day 8 using Image J software (National Institutes of Health). Tumorspheres continued to grow and started to form organoids with crypt-like structures on days 10-11 post-plating. The number of organoids formed was quantified on day 15 and organoids were harvested for RNA and DNA extraction. Genotyping on organoid DNA for floxed and VC-recombined IR gene was performed as previously described [273]. Organoids were passaged and treated with 0 or 10 mM metformin (Aurobindo Pharma) the following day. Metformin was added in F medium, which was replaced every two days. Tumorspheres were imaged at days 2 and 4 after the start of metformin treatment and diameter was measured as described above.

IGF1 treatment of tumor CEC monolayers and western blot

CR-CTECs were plated on 12-well transwell plates with F medium plus 10 μM Y-27632 and with feeders at the bottom. Cells were allowed to grow for 2 days. Prior to signaling, cells were transferred to feeder-free wells and serum-starved for 4 hours in DMEM plus 10 μM Y-27632. Stimulation with IGF1 (20 ng/ml, Tercica, Inc) in serum-free DMEM was performed for 20 minutes. An IGF1 concentration of 20 ng/ml (2.8 nM) is only able to activate IGF1R and not IR [103]. Hot Laemmli blue buffer was added to treated and untreated transwell filters and protein samples were collected, vortexed, and run on a NuPAGE 4–12% Bis-Tris 1.0 mm gel (Life Technologies). Gel was transferred to a 0.45 mm pore PVDF membrane (Millipore), which was blocked with Blocker Casein in TBS (Thermo Fisher Scientific) for 1 hour. Primary antibodies used were phospho-AKT1 (Ser 473, Cell Signaling), AKT (Cell Signaling), and β-actin (Sigma Aldrich). Secondary antibody used was Dylight 800 goat anti-rabbit IgG (Thermo Scientific). Protein was visualized with an Odyssey CLx Infrared Imager (Version 3, LI-COR) and band intensity was measured by densitometry with ImageJ software (National Institutes of Health).

RNA extraction, reverse transcription, and quantitative real-time PCR

RNA was isolated from colon tumors and organoids using the RNeasy kit (Qiagen) and cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit and RNase inhibitor (Applied Biosystems) according to the manufacturer's protocol. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using the 7500 Real-Time PCR System (Applied Biosystems) and primer/probes for *Igf1* (Mm00439560_m1), *Igf2* (Mm00439564_m1), *Stat3* (Mm01219775_m1), *Tnf* (Mm00443258_m1), and *Actb* as the invariant control (Mm00607939_s1). Samples were run in duplicates and concentrations were determined based on a standard curve made with cDNA pooled from all samples studied.

Statistical analysis

Data were collected from two independent experiments and expressed as mean ± SEM. For WT-IR$^{fl/fl}$ and VC-IR$^{\Delta/\Delta}$ groups, n ≥ 7 for survival and tumor number, and n = 7 for tumor gene expression data. Tumorspheres and organoids used during *in vitro* experiments were prepared from tumor cells from only 1 pair of animas (n = 1), but replicate studies were performed on lines established from these tumors. Ongoing studies aim to increase the sample size for the cell culture experiments. Survival data were analyzed using the Log-rank (Mantel-Cox) test. Tumor number in WT-IR$^{fl/fl}$ *versus* VC-IR$^{\Delta/\Delta}$ groups were compared using unpaired t-test. Gene expression in tumor tissue was analyzed by paired t-test. All analyses were performed using GraphPad Prism 6 software (La Jolla, CA) and $P < 0.05$ was considered statistically significant.

Results

Loss of IR in CECs leads to decreased survival and increased number of colon tumors after AOM-DSS treatment

WT-IR$^{fl/fl}$ and VC-IR$^{\Delta/\Delta}$ mice were given a single AOM injection followed by three DSS cycles, each separated by 14 days of water drinking, and euthanized 14 days after the last DSS cycle. Survival

was significantly decreased in the VC-IR$^{\Delta/\Delta}$ group (P = 0.03), particularly during the last two weeks of the experiment (Figure 3.2A). Consistent with our hypothesis, VC-IR$^{\Delta/\Delta}$ mice had significantly more tumors than WT-IR$^{fl/fl}$ littermates (18.3 ± 1.4 *versus* 9.3 ± 1.6, P = 0.002, Figure 3.2B-D). These data indicate that loss of IR in CECs promotes colorectal tumorigenesis and decreases survival following AOM-DSS treatment.

Preliminary data indicate that loss of IR in CECs promotes tumorsphere and organoid formation and IGF1-induced activation of AKT *in vitro*

Confluent WT-IR$^{fl/fl}$ and VC-IR$^{\Delta/\Delta}$ CR-CTEC colonies were removed from feeders and plated in matrigel. Spheroid/tumorsphere diameter was measured on day 8 and the number of organoids formed was quantified on day 15 post-plating. Compared with the WT-IR$^{fl/fl}$ group, VC- IR$^{\Delta/\Delta}$ tumorspheres had a much larger mean diameter (686.2 ± 81.6 μm *versus* 334.8 ± 45.6 μm) and formed more organoids (19 *versus* 6, Figure 3.3A-C). Importantly, organoids from VC-IR$^{\Delta/\Delta}$ mice retained complete VC-induced IR gene disruption, validating stability and epithelial phenotype (Figure 3.3D). We next asked if the remaining IGF1R would cause VC-IR$^{\Delta/\Delta}$ CECs to be more responsive to IGF1 stimulation. To address this question, we plated CR-CTEC onto transwells and added 20 ng/ml IGF1. After 20 minutes, IGF1 treatment induced an increase in AKT phosphorylation of WT-IR$^{fl/fl}$ tumor cells. Interestingly, IGF1-induced AKT phosphorylation was further increased in VC-IR$^{\Delta/\Delta}$ tumor cells (Figure 3.3E-F). Together, these preliminary *in vitro* data indicate that tumor CECs lacking IR have an intrinsic increase in growth capacity and enhanced sensitivity to IGF1-induced activation of AKT relative to tumor CECs with intact IR.

Preliminary results show that treatment with the insulin-sensitizing agent metformin decreases growth rate of VC-IR$^{\Delta/\Delta}$ tumorspheres to basal levels observed in the WT-IR$^{fl/fl}$ group

Tumor CECs in matrigel were treated with 0 or 10 mM metformin and diameter of tumorspheres formed were measured 2 and 4 days after the start of treatment (Figure 3.4). Metformin is an insulin-

sensitizing drug widely used for the treatment of diabetes and multiple studies have reported that metformin has strong anti-tumorigenic effects [200]. Metformin treatment on WT-IR$^{fl/fl}$ tumor CECs reduced tumorsphere diameter by 2.1 ± 0.4 fold on day 4. In the VC-IR$^{\Delta/\Delta}$ group, tumorsphere diameter was dramatically decreased by 2.7 ± 0.4 fold and growth rate was comparable to that observed in the untreated WT-IR$^{fl/fl}$ group (Figure 3.4). These preliminary data indicate that when IR signaling is lost, metformin may be able to reduce tumor cell growth rate to that of cells with intact IR.

Igf1 and *Igf2* mRNAs in tumor tissue are increased in VC-IR$^{\Delta/\Delta}$ *versus* WT-IR$^{fl/fl}$ mice and tumor organoids show a similar expression pattern

Quantitative real-time PCR was performed on colon tumor tissue and organoids from WT-IR$^{fl/fl}$ and VC-IR$^{\Delta/\Delta}$ mice. *Igf1* and *Igf2* are known to be overexpressed in cancer [82,243]. Tumor tissue from VC-IR$^{\Delta/\Delta}$ mice had significantly higher *Igf2* and a non-significant trend towards increased *Igf1* mRNA levels relative to WT-IR$^{fl/fl}$ animals (Figure 3.5A-B). Levels of the pro-inflammatory mediators *Stat3* and *Tnf* (which encodes for TNF-α) did not change across genotypes (Figure 3.5C-D). This gene expression profile appeared to be recapitulated in organoids derived from tumor cells. Our data suggest that increased local production of IGF1 and IGF2 may contribute to enhanced colon tumor growth in VC-IR$^{\Delta/\Delta}$ mice likely via IGF1R signaling.

Discussion

This study aimed to directly define the role of IR in colorectal tumorigenesis using mice with IEC-specific deletion of IR and the AOM-DSS model of inflammation-associated CRC. Our novel data showed that loss of IR in CECs dramatically enhanced colon tumor development *in vivo* and preliminary data indicated that IR loss promotes growth of tumor cells *in vitro*. Defining mechanisms linked to the tumor-promoting effects of IR loss is highly relevant to understanding whether and how diminished insulin signaling in CECs may impact risk of colon adenomas or cancer in humans.

Numerous studies have reported increased expression of the IR-A isoform in cancer and linked

IR-A to tumor growth [90,92,107,267,276]. However, it is important to note that a majority of reports use traditional non-quantitative PCR to demonstrate that IR-A is the primary IR isoform expressed and less evidence (e.g. by qRT-PCR or assays at the protein levels) confirms quantitative increases in IR or IR-A expression. Our recent findings demonstrated reduced expression of total IR mRNA in small intestine and colon tumors of $Apc^{Min/+}$ mice was due to decreased IR-B [97]. In addition, we demonstrated that IR-B expression is low or barely detectable in aggressively growing human colon cancer cell lines which express IR-A [97]. In a recent human study, we also showed that reduced IR-B predicts increased colorectal adenoma risk in humans with elevated plasma insulin [269]. Brierley et al. found that knockdown of IR-A in a CRC cell line that does not express IR-B potentiated IGF1R signaling and cancer cell viability [104]. These data, our current data that deletion of IR in CECs promotes colon tumorigenesis *in vivo*, and our previous findings that reductions in IR-B accompany and predict risk of pre-cancerous adenomas[269] strongly support a protective effect of IR against colon tumor growth. Furthermore, our preliminary *in vitro* data showed that tumor CECs with deleted IR had enhanced growth in matrigel relative to CECs with intact IR, indicating that loss of IR confers an intrinsic growth advantage to colon tumor epithelial cells.

To directly test if IR loss enhanced IGF1R activation, we treated WT-$IR^{fl/fl}$ and VC-$IR^{\Delta/\Delta}$ tumor cells grown on transwells with a dose of IGF1 considered sufficient to activate IGF1R but not IR[103]. This preliminary experiment showed that phosphorylation of AKT, a key downstream mediator of IGF1R and IR signaling, was increased in VC-$IR^{\Delta/\Delta}$ *versus* WT-$IR^{fl/fl}$ tumor CECs treated with IGF1. This suggests that tumor cells lacking IR are more responsive to IGF1 treatment, which likely reflects enhanced signaling through the remaining IGF1R. Furthermore, gene expression analyses in tumor tissue showed that, by a yet unclear mechanism, mRNA levels of *Igf1* and *Igf2* were up-regulated in VC-$IR^{\Delta/\Delta}$ tumors relative to WT-$IR^{fl/fl}$ tumors. Therefore, increased local expression of *Igf1* and *Igf2* could further contribute to enhanced activation of IGF1R when IR signaling is lost. The increase in *Igf2* is of particular interest because the maternal allele of the *IGF2* gene is usually silenced by imprinting, and increased IGF2 expression due to loss of imprinting is a common event in CRC [277,278]. Importantly, WT-$IR^{fl/fl}$ and

VC-IR$^{\Delta/\Delta}$ organoids showed a similar gene expression pattern, suggesting that the matrigel tumor culture system used here may represent a useful tool to study mechanisms of tumor growth *in vitro* that are difficult to address in the intestine *in vivo*. Together, IGF1R may be hyperactivated due to IR deletion, leading to enhanced cell growth and tumorigenesis. Future studies will test whether tumors from VC-IR$^{\Delta/\Delta}$ mice exhibit increased proliferation, reduced apoptosis, or activation of other mediators linked to IGF1R signaling and test directly if re-expression of IR-B or IR-A or inhibition of IGF1R reduces growth of VC-IR$^{\Delta/\Delta}$ tumor cells.

Insulin resistance and obesity are associated with impaired activation of AMP kinase (AMPK) which is improved by metformin, a widely used anti-diabetic drug with promising protective roles against CRC [279-281]. Our preliminary data demonstrated that metformin decreased growth of both WT-IR$^{fl/fl}$ and VC-IR$^{\Delta/\Delta}$ tumorspheres and restored growth of VC-IR$^{\Delta/\Delta}$ tumor cells to similar levels as in WT-IR$^{fl/fl}$ cells. These data suggest that altered AMPK signaling may contribute to enhanced tumor growth when IR is dysfunctional. The ability of metformin to reverse enhanced tumor growth due to loss of IR signaling has implications for cancer-protective roles in type 2 diabetes.

In summary, our work provided novel evidence that loss of IR promotes colon tumorigenesis and tumor growth *in vivo* and *in vitro*. These findings add direct support for a growing body of evidence that robust IR signaling may be crucial for improved prevention of colorectal cancer and therefore have major clinical relevance. Confirmation that loss of IR promotes colon tumors would highlight the clinical significance of strategies to preserve and better monitor IR signaling. Further studies aimed at defining mediators of enhanced tumorigenesis due to loss of IR will improve our ability to study the impact of impaired IR signaling on risk of pre-cancerous or cancerous lesions in the colon.

Figures

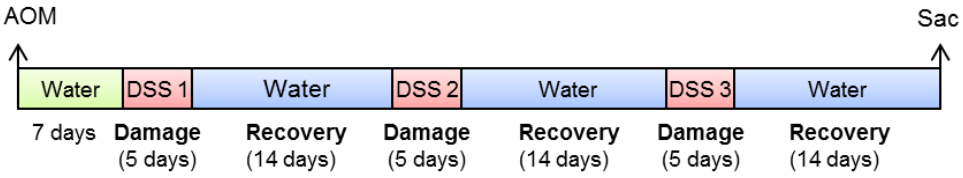

Figure 3.1: Experimental design.
Animals were given a single intraperitoneal injection of AOM followed by 3 treatments consisting of 2% DSS for 5 days. Between each DSS treatment, there was a recovery period of 14 days in which the mice were given water. Mice were sacrificed 14 days after the third DSS cycle.

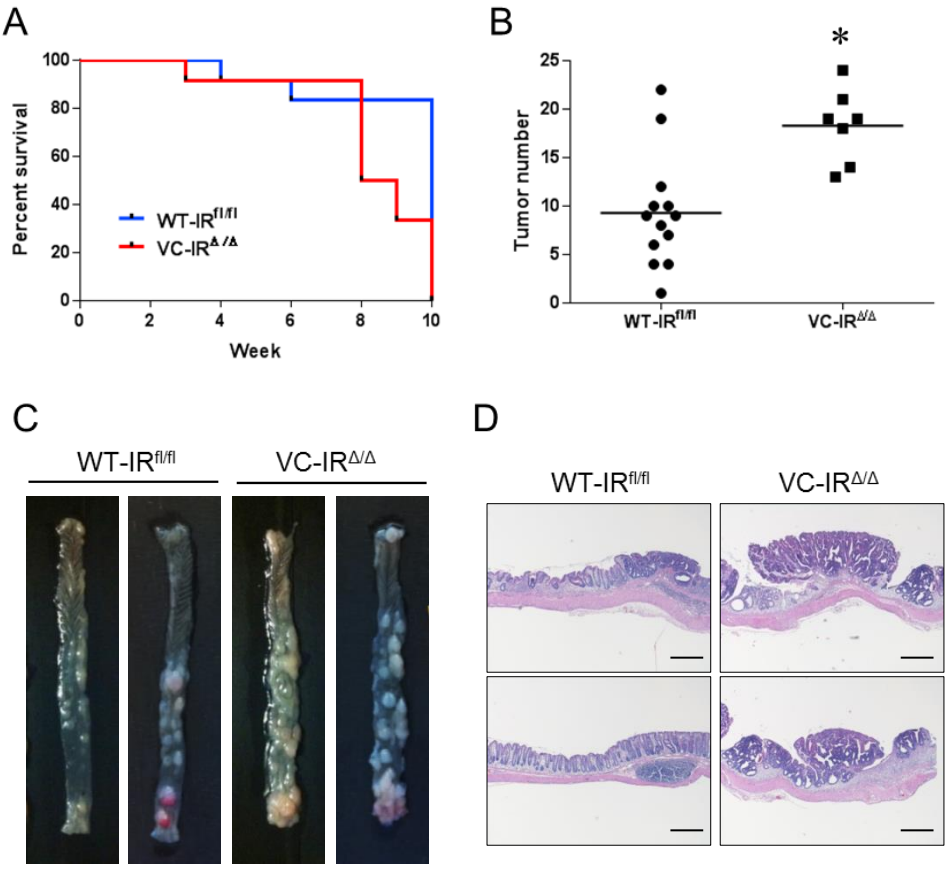

Figure 3.2: VC-IR$^{\Delta/\Delta}$ mice showed increased mortality and number of colon tumors resulting from AOM-DSS treatment.

(A) Survival curve showing the percent survival for each group over time. Data were analyzed using the log-rank (Mantel-Cox) test, $P = 0.03$. (B) Quantification of colonic tumors in WT-IR$^{fl/fl}$ and VC-IR$^{\Delta/\Delta}$ animals. Data expressed as mean ± SEM ($n \geq 7$), $*P < 0.05$, unpaired t-test. C-D: Representative images of (C) tumors in fresh colon specimens and (D) H&E images of tumor histology from WT-IR$^{fl/fl}$ and VC-IR$^{\Delta/\Delta}$ mice after AOM-DSS treatment (for panel D, 4x objective, black scale bar = 500 μm).

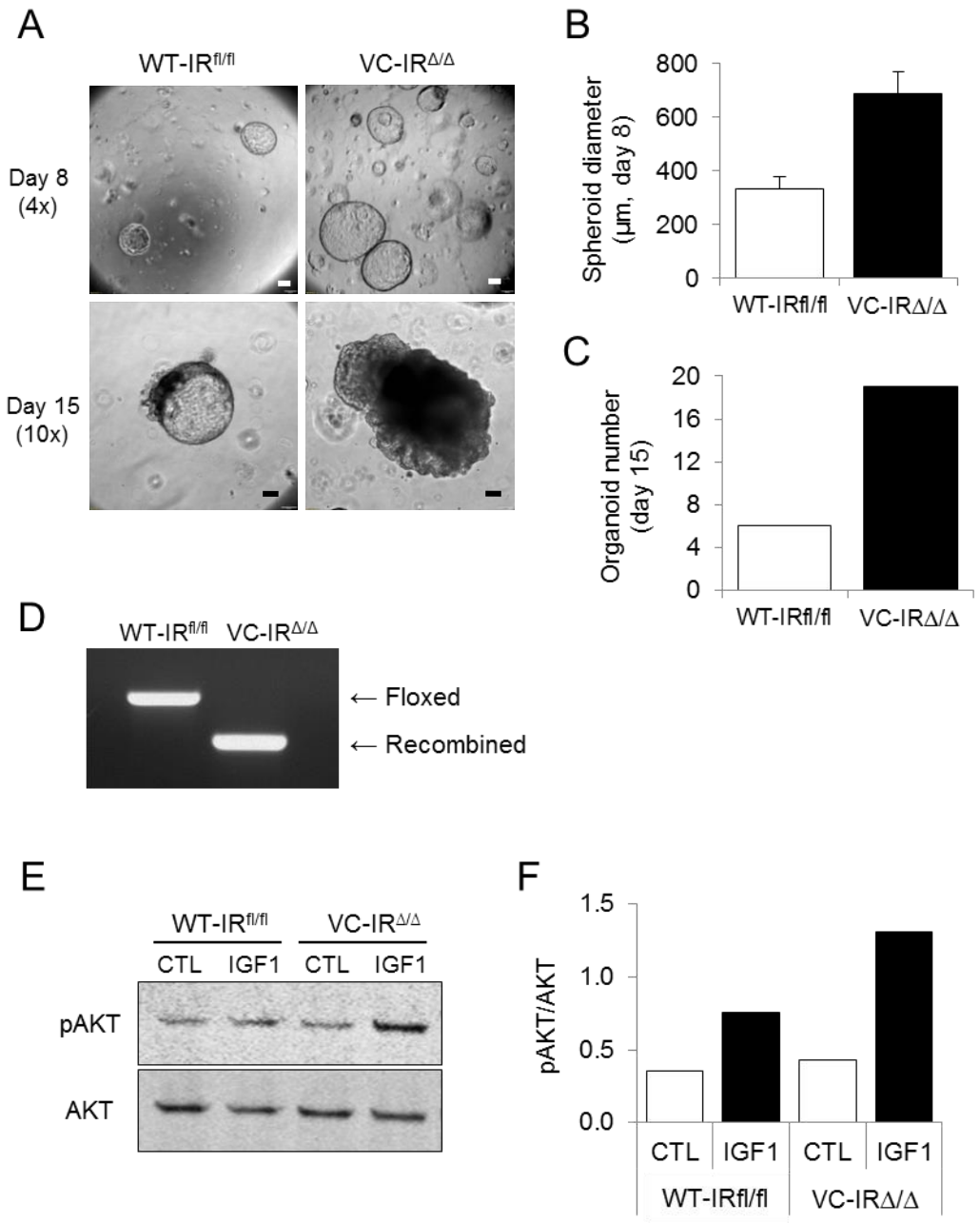

Figure 3.3: Loss of IR enhances tumorsphere growth and organoid formation in matrigel and promotes IGF1-induced AKT phosphorylation of tumor CECs.

(A) Representative images showing tumorspheres on day 8 (top row) and organoids on day 15 (bottom row) after plating tumor-derived CECs in matrigel (white scale bars = 200 μm, black scale bars = 100 μm). B-C: Graphs with data for (B) tumorsphere diameter on day 8 and (C) number of organoids formed on day 15 post-plating. Data in panel B are representative of 10 tumorspheres per mouse and expressed as mean ± SEM. (D) Gel image showing complete villin-Cre-induced recombination of the IR gene in VC-IR$^{\Delta/\Delta}$ and not WT-IR$^{fl/fl}$ organoids after passage. E-F: (E) Western blot and (F) protein quantification by densitometry indicating enhanced AKT phosphorylation in VC-IR$^{\Delta/\Delta}$ tumor CECs 20 minutes after treatment with 20 ng/ml IGF1.

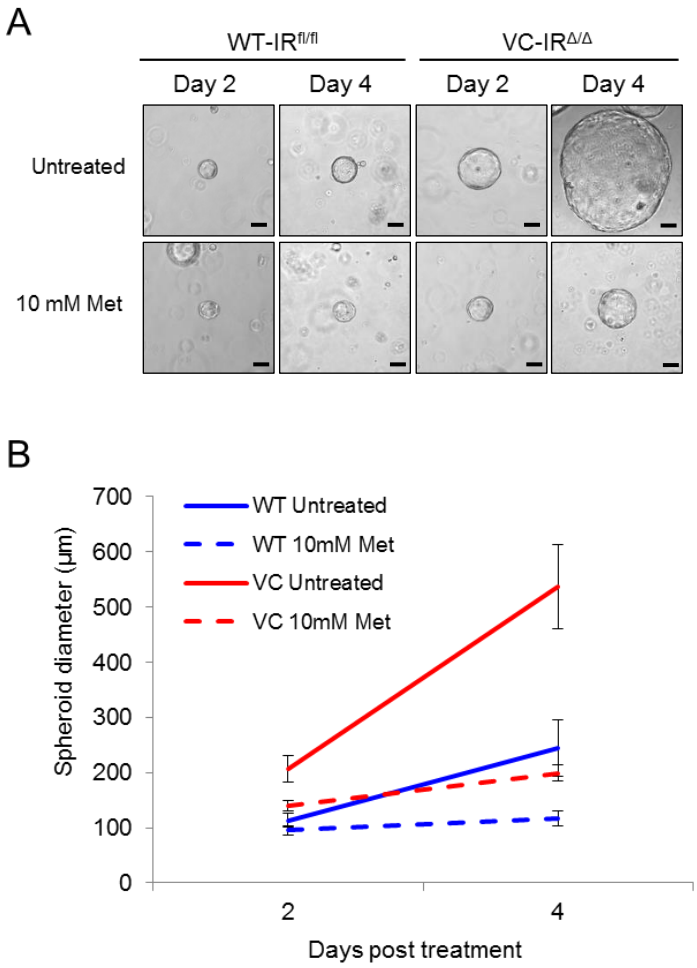

Figure 3.4: Metformin treatment restores growth of VC-IR$^{\Delta/\Delta}$ tumorspheres to levels observed in WT-IR$^{fl/fl}$ tumorspheres.

(A) Representative images of tumorspheres treated with 0 or 10 mM metformin (Met) for 2 or 4 days (10x objective, scale bars = 100 µm). (B) Diameter measured in untreated and treated WT-IR$^{fl/fl}$ and VC-IR$^{\Delta/\Delta}$ tumorspheres on days 2 and 4 after the start of metformin treatment. Data are representative of ≥ 7 tumorspheres per group and are expressed as mean ± SEM.

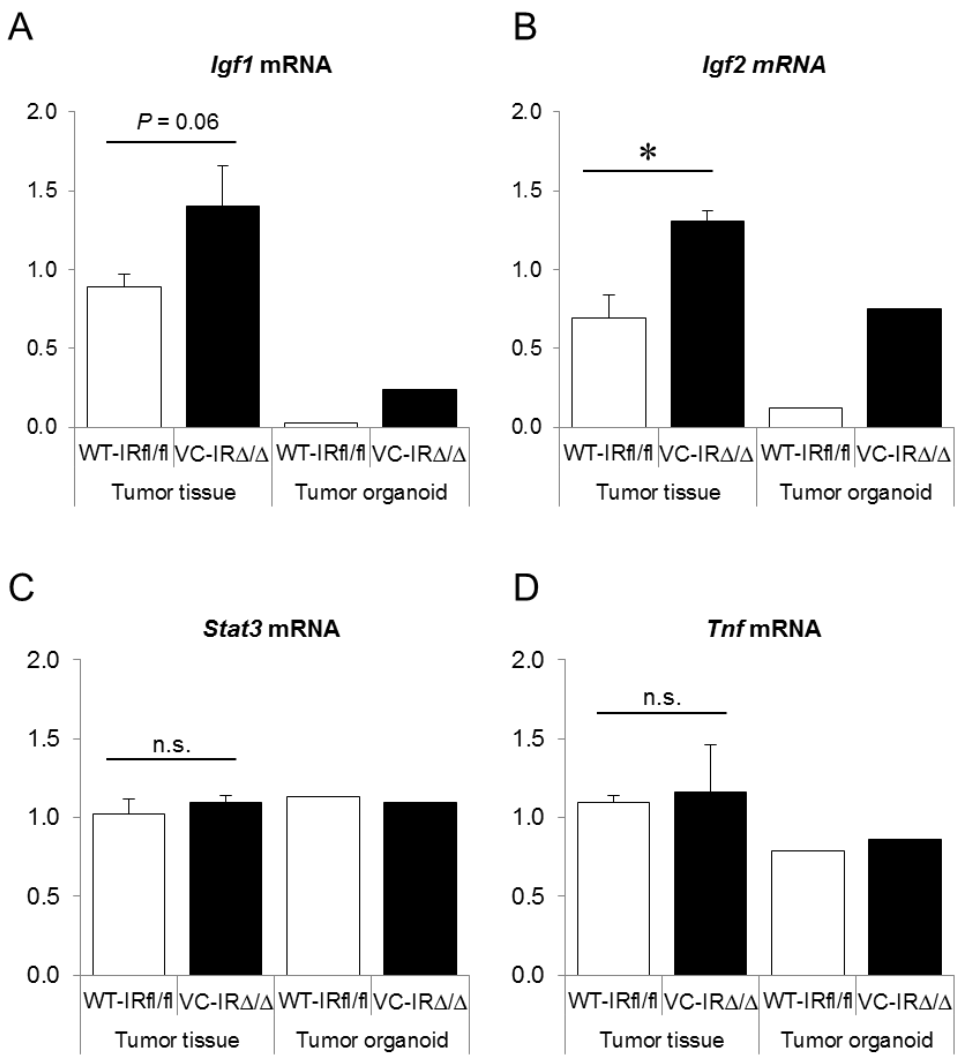

Figure 3.5: Colon tumors and organoids from VC-IR$^{\Delta/\Delta}$ mice show increases in *Igf1* and *Igf2* mRNA levels and no change in *Stat3* or *Tnf* mRNAs relative to WT-IR$^{fl/fl}$ mice.
Gene expression data obtained by qRT-PCR showing mRNA levels of (A) *Igf1*, (B) *Igf2*, and the pro-inflammatory mediators (C) *Stat3* and (D) *Tnf* in tumor tissue and organoids (harvested 15 days post-plating) from WT-IR$^{fl/fl}$ and VC-IR$^{\Delta/\Delta}$ mice. Data for tumor tissue were expressed as mean ± SEM (n = 4), *$P < 0.05$, n.s.: no significance, paired t-test.

CHAPTER 4: OBESITY AND THE INSULIN RECEPTOR, BUT NOT THE IGF1 RECEPTOR, PROMOTE RESISTANCE OF COLON EPITHELIAL CELLS TO RADIATION-INDUCED APOPTOSIS

Introduction

Obesity affects 35% of the adult population of the United States and 600 million adults worldwide [176,282]. Obesity can lead to hyperinsulinemia and insulin resistance, which can progress to type 2 diabetes (T2D), and these metabolic disorders have been strongly linked to risk of a number of cancers [210,244,283,284], including colorectal cancer (CRC) [187,241,285,286]. Hyperinsulinemia is a compensatory response to impaired insulin signaling and may contribute to increased CRC risk, decreased response to chemotherapy or radiotherapy, and increased CRC recurrence and mortality associated with obesity and T2D [189-192,287]. However, the mechanisms underlying CRC risk and poor treatment outcome during obesity or hyperinsulinemia remain unclear.

Current views support the concept that tumors can arise due to survival and subsequent expansion of genetically damaged stem or progenitor cells [288,289]. Apoptosis is a programmed cell death that is used by the body to eliminate unwanted cells with DNA damage, which may otherwise contribute to neoplasia. The frequency of basal, spontaneous apoptosis in the non-challenged small intestinal epithelium is very low [230,233,235]. Spontaneous apoptosis in the colonic epithelium is even rarer, consistent with relatively high levels of the anti-apoptotic protein BCL-2 and low levels of the pro-apoptotic p53 [231-234]. Therefore, models of DNA damage induced by low-dose ionizing radiation (1-6 Gy) with evaluation of maximal apoptosis at 3-4.5 hours post-radiation have served as useful systems to study apoptosis in the intestinal epithelium [113,230,235,290]. Whereas in the small intestine both spontaneous and radiation-induced apoptosis occur primarily at the crypt base where stem cells reside, apoptosis in the colon is scattered along the crypt and is not associated with the position of putative stem cells [230,233].

Our group and others have reported that insulin-like growth factor 1 (IGF1) inhibits radiation-induced apoptosis in the intestinal crypts [113,235,290]. IGFs potently stimulate intestinal growth [112,119,123,127] and are widely considered mediators of survival or proliferation of cancer cells [180,243]. Levels of bioavailable IGF1 can be increased by elevated plasma insulin, which inhibits hepatic production of IGF binding protein 1 (IGFBP1) [153,291]. Both insulin and IGF1 can exert mitogenic and anti-apoptotic actions in normal and cancer cells via the insulin receptor (IR) or the IGF1 receptor (IGF1R) [77,86,119,243], which belong to the receptor tyrosine kinase family and are highly homologous in structure [83]. Studies on the specific roles of IGF1R *versus* IR in proliferation and anti-apoptosis in the context of elevated insulin have been challenging because dependent on their levels, IGF1 and insulin can each activate both IGF1R and IR.

Traditionally, IGF1R has been viewed as the main mediator of the trophic, anti-apoptotic, and pro-tumorigenic actions of IGFs and IGF1R overexpression has been reported in colorectal adenocarcinomas [78,79,141,142,292]. In contrast, IR has been considered to play a larger role in mediating the metabolic actions of insulin [80,271,293]. However, the IR gene undergoes pre-mRNA splicing that leads to expression of two functionally-distinct IR isoforms, IR-A and IR-B. IR-A lacks exon 11, mediates growth of fetal and tumor cells, and binds both insulin and IGFs, particularly IGF2, with high affinity [90,92]. IR-B contains exon 11 which alters the structure of the ligand binding α-subunit and confers high affinity for insulin *versus* IGFs [90,107]. IR-B is considered the major mediator of insulin action on nutrient storage and metabolism [77,107]. A concept that IR may promote tumor growth is based on evidence that IR-A is highly expressed in cancer cells or tumors and studies showing that IR promoted tumor cell survival or growth when IGF1R was inhibited [97,107,146,147,263,264]. In contrast, knockdown of IR-A in the colon cancer cell line SW480 promoted cell viability and this was associated with enhanced IGF1R phosphorylation [104].

Our prior human studies found that high plasma insulin and low apoptosis in normal rectal mucosa were consistently associated with colorectal adenomas in multiple patient cohorts [155,165,186,294]. Therefore, defining the roles of obesity-associated hyperinsulinemia and IGF1R *versus* IR in promoting survival of genetically damaged colon epithelial cells (CECs) is relevant to mechanisms by which obesity

may promote risk of early stage colon tumors. In this study, we used a model of high fat diet (HFD)-induced obesity and hyperinsulinemia challenged with DNA damage caused by low-dose radiation to directly evaluate the impact of obesity on apoptosis of genetically damaged CECs. These studies were performed in mice with genetic disruption of IGF1R or IR in intestinal epithelial cells (IEC) and littermate controls to more directly define the roles of IGF1R or IR in radiation-induced CEC apoptosis during obesity and hyperinsulinemia. We hypothesized that obesity/hyperinsulinemia would lead to decreased apoptosis of genetically damaged CECs. We also hypothesized that any anti-apoptotic effects of obesity and hyperinsulinemia would be prevented or attenuated by IGF1R deletion and not IR deletion. Our results provide novel evidence that obesity and hyperinsulinemia promote reduced apoptosis of CECs following DNA damage. Surprisingly, IR but not IGF1R deletion increased apoptosis in lean and obese mice but did not prevent the reduction in apoptosis observed during obesity.

Materials and methods

Animals

Mice with loxP sites flanking exon 3 of the *Igf1r* gene ($IGF1R^{fl/fl}$) were obtained from Dr. Argiri Efstratiadis (Columbia University, NY) and have been described by Xuan et al. and Zhang et al. [295,296]. Mice with loxP sites flanking exon 4 of the *Insr* gene ($IR^{fl/fl}$) were provided by Dr. Ronald Kahn and described by Brüning et al. [271]. To generate mice with IEC-specific IGF1R or IR gene disruption, $IGF1R^{fl/fl}$ and $IR^{fl/fl}$ mice were crossed with villin-Cre (VC) mice expressing a Cre recombinase transgene driven by the villin promoter (The Jackson Laboratory, Bar Harbor, ME) [272]. The VC transgene is expressed throughout both small intestinal and colonic epithelium. All mice were on a C57BL/6 background. Study animals were derived by cross-breeding WT-$IGF1R^{fl/fl}$ and VC-$IGF1R^{\Delta/\Delta}$ mice or WT-$IR^{fl/fl}$ and VC-$IR^{\Delta/\Delta}$ mice. Genotyping on tail DNA was performed as previously described [273,296]. Animals were housed in a pathogen-free facility at the University of North Carolina (Chapel Hill, NC), and food and water were provided *ad libitum*. All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of North Carolina.

Diet-induced obesity

WT-IGF1R$^{fl/fl}$ and VC-IGF1R$^{\Delta/\Delta}$ mice or WT-IR$^{fl/fl}$ and VC-IR$^{\Delta/\Delta}$ male mice (10-12 week old) were assigned to either a standard rodent chow control (CTL) diet (14% Kcal from fat) or high fat diet (HFD, 45% Kcal from fat, Research Diets, New Brunswick, NJ). In C57BL/6 mice this HFD leads to obesity, hyperinsulinemia, and insulin resistance within 14-16 weeks of onset of diet [67,297]. Male mice were used because our prior studies have demonstrated more reproducible and consistent development of obesity and hyperinsulinemia in males versus females on C57BL/6 background. In the current study, after 16 weeks on CTL diet or HFD, blood was collected from the tail vein after an overnight fast and plasma was isolated by centrifugation at 600 rcf for 6 minutes. Fasting blood glucose levels were measured using a OneTouch Ultra 2 glucometer (LifeScan, Milpitas, CA). Mice were given CTL diet or HFD for two additional weeks before radiation and euthanasia.

Radiation and tissue harvest

After 18 weeks on diets, mice were anesthetized with isoflurane (Baxter, Deerfield, IL) and abdominally irradiated with a single dose of 5 Gy using an XRad 320 (Precision X-Ray, North Branford, CT) at a rate of 1.07 Gy/min. Euthanasia was carried out with a lethal dose of Nembutal (150 μg/g) given via intraperitoneal injection 4 hours post-radiation, a time corresponding to maximal apoptosis of (IEC) after radiation-induced DNA damage [12,113,237,298,299]. The colon was collected and flushed with cold 1x phosphate-buffered saline pH 7.4. All mesentery fat was removed and discarded. Length and weight of entire colon were recorded. As illustrated in Figure 4.1, for the first group of mice ($n \geq 5$), entire colon was splayed open and fixed in 4% paraformaldehyde (PFA) overnight for histological scoring of apoptosis. For the second group ($n \geq 3$), the most proximal and distal ~1-cm segments of the colon were fixed in 10% zinc formalin overnight to confirm histological apoptosis data obtained in the first group. The remaining of the tissue was splayed opened and 3-4 cm of the distal colon were used for CEC isolation for gene and protein expression experiments. The distal colon was used for histological and

biochemical analyses because in mouse models of CRC, tumors develop mainly in the distal colon and very rarely in the proximal portion.

Blood and plasma measurements

Fasting plasma insulin was measured by ELISA (Mercodia, Uppsala, Sweden). Insulin sensitivity was estimated by calculating homeostatic model assessment (HOMA) values using the formula Fasting Glucose (mmol/L) × Fasting Insulin (mU/L) / 22.5. Higher HOMA values indicate decreased insulin sensitivity. Blood was collected by cardiac puncture at the time of euthanasia and the plasma was acid-ethanol extracted to remove IGF binding proteins as previously described [300]. IGF1 levels in plasma were measured by ELISA (R&D, Minneapolis, MN).

Immunohistochemistry and histological analyses

Histological sections were visualized with an Axio Imager.A2 bright field microscope (Zeiss; Thornwood, NY, USA) and a ProgRes CF Scan camera (Jenoptik; Jena, Thuringia, Germany). Following overnight fixation in 10% zinc formalin, closed, cross sections were embedded in paraffin for hematoxylin and eosin (H&E) staining. Crypt depth was measured in 20 well-oriented crypts for each animal using the software ProgRes Capture Pro 2.7. Splayed opened colons fixed in 4% PFA were cryopreserved by two subsequent overnight incubations in 10% and 30% sucrose at 4ºC. Tissues were embedded in Optimal Cutting Temperature compound (Sakura, Torrance, CA) and allowed to freeze on dry ice. Six-µm thick sections were placed on microscope slides and baked at 37ºC overnight and then at 60ºC for 2 hours. Heat-induced epitope retrieval (HIER) was performed using HIER Buffer L pH 6.0 (Thermo Scientific, TA-135-HBL). Slides were washed in distilled water and incubated in 3% hydrogen peroxide for 10 min at room temperature, and blocked with 10% normal goat serum for 1 hr. Slides were incubated with a cleaved caspase-3 antibody (Cell Signaling Technology, Danvers, MA) at 4ºC overnight. Incubation in Biotinylated Goat Anti-Rabbit antibody (Jackson Immunoresearch, West Grove, PA) was performed for 1 hour at room temperature. Apoptosis was assessed by quantifying cells positive for

cleaved caspase-3 staining in 40 well-oriented crypts for each mouse. The total number of cells per crypt was quantified based on hematoxylin-stained nuclei. The apoptotic indexes were determined for each crypt by calculating the percentage of cleaved caspase-3 positive cells relative to the total number of cells.

Colon epithelial cell isolation

Distal colon segments were placed in a solution containing 30mM EDTA/1.5mM DTT/1xPBS for 20 minutes on ice. The tissues were transferred to a 30mM EDTA/1x PBS solution and incubated in a 37ºC water bath for 10 minutes. Samples were shaken vigorously for 1 minute to separate the epithelium from the submucosal and muscularis layers and were centrifuged at 1,750 rpm for 5 minutes. Following two washes with 1x PBS, samples were pelleted and resuspended in RLT buffer (Qiagen, Valencia, CA) containing 1:100 2-mercaptoethanol (Gibco, Grand Island, NY) for RNA extraction or flash frozen for protein extraction.

Protein extraction and Western blot

Isolated CECs were lysed with hot Laemmli buffer, boiled, and sonicated. Protein samples were run on NuPAGE 4–12% Bis-Tris 1.0 mm gels (Life Technologies, Carlsbad, CA) and transferred to a 0.45 mm pore PVDF membrane (Millipore, Billerica, MA). Membranes were blocked with Blocker Casein in TBS (Thermo Fisher Scientific, Waltham, MA) for 1 hour and incubated in primary antibodies at 4ºC overnight. Primary antibodies used were IR-β (Santa Cruz Biotechnology, Santa Cruz, CA), IGF1R-β, p53 and pH2AX (Cell Signaling Technologies, Danvers, MA), and β-actin (Sigma Aldrich, St. Louis, MO) as the loading control. Membranes were washed with 1x TBS, 0.1% Tween buffer and incubated in Dylight 800 goat anti-rabbit or anti-mouse IgG secondary antibodies (1:15,000, Pierce, ThermoScientific, Rockford, IL) at room temperature for 2 hours. Protein was visualized with an Odyssey CLx Infrared Imager (Version 3, LI-COR, Lincoln, NE) and band intensity was measured with ImageJ software (National Institutes of Health).

RNA isolation and high throughput real time PCR

RNA was isolated from CECs using the RNeasy kit (Qiagen, Valencia, CA) according to manufacturer's instructions. High quality RNA as determined by the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) was utilized for high throughput gene expression analyses using the BioMark HD System (Fluidigm, San Francisco, CA) as previously described[301] based on the manufacturer's protocol. An RNA pool derived from pooling equal amounts of all samples studied was used as the reference sample. All test mRNAs were normalized to the mRNA encoding the housekeeping gene hydroxymethylbilane synthase (*Hmbs*). TaqMan primer/probes for data presented here include Mm01143545_m1 (*Hmbs*), Mm00432051_m1 (*Bax*), Mm00477631_m1 (*Bcl2*), Mm00480750_m1 (*Perp*), and Mm00438168_m1 (*Cdkn1b*) and were purchased from Applied Biosystems (Carlsbad, CA).

Statistical analysis

Data were collected from multiple independent experiments, each consisting of 4-8 mice. Subsets of mice were used for different experiments. Data were expressed as mean ± SEM. Morphological measurements and quantification of apoptosis, protein, and mRNA levels were analyzed by two-way analysis of variance (ANOVA) for main effects of diet or genotype and interactions between diet and genotype. Multiple comparisons were performed by Tukey's test. All analyses were performed using GraphPad Prism 6 software (La Jolla, CA) and $P < 0.05$ was considered statistically significant.

Results

Complete genetic disruption of IGF1R or IR in IEC of VC-IGF1R$^{\Delta/\Delta}$ and VC-IR$^{\Delta/\Delta}$ mice

Figure 4.2 shows a PCR gel confirming that VC-IGF1R$^{\Delta/\Delta}$ and VC-IR$^{\Delta/\Delta}$ mice are homozygous for the recombined allele of IGF1R or IR, respectively, specifically in IEC. Only floxed but intact alleles are observed in serosa and liver of VC mutants and IEC of WT-IGF1R$^{fl/fl}$ and WT-IR$^{fl/fl}$ controls.

IGF1R or IR loss in IEC does not impact weight gain, hyperinsulinemia, hyperglycemia, or insulin insensitivity resulting from long term HFD feeding

Table 4.1 demonstrates that HFD-fed animals had increased body weight gain and fasting levels of blood glucose and plasma insulin compared to controls. Animals on HFD also had higher HOMA values, indicating decreased insulin sensitivity. There were no significant differences across genotypes in any of these metabolic consequences of HFD. Plasma IGF1 levels did not differ between diet groups or genotypes. Our data therefore demonstrate that IEC loss of IGF1R or IR had no significant effects on the metabolic phenotype associated with HFD feeding for 18 weeks. It should be noted that body weight gain in HFD-fed mice *versus* controls differs between the IGF1R and IR mouse colonies. However, values for body weight gain within control and HFD animals of the same colony were very consistent.

Loss of IGF1R in CECs does not impact basal colon phenotype in obese or lean mice

We next examined the effects of obesity and IGF1R receptor deletion on colon morphology and morphometry. Western immunoblot on isolated CECs confirmed complete absence of IGF1R protein in VC-IGF1R$^{\Delta/\Delta}$ animals and no change in IGF1R levels during obesity (Figure 4.3A). Obese mice had lighter and shorter colons, and disruption of IGF1R did not impact this phenotype (Figure 4.3B-C). Colon crypt depth was unaffected by either obesity or loss of IGF1R (Figure 4.3D). These data suggest that morphology and morphometry of the colon is not altered by epithelial loss of IGF1R in lean or obese mice.

IGF1R loss in CECs does not cause a compensatory increase in IR protein

Since no differences were observed in gross or histological morphology of colons lacking epithelial IGF1R, we asked if IR protein levels were increased in response to IGF1R loss. Expression of IR was similar in both obese and lean WT-IGF1R$^{fl/fl}$ mice and IGF1R loss did not significantly affect IR protein levels in either diet group (Figure 4.4).

Obesity promotes decreases in radiation-induced apoptosis of CECs and this is unaffected by IGF1R deletion

We hypothesized that diet-induced obesity and hyperinsulinemia would decrease the ability of colonic epithelial cells to undergo apoptosis following DNA damage, and these effects would be mediated by IGF1R. Quantification of cleaved caspase-3 positive cells in colon crypts revealed that obese WT-IGF1R$^{fl/fl}$ animals had significantly lower apoptosis than their lean counterparts at 4 hours after 5 Gy radiation (Figure 4.5A-B). Surprisingly, no difference in the apoptotic index was observed between WT-IGF1R$^{fl/fl}$ and VC-IGF1R$^{\Delta/\Delta}$ mice in either lean or obese groups (Figure 4.5A-B). Phospho-H2AX, a well-established biomarker of DNA damage, was assessed by western immunoblot to verify that obesity or IGF1R deletion did not affect DNA damage. Levels of pH2AX were similar in all groups (Figure 4.5C-D). Together, these findings indicate that obesity and hyperinsulinemia promote resistance of CECs to apoptosis after radiation-induced DNA damage and, surprisingly, IGF1R loss did not affect these results.

Loss of IR in CECs does not affect basal colon phenotype and does not alter IGF1R protein expression in obese or lean mice

Figure 4.6A confirms IR deletion in CECs from VC-IR$^{\Delta/\Delta}$ mice and shows that IR protein levels in WT-IR$^{fl/fl}$ animals are unaltered during obesity. Consistent with findings in the IGF1R mouse colony, obesity was associated with lighter and shorter colons in both WT-IR$^{fl/fl}$ and VC-IR$^{\Delta/\Delta}$ mice (Figure 4.6B-C). Colon crypt depth was similar across all groups (Figure 4.6D) and neither obesity nor IR loss affected IGF1R protein levels (Figure 4.7). These data indicate that loss of IR in CECs has no detectable effects on colon morphology or morphometry and does not lead to a compensatory increase in IGF1R protein.

IR loss promotes apoptosis of genetically damaged CECs in lean and obese animals

We next examined if the anti-apoptotic effects of obesity or hyperinsulinemia on CECs with DNA damage were mediated by IR rather than IGF1R. Obese WT-IR$^{fl/fl}$ mice had lower levels of apoptosis than lean WT-IR$^{fl/fl}$ mice (Figure 4.8A-B), consistent with findings in the IGF1R group.

Interestingly, lean VC-IR$^{\Delta/\Delta}$ mice had significantly higher levels of apoptosis than lean WT-IR$^{fl/fl}$ mice, providing evidence that IR loss increases CEC apoptosis. In the obese group, VC-IR$^{\Delta/\Delta}$ mice also had higher apoptosis than WT-IR$^{fl/fl}$ animals, further indicating anti-apoptotic roles of IR. However, apoptosis levels in obese VC-IR$^{\Delta/\Delta}$ mice remained significantly lower than in lean VC-IR$^{\Delta/\Delta}$ mice (Figure 4.8A-B). Levels of the DNA damage marker pH2AX were not affected by diet or genotype (Figure 4.8C-D). Overall, our results show that disruption of IR promotes apoptosis of genetically damaged CECs in both lean and obese mice. However, obesity is still associated with reductions in apoptosis in mice lacking IR.

Obesity and IR loss impact protein expression of p53 and mRNA levels of *Perp* and *Cdkn1b* in genetically damaged CECs

We next sought to investigate potential molecular mediators influenced by obesity and IR that may contribute to resistance of CECs to apoptosis after DNA damage. CECs were isolated from WT-IR$^{fl/fl}$ and VC-IR$^{\Delta/\Delta}$ mice and levels of pro-apoptotic and anti-apoptotic regulators were assessed. We first examined p53, a key sensor of DNA damage that is a required mediator of apoptosis in the intestinal crypts within the initial hours following radiation [231,238]. At 4 hours after 5 Gy radiation, we observed a non-significant trend ($P = 0.08$) for reduced p53 in CECs of obese *versus* lean WT-IR$^{fl/fl}$ animals (Figure 4.9A-B). Both lean and obese VC-IR$^{\Delta/\Delta}$ mice had significantly increased p53 protein levels compared with their WT-IR$^{fl/fl}$ counterparts (Figure 4.9A-B). Next, we performed high throughput qRT-PCR on regulators of apoptosis and cell cycle progression. We found no changes in mRNA levels of pro-apoptotic *Bax* or anti-apoptotic *Bcl2* in obese *versus* lean mice across genotypes (Figure 4.9C-D). However, mRNA levels of the pro-apoptotic *Perp* (p53 apoptosis effector related to PMP-22) and the tumor suppressor *Cdkn1b* (which encodes for p27) were significantly down-regulated in CECs of obese WT-IR$^{fl/fl}$ and VC-IR$^{\Delta/\Delta}$ mice *versus* lean mice of the same genotype (Figure 4.9E-F). Both *Perp* and *Cdkn1b* mRNAs were significantly higher in lean VC-IR$^{\Delta/\Delta}$ mice than in lean WT-IR$^{fl/fl}$ controls. These data show a potential contribution of p53 and p53-regulated genes to the differential apoptosis response of CECs during obesity and IR deletion.

Discussion

This study aimed to investigate the effects of obesity on apoptosis of colonic epithelial cells (CECs) after radiation-induced DNA damage and test our original hypothesis that IGF1R loss would have an impact on these effects. We used a mouse model of HFD-induced obesity and hyperinsulinemia with genetic deletion of IGF1R specifically in intestinal epithelial cells (IEC). We found that 4 hours after DNA damage induced by 5 Gy radiation, obesity led to reduced CEC apoptosis and this was unaffected by IGF1R loss. Given this surprising result, we next studied mice with IEC-specific deletion of IR to test if IR contributed to the anti-apoptotic effects of obesity. We found that IR loss significantly increased apoptosis of genetically damaged CECs in both lean and obese mice but did not prevent the reduction in apoptosis in obese animals. Together, our studies on two independent mouse colonies showed that obesity and hyperinsulinemia decrease apoptosis of CECs after radiation-induced DNA damage. Furthermore, we provide novel and unexpected evidence that loss of IR, but not IGF1R, increases apoptosis of genetically damaged CECs in lean or obese mice, indicating novel anti-apoptotic roles of IR in the colon.

A large body of clinical data strongly links obesity, hyperinsulinemia, insulin therapies, and diabetes to CRC risk [155,187,285,286,294,302], and considerable interest in the role of the insulin/IGF system in mediating this risk has emerged. Due to the structural similarities and overlapping functions of IGF1R and IR, defining their individual roles in growth and cell death in different tissues has been challenging. We therefore generated mice with IEC-specific disruption of IGF1R or IR in the intestinal epithelium. The colons of these mice show no obvious basal phenotype, as demonstrated by the lack of effects on colon length, weight and crypt depth with loss of either receptor. These mouse models were first used to evaluate the early effects of obesity on apoptosis of genetically damaged cells in normal colonic epithelium which may reflect an important mechanism to remove cells that could harbor potentially oncogenic mutations. Our findings in two independent experiments on two independent colonies of mice with diet-induced obesity and hyperinsulinemia provide, to our knowledge, new, direct evidence that obesity and hyperinsulinemia are associated with reduced apoptosis of genetically damaged cells. This

preclinical evidence supports our findings in humans linking increased plasma insulin to reduced apoptosis in normal rectal mucosa, which in fact predicted risk of colorectal polyps [155,165].

The role of IR in the gut epithelium has been largely ignored, and significantly more attention has been focused on IGF1R due to its known role in growth and cancer [119,180,292]. Circulating "free" IGF1, which increases during elevated plasma insulin due to inhibition of hepatic production of IGFBPs [153], has been associated with normal and aberrant growth of colon [112,303,304]. In line with this concept, IGF1R has been considered a critical mediator of the proliferative and anti-apoptotic effects of elevated insulin or IGF1. Our findings that loss of IGF1R did not impact radiation-induced apoptosis of CECs are therefore unexpected and may suggest that, in the colon, IGF1R is not essential for anti-apoptotic actions. Loss of IR, however, led to a dramatic increase in apoptosis of genetically damaged CECs. A direct role for IR in apoptosis has been previously described in primary hepatocypes of newborn mice and in mouse embryo fibroblasts [305,306]. Here, we provide the first direct evidence that IR exerts anti-apoptotic actions in the adult colon epithelium.

Our studies indicated that the increase in apoptosis associated with IR deletion occurred regardless of whether animals were lean or obese, since apoptosis levels in obese VC-IR$^{\Delta/\Delta}$ mice still remained lower than in lean VC-IR$^{\Delta/\Delta}$ littermates. This suggests that factors other than insulin signaling may mediate the anti-apoptotic effects of obesity. When we examined protein expression in CECs of WT-IR$^{fl/fl}$ animals, we found a non-significant, consistent decrease in p53 with obesity. Relative to WT-IR$^{fl/fl}$ mice, loss of IR led to a slight but significant increase in p53 protein in both obese and lean groups. These small differences in protein expression may reflect the fact that the apoptosis phenotype we observed histologically is restricted to a few cells per crypt (2-4 cells in lean mice and 1-2 cells in obese mice) and examining total CECs may therefore mask any changes in protein levels in crypt cells undergoing apoptosis. A study on mouse embryonic fibroblasts showed that, after DNA damage, IGF1 treatment induced p53 protein degradation via the MAPK pathway [307]. However, whether these effects were mediated by IGF1R or IR-A was not investigated. It could therefore be speculated that in the colon epithelium, where IR-A is the predominant isoform [294], increased protein levels of p53 in VC-IR$^{\Delta/\Delta}$ mice

may result from loss of IR-A signaling. Analyses of CEC mRNA revealed that *Perp* and *Cdkn1b* were increased in lean mice lacking IR and decreased in obese mice regardless of genotype. *Perp* is transcriptionally induced by p53 in normal and cancer cells specifically during apoptosis [308-310]. *Cdkn1b* encodes the tumor suppressor p27, which promotes cell cycle arrest at G_1 by inhibiting cyclin-dependent kinases, is down-regulated in tumors from p53 null mice, and can be up-regulated by p53 to protect normal IEC from the cytotoxic effects of chemotherapy [311-313]. These data showing changes in *Perp* and *Cdkn1b* mRNA levels in genetically damaged CECs of obese and lean VC-IR$^{\Delta/\Delta}$ mice are consistent with the anti-apoptotic effects of obesity and IR observed by histology. We note that in mice lacking IR, *Perp* and *Cdkn1b* mRNAs are significantly decreased with obesity, but p53 protein is not. This discrepancy could be due to the fact that the western blot experiments used whole cell lysates and therefore do not provide information on p53 nuclear translocation, which is required to activate gene transcription after DNA damage. Alternatively, elevated p53 may reflect a mechanism by which loss of IR increases apoptosis while reduced *Perp* and *Cdkn1b* mRNAs reflect other pathways that maintain apoptosis at low levels during obesity. This would cause obese VC-IR$^{\Delta/\Delta}$ mice to have higher apoptosis than obese WT-IR$^{fl/fl}$ mice but lower apoptosis than their lean VC-IR$^{\Delta/\Delta}$ counterparts.

Our study is to our knowledge the first to directly show that obesity and hyperinsulinemia promote resistance of CECs to apoptosis after DNA damage. Furthermore, we provide novel evidence that IR and *not* IGF1R normally protects genetically damaged CECs from apoptosis. Overall, our work suggests that: (i) the mediators of obesity-associated reductions in apoptosis should be further explored as they may represent a potential early mechanism driving colon tumorigenesis and (ii) more attention should be given to the physiological roles of IR in cell death and survival in the colon crypts to better understand the mechanisms underlying normal or aberrant colon growth.

Figures and Tables

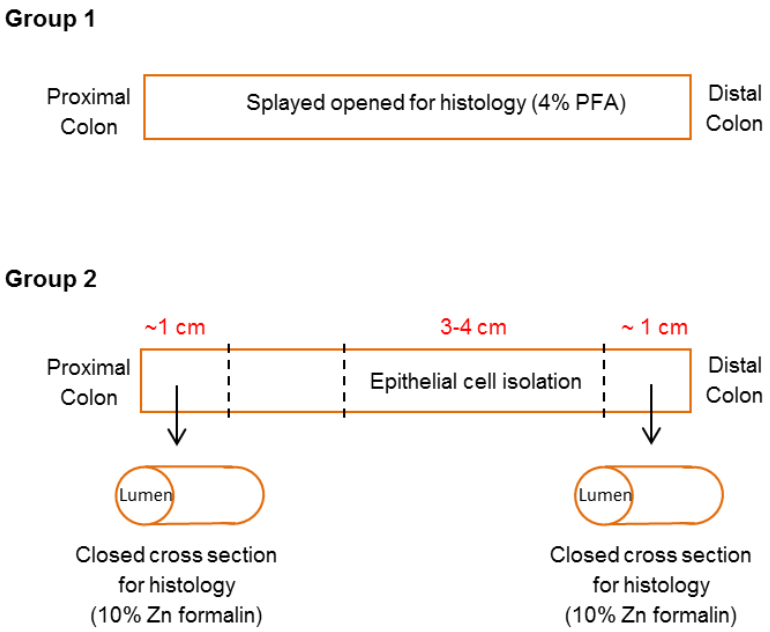

Figure 4.1: Colon tissue harvest.
First group of animals: the entire colon was splayed opened and fixed in 4% paraformaldehyde (PFA) for histological assessment of apoptosis. Second group of mice: one closed piece of proximal and one closed piece of distal colon (~1cm each) were fixed in 10% Zn formalin to confirm apoptosis scoring, and 3-4 cm of the distal colon were splayed opened and used for epithelial cell isolation.

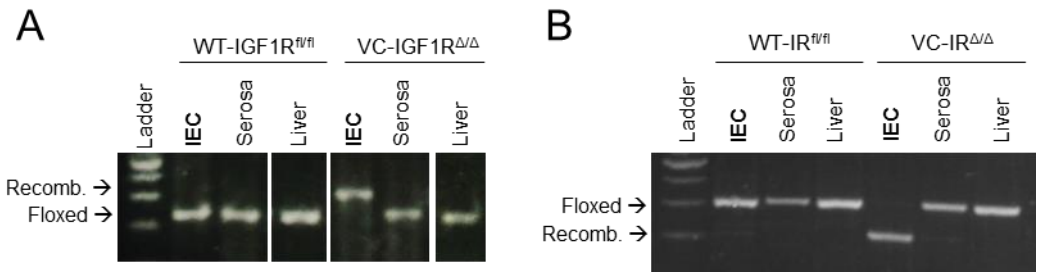

Figure 4.2: Intestinal epithelial cell (IEC)-specific villin-Cre mediated recombination of *Igf1r* and *Insr* genes in VC-IGF1R$^{\Delta/\Delta}$ and VC-IR$^{\Delta/\Delta}$ mice, respectively.

PCR gels for floxed regions in exon 3 of the *Igf1r* gene and exon 4 of the *Insr* gene in IEC, intestinal serosa, and liver DNA from (A) WT-IGF1R$^{fl/fl}$ and VC-IGF1R$^{\Delta/\Delta}$ mice and (B) WT-IR$^{fl/fl}$ and VC-IR$^{\Delta/\Delta}$ mice. Only mice carrying the villin-Cre recombinase are homozygous for the recombined (recomb.) alleles specifically in IEC and not in other tissues.

Table 4.1: IEC-specific loss of IGF1R or IR does not affect obesity-associated changes in body weight, blood glucose, plasma insulin, and insulin sensitivity.

	WT-IGF1R$^{fl/fl}$		VC-IGF1R$^{\Delta/\Delta}$		WT-IR$^{fl/fl}$		VC-IR$^{\Delta/\Delta}$	
	CTL	HFD	CTL	HFD	CTL	HFD	CTL	HFD
Body weight gain (%)	39.5 ±10.0	101.6 ±4.2[a]	31.2 ±8.1	93.9 ±10.5[a]	24.9 ±1.7	70.4 ±6.2[a]	25.7 ±3.2	71.0 ±5.8[a]
Fasting blood glucose (mg/dl)	127.1 ±11.3	164.6 ±16.4	127.6 ±17.3	167.1 ±13.8	108.2 ±2.3	151.8 ±11.3[a]	108.7 ±8.8	167.5 ±10.4[a]
Fasting plasma insulin (µg/l)	0.2 ±0.03	1.1 ±0.2[a]	0.3 ±0.1	1.3 ±0.3[a]	0.27 ±0.03	1.16 ±0.23[a]	0.27 ±0.07	1.14 ±0.19[a]
HOMA	2.3 ±0.4	13.9 ±3.0[a]	2.5 ±0.9	15.0 ±3.89[a]	2.0 ±0.2	12.6 ±2.7[a]	2.2 ±0.8	15.4 ±2.8[a]
Plasma IGF1(ng/ml)	31.8 ±11.2	46.7 ±3.8	40.8 ±4.0	37.1 ±6.6	49.3 ±2.6	52.2 ±4.5	49.7 ±3.3	51.2 ±2.2

a: $P < 0.05$ vs. CTL same genotype
Two-way ANOVA, Tukey's multiple comparisons test
$n \geq 5$

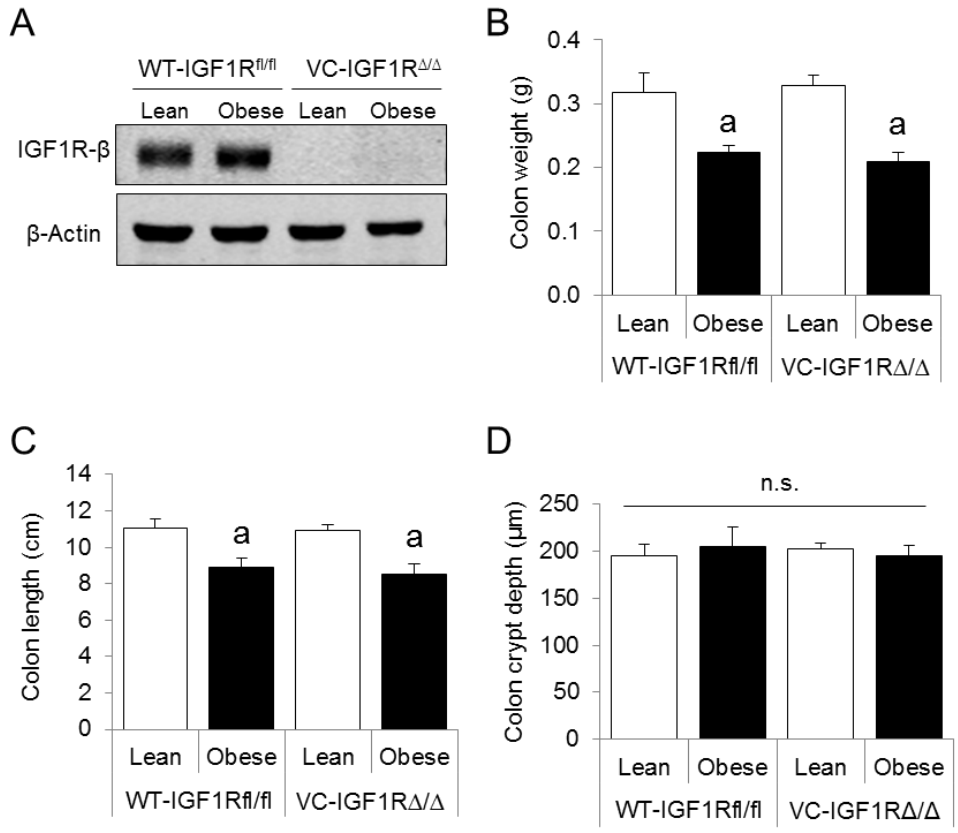

Figure 4.3: Obesity does not affect IGF1R protein expression in CECs of WT-IGF1R$^{fl/fl}$ mice and IGF1R loss does not alter colon weight, length, or crypt depth in lean or obese mice.
(A) Representative western blot for IGF1R protein in isolated CECs of lean and obese WT-IGF1R$^{fl/fl}$ and VC-IGF1R$^{\Delta/\Delta}$ mice. B-D: Measurements of colon (B) weight, (C) length and (D) crypt depth in each group. Western blot is representative of $n \geq 3$. Data in bar graphs represent mean ± SEM ($n \geq 4$). a: $P < 0.05$ *versus* lean same genotype, n.s.: no significance, two-way ANOVA with Tukey's multiple comparisons test.

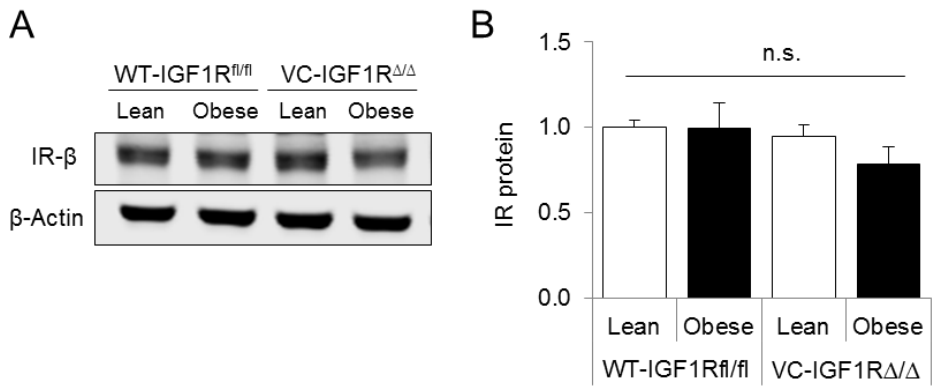

Figure 4.4: Neither obesity nor IGF1R deletion impact IR protein levels in CECs.
(A) Representative western blot showing IR protein in isolated CECs of lean and obese WT-IGF1R$^{fl/fl}$ and VC-IGF1R$^{\Delta/\Delta}$ animals. (B) Quantification of IR protein levels relative to β-actin expressed as mean ± SEM ($n \geq 3$), n.s.: no significance, two-way ANOVA.

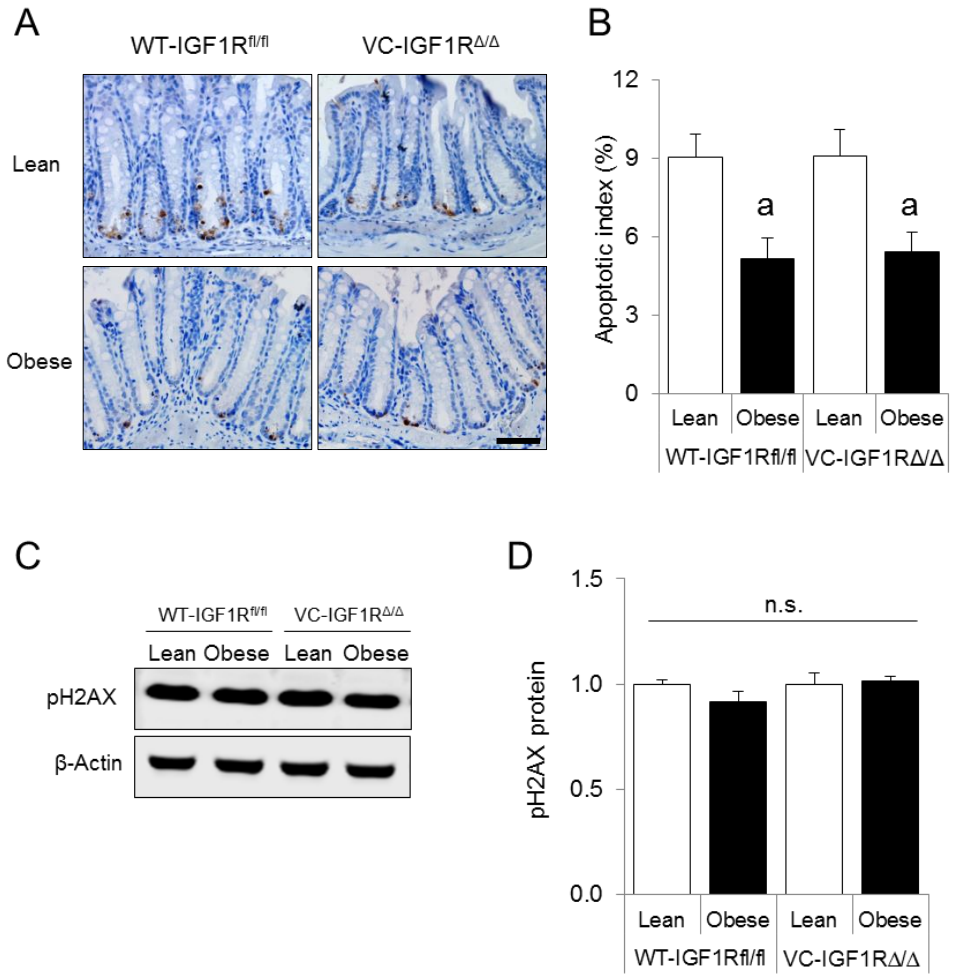

Figure 4.5: Reduced apoptosis of genetically damaged CECs in obese mice with intact IGF1R or IGF1R deletion and no changes in levels of the DNA damage marker pH2AX across groups.
(A) Representative images showing cleaved caspase-3 staining in distal colon of lean and obese WT-IGF1R$^{fl/fl}$ and VC-IGF1R$^{\Delta/\Delta}$ mice (40x objective, scale bar = 50 µm). (B) Apoptotic index calculated using the formula: number of cleaved caspase-3 positive cells/total number of cells x 100. Data represent mean ± SEM ($n \geq 5$). (C) Representative western blot showing pH2AX protein in isolated CECs of lean and obese WT-IGF1R$^{fl/fl}$ and VC-IGF1R$^{\Delta/\Delta}$ animals. (D) Quantification of pH2AX levels relative to β-actin expressed as mean ± SEM ($n \geq 3$). a: $P < 0.05$ *versus* lean same genotype, n.s.: no significance, two-way ANOVA with Tukey's multiple comparisons test.

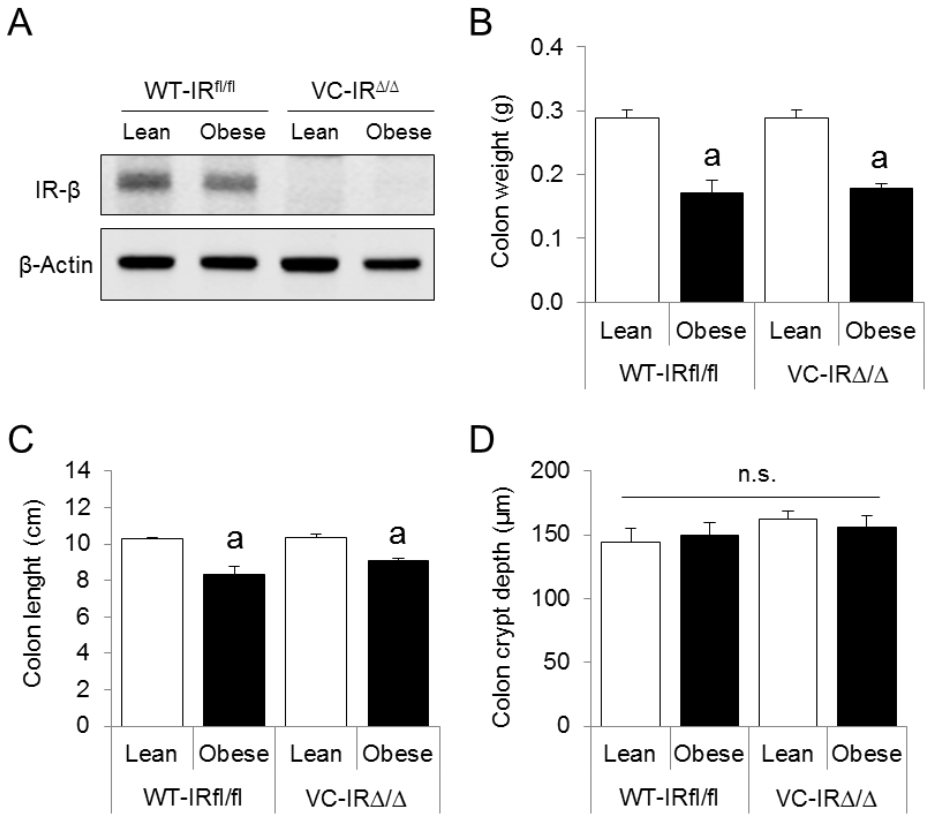

Figure 4.6: Obesity does not affect IR protein expression in CECs of WT-IR$^{fl/fl}$ mice and IR deletion does not affect measures of colon growth.

(A) Representative western blot for IR in isolated CECs of lean and obese WT-IR$^{fl/fl}$ and VC-IR$^{\Delta/\Delta}$ mice. B-D: Measurements of colon (B) weight, (C) length and (D) crypt depth in each group. Western blot is representative of n = 4. Quantitative data were expressed as mean ± SEM (n ≥ 4). a: $P < 0.05$ *versus* lean same genotype, n.s.: no significance, two-way ANOVA with Tukey's multiple comparisons test.

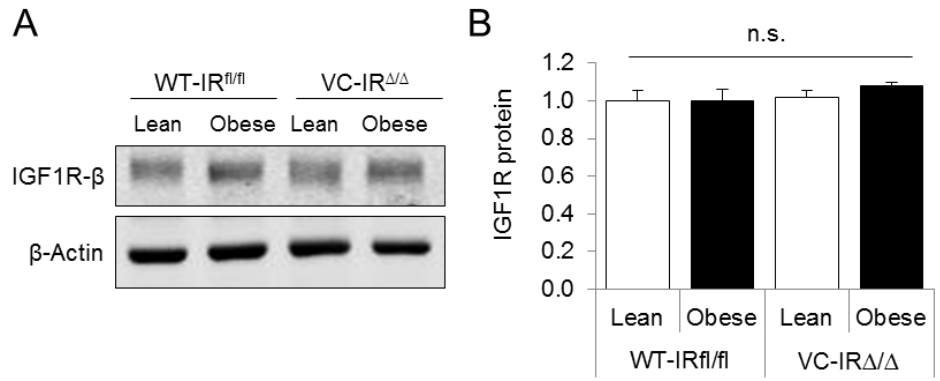

Figure 4.7: IR loss does not impact IGF1R protein levels in CECs of lean or obese mice.
(A) Representative western blot showing IGF1R protein levels in isolated CECs of lean and obese WT-IR$^{fl/fl}$ and VC-IR$^{\Delta/\Delta}$ animals. (B) Quantification of IGF1R protein relative to β-actin expressed as mean ± SEM (n = 4), n.s.: no significance, two-way ANOVA with Tukey's multiple comparisons test.

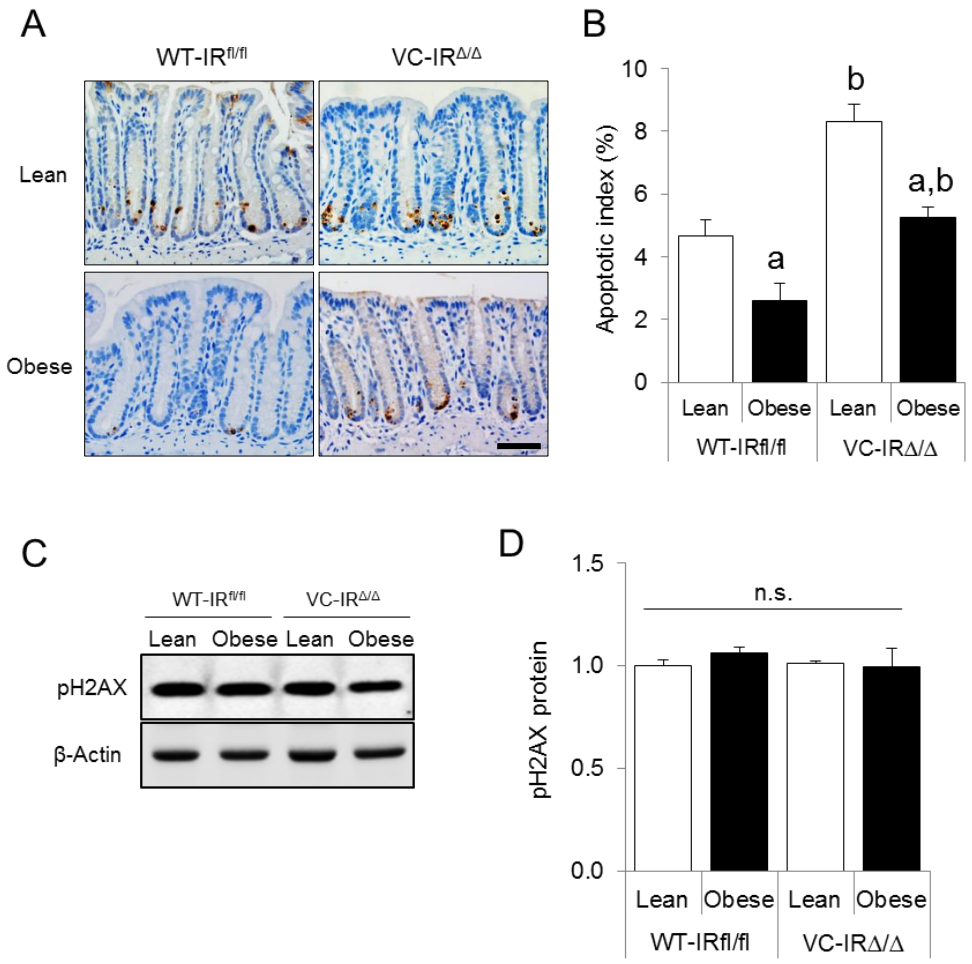

Figure 4.8: IR loss increases apoptosis of genetically damaged CECs in lean and obese mice and levels of pH2AX do no change across groups.

(A) Representative images showing cleaved caspase-3 staining in distal colon of lean and obese WT-IR$^{fl/fl}$ and VC-IR$^{\Delta/\Delta}$ mice (40x objective, scale bar = 50 μm). (B) Apoptotic index calculated using the formula: number of cleaved caspase-3 positive cells/total number of cells x 100. Data were expressed as mean ± SEM ($n \geq 5$). (C) Representative western blot showing pH2AX protein in isolated CECs of lean and obese WT-IR$^{fl/fl}$ and VC-IR$^{\Delta/\Delta}$ animals. (D) Quantification of pH2AX expression relative to β-actin expressed as mean ± SEM ($n = 4$). a: $P < 0.05$ *versus* lean same genotype, b: $P < 0.05$ *versus* WT same diet group, two-way ANOVA with Tukey's multiple comparisons test.

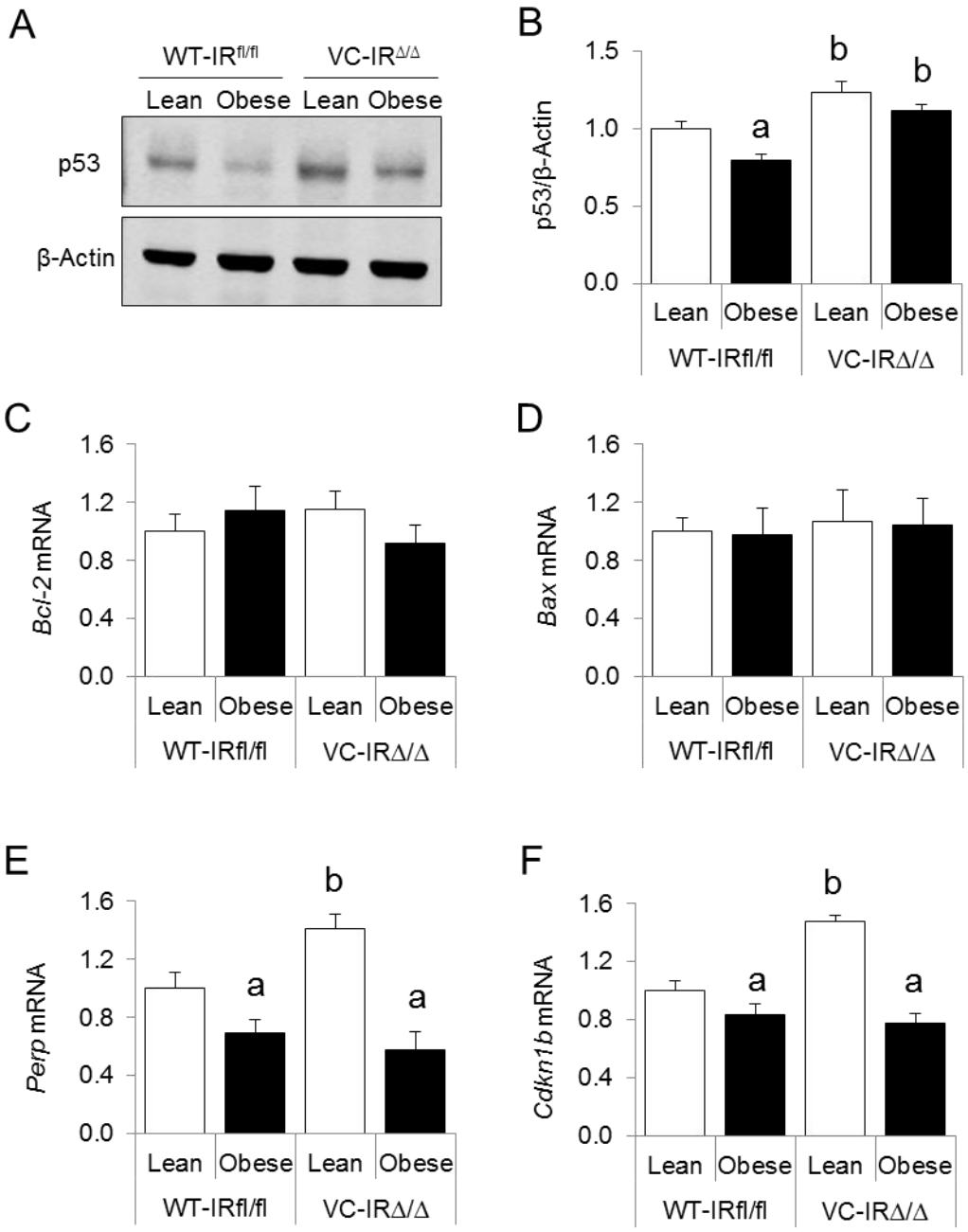

Figure 4.9: Obesity and IR deletion alter levels of p53 protein and p53-regulated mRNAs.
(A) Western blot showing p53 protein expression in isolated CECs of lean and obese WT-IR$^{fl/fl}$ and VC-IR$^{\Delta/\Delta}$ mice. (B) Quantification of p53 protein levels relative to β-Actin by densitometry. C-F: mRNA levels of (C) anti-apoptotic *Bcl-2*, (D) pro-apoptotic *Bax*, (E) pro-apoptotic *Perp*, and (F) tumor suppressor *Cdkn1b*. Bar graphs in panels B-F show fold change *versus* lean WT-IR$^{fl/fl}$ control. Data were expressed as mean ± SEM (n = 4 in duplicates for protein data and n ≥ 4 for mRNA data). a: $P < 0.05$ *versus* lean same genotype, b: $P < 0.05$ *versus* WT same diet group, two-way ANOVA with Tukey's multiple comparisons test.

CHAPTER 5: NOVEL ROLES FOR IR IN CRC RISK AND COLON APOPTOSIS: SIGNIFICANCE AND FUTURE AREAS OF INVESTIGATION

The first evidence for presence of insulin receptors in the mammalian colon was reported in 1985[314]. However, the function of IR in the intestine has been largely ignored, probably because the intestine does not rely on insulin for carbohydrate metabolism [315]. Thus, IR has been primarily studied in 'traditional' targets of the metabolic actions of insulin such as skeletal muscle, adipose tissue, and liver, as well as in the insulin-producing β-cells of the pancreas [273,316-318]. In contrast, significant attention has been given to intestinal IGF1R since it is generally considered the major mediator of the effects of IGF1, IGF2, or elevated insulin on growth, anti-apoptosis, regeneration, and cancer [78,112,120,129,130,203,235]. However, it is only recently that we and others have been able to generate mouse models with genetic deletion of IR or IGF1R in intestinal epithelial cells to more directly define the roles of these two related receptors.

In 1999, Frasca et al. provided evidence for increased IR-A expression in human colon cancer tissues [90]. Additionally, human studies have found positive correlations between elevated plasma insulin and risk of colorectal adenomas and cancer [155,186,187]. These lines of evidence and the fact that both IGF1 and insulin can signal through either IGF1R or IR prompted us to investigate the specific roles of each receptor in the early stages of colorectal carcinogenesis. Distinguishing functional roles of IGF1R and IR *in vivo* has been a challenge due to their structural and functional similarities. The mouse models with IEC-specific deletion of IGF1R or IR used in our studies are therefore powerful tools to directly define *in vivo* contributions of each receptor in distinct physiological and pathophysiological contexts. The fact that the IEC-IGF1R and IEC-IR deletion mutants had no detectable basal phenotype permitted us to challenge them with damage or tumor inducing stimuli to define their roles in disease-relevant situations.

This dissertation aimed to first define the expression levels of IGF1R, IR, and IR isoforms in the normal mucosa of patients with or without colorectal adenomas, in order to establish whether mRNA levels of these receptors could predict adenoma risk, particularly during elevated plasma insulin. In the second portion of this dissertation, IEC-specific IR knockout mice treated with AOM-DSS were used to determine the impact of IR loss on tumorigenesis. The third part of this work examined DNA damage, the earliest step in tumor initiation. The goal was to evaluate the effects of loss of IGF1R or IR on apoptosis of genetically damaged colon epithelial cells (CECs) during diet-induced obesity or hyperinsulinemia. This final chapter will discuss the overall findings, future directions for research, and the clinical significance of this work.

Hyperinsulinemia and colorectal adenoma risk

IR-A and IR-B isoforms or IGF1R as predictive biomarkers

The human study in the first part of this dissertation reported two main novel findings (Figure 5.1): (i) *IGF1R* mRNA is modestly but significantly down-regulated in the normal mucosa of adenoma cases compared with adenoma-free controls and (ii) increased IR-A:IR-B ratio is associated with increased risk of adenomas in patients in the upper half of plasma insulin. The latter appeared to be due to reduced IR-B and maintained IR-A expression, as observed semi-quantitatively based on RT-PCR. These results provide evidence that IR-B protects against early stage colon tumorigenesis in humans with elevated plasma insulin.

The findings that low *IGF1R* mRNA predicted adenoma risk were unexpected and may suggest that overexpression of *IGF1R* in carcinogenesis may become more relevant at later stages in the adenoma-cancer sequence. Since IGF1 is known to negatively feedback on IGF1R receptor expression in a number of cell types and its production is elevated in colon adenomas in preclinical models [97,319-321], it is possible that local IGF1 secreted by adenomas act in a paracrine manner on the surrounding normal mucosa to down-regulate IGF1R in cases. Increased circulating levels of free IGF1 could also contribute

to this down-regulation, but plasma IGF1 was not measured in this study and prior studies did not report elevated plasma IGF1 in adenoma patients [155]. We considered that high insulin may drive reduced *IGF1R* but *IGF1R* levels were elevated regardless of plasma insulin [269]. Thus the mechanism and significance of low *IGF1R* mRNA in adenoma cases remains to be further evaluated.

Interestingly, IR-A:IR-B ratios were increased in cases *versus* controls with high plasma insulin. This relationship was strengthened by logistic regression analyses showing that as IR-A:IR-B ratios increased, patients with elevated insulin were more likely to have adenomas than patients with low insulin. Rather than increased IR-A, these alterations in IR-A:IR-B ratios seemed to be dictated by reductions in IR-B. This suggests that insulin-induced regulation of IR isoform expression may be relevant to the mechanisms underlying adenoma risk. Total *IR* mRNA did not differ between cases and controls, suggesting that the relative amounts of IR isoforms should be the focus of future studies examining IR.

One question that remained unanswered by this study is whether adenomas express higher IR-A:IR-B ratios during normal or high plasma insulin. This is unknown in humans, but our recent publication showed increased IR-A:IR-B ratio in adenomas from $Apc^{Min/+}$ mice due to reduced IR-B [97]. Consistent with these findings, preliminary data obtained during this work showed that, compared with normal colon epithelium from untreated mice, adenomas from AOM-DSS mice had increased IR-A:IR-B ratio resulting from decreased IR-B (Figure 5.2).

Together, this work leads to a new area in colorectal cancer prevention research that should be further explored to define if altered IR-A, IR-B, or IGF1R expression in normal colon biopsies better predict colorectal adenoma risk, particularly in patients with hyperinsulinemia or insulin resistance. Thus, identification of those patients at greatest risk for CRC who would benefit from more frequent colonoscopy-based surveillance could help decrease CRC morbidity and mortality.

Regulation of IR isoform pre-mRNA splicing

There is currently limited information on the mechanisms controlling pre-mRNA splicing of IR isoforms. It is well established, however, that CUG RNA binding protein 1 (CUGBP1), muscleblind proteins (MBNL), and serine/arginine-rich splicing factors (SRSFs) regulate IR alternative splicing in muscle (Figure 5.3), and more recently we found that they also play a role in the intestine [97]. Most of the evidence for roles of these proteins in IR isoform expression resulted from studies on myotonic dystrophy (DM), an autosomal dominant muscle disease that is characterized by expanded CUG repeats in the RNA encoding DM protein kinase (DMPK) [322]. Patients show insulin resistance in skeletal muscle and this has been associated with increases in CUGBP1 that favor IR-A expression [322]. In contrast, MBNL1 and MBNL2 favor generation of IR-B (Figure 5.3), and this is inhibited by CUGBP1 [323]. *In vitro* studies demonstrated that MBNL1 and SRSF3 promote exon 11 inclusion and expression of IR-B by binding to intronic and exonic splicing enhancers, respectively [324,325]. CUGBP1 promotes exon 11 exclusion and expression of IR-A by binding to intronic and exonic splicing silencers [325]. Consistent with this evidence, our recent work showed that in differentiated Caco2 colorectal cancer cells, which express higher IR-B than in the undifferentiated state, CUGBP1 transcript levels were decreased and MBNL2 levels were increased [97]. In contrast, in colon and small intestinal adenomas from $Apc^{Min/+}$ mice where IR-A predominates, MBNL2 expression was reduced [97]. Studies on human liver recently demonstrated that compared with normal hepatocytes where IR-B is by far the predominant isoform, hepatocellular carcinomas showed increased relative expression of IR-A, which correlated with increased CUGBP1 levels [276]. Interestingly, CUGBP1-induced expression of IR-A was regulated by the oncogenic EGFR/MAPK pathway [276].

Expression patterns of these splicing factors in human intestine have not been examined. Measuring the levels of splicing factor mRNAs in the human biopsies used in our studies could provide insight into mechanisms by which high plasma insulin up-regulates IR-A:IR-B ratio to promote adenoma risk. In biopsies from cases with elevated insulin, which show increased IR-A:IR-B ratio due to reduced IR-B, we would anticipate decreased MNLBs and increased or maintained CUGBP1. More direct

mechanistic studies on whether elevated insulin itself or insulin resistance affects IR isoform splicing could be addressed *in vivo or in vitro*. Using the conditional reprogramming method described in Chapter 3, cells from normal colon mucosa of patients with or without adenomas, or human adenoma cells, if available, could be plated on feeders or matrigel to evaluate their proliferative capacity and the relative abundance of IR isoforms with or without insulin treatment. Knockdown of IR by shRNA and re-expression of IR-A or IR-B would test whether insulin-mediated cell growth and survival are enhanced by IR-A or attenuated by IR-B. Alternatively, splicing factors could be modulated by transfecting cells with lentiviral constructs expressing shRNAs for *MBNL2* and *CUGBP1*, which are available in our laboratory. If increased IR-A levels relative to IR-B prove to be a driver of adenoma cell growth and anti-apoptosis, treatment with EGFR inhibitors to reduce IR-A expression and favor IR-B would be of interest. EGFR inhibitors are currently used in combination with chemotherapy for the treatment of CRC[32], and an effect on IR splicing in colonocytes or cells from adenomas would provide insight into mechanisms by which EGFR inhibitors may prevent or treat CRC. Additionally, given the role of EGFR in IR splicing in liver[276] and our previous studies demonstrating synergistic proliferative effects of EGF and IGF in intestinal epithelial cells [326], it would be of interest to test EGF and IGF in combination to assess if they more potently down-regulate IR-B or affect splicing factors.

Mechanisms of decreased colon epithelial cell apoptosis during obesity/hyperinsulinemia

Chapter 4 of this dissertation described the impact of obesity and IR on apoptosis of CECs after radiation-induced DNA damage. We provided evidence that obesity and hyperinsulinemia were associated with decreased apoptosis in the colonic crypts and, importantly, this was confirmed in two independent mouse colonies. This supports a previous human study where elevated plasma insulin correlated with decreased apoptosis in normal rectal mucosa [155]. A somewhat similar finding in mice was reported after 5 injections of AOM, where AOM-induced apoptosis was decreased in animals that had been on high fat diet (HFD) for 7 or 15 weeks before the last AOM dose [327]. However, mice did not

appear to be obese or hyperinsulinemic, and the authors attributed reduced AOM-induced apoptosis to the lower per-body-weight dose of AOM that the obese mice had received [327].

We originally hypothesized that the growth-promoting IGF1R would be required for any anti-apoptotic effects of obesity/hyperinsulinemia. Unexpectedly, genetic deletion of IGF1R in CECs did not impact apoptosis in lean or obese mice. We then asked if IR played a role in mediating these effects. Although IR loss did increase apoptosis in both lean and obese animals, which was an exciting and novel finding, apoptosis remained lower in obese mice relative to lean mice lacking IR. These data imply that IR is not required for the reductions in apoptosis associated with obesity/hyperinsulinemia. The current section will discuss potential mechanisms that may explain lower radiation-induced apoptosis in obese *versus* lean mice whether or not IR is present, as summarized in Figure 5.4.

IGF1R hyperactivation due to IR deletion

One hypothesis we could formulate is that in the absence of IR, elevated insulin as occurs in our obese model exerts anti-apoptotic signals through the remaining IGF1R (Figure 5.4). Although IGF1R protein levels did not increase to compensate for IR loss, we cannot rule out a compensatory mechanism by increased IGF1R activation rather than expression. This could be possible given that IR deletion enhanced tumor cell growth and increased IGF1-induced AKT activation as suggested in our studies in Chapter 3. Evaluating phosphorylation of IGF1R or downstream mediators in the intestinal epithelium *in vivo* is difficult because signaling pathways are activated during handling and harvest of the intestine and almost certainly affected by epithelial cell isolation methods. *In vitro* studies in conditionally reprogrammed CECs isolated from WT-IR$^{fl/fl}$ and VC-IR$^{\Delta/\Delta}$ provide an alternate approach.

AMP-activated protein kinase

AMP-activated protein kinase (AMPK) is a key metabolic sensor that is activated in response to increased intracellular AMP/ATP ratios, when energy in the cell is low [328]. AMPK activation induces energy-producing processes such as glucose uptake, glycolysis, and fatty acid oxidation, and inhibits

energy-consuming processes such as gluconeogenesis, lipogenesis, and glycogen synthesis as well as, in some organs, cell proliferation [201,329]. It is well known that AMPK action is decreased in situations of insulin resistance and obesity [330], and activation of AMPK by drugs such as metformin improves glucose uptake in muscle and inhibits hepatic gluconeogenesis during type 2 diabetes [200].

Moreover, a role for AMPK in modulating the DNA damage response has been reported [329]. *In vitro* studies suggest that AMPK activation sensitizes cancer cells to chemotherapy or radiation by blocking cell cycle progression via p53, p21, and p27 induction as well as inhibiting the pro-survival mammalian target of rapamycin (mTOR) pathway [329]. Therefore, AMPK has been implicated in cell cycle arrest and apoptosis following DNA damage [329]. These mechanisms should be examined in our studies, as AMPK activity in the intestine could be reduced as a consequence of obesity and insulin resistance, thereby contributing to decreased radiation-induced apoptosis of CECs in obese mice (Figure 5.4). It should be noted that AMPK activates the cell cycle inhibitor p27, and we found p27 mRNA to be down-regulated in CECs from obese mice. However, no significant changes were observed in markers of S-phase (5-ethynyl-2'-deoxyuridine, EdU) or M-phase (phospho-histone 3, pH3) in obese mice (Figure 5.5). The role of AMPK in the colon has not been well explored, but a study reported that in mice fed a high-energy diet, AMPK activation by metformin decreased tumor growth of xenografts derived from a CRC cell line [279]. This was associated with decreased phosphorylation of AKT signaling and increased expression of apoptosis markers in tumors cells [279]. Moreover, activation of AMPK was associated with reductions in AOM-induced ACF in the *db*/*db* mouse model of obesity and diabetes treated with the anti-oxidant curcumin or the cholesterol-lowering drug pitavastatin [331,332]. Therefore, metformin treatment prior to radiation may sensitize CECs of obese animals to radiation-induced apoptosis, as was previously shown in cancer cells [200]. If in obese mice metformin-induced AMPK activation increased apoptosis of genetically damaged CECs back to levels observed in lean mice, this would be exciting mechanistic evidence for roles of impaired AMPK action. Furthermore, these experiments would support a potential mechanism for the anti-tumor effects of metformin, specifically, an effect to prevent genetically damaged cells from expanding and forming pre-neoplastic lesions in the colon.

Microbiota

The gut microbiota is an important regulator of energy balance and a key factor contributing to development of obesity, as demonstrated by the fact that germ-free (GF) mice do not become obese after HFD feeding [71]. An increase in the proportion of *Firmicutes* relative to *Bacteroidetes* phyla during obesity has been well documented in both mice and humans [69,333]. *Firmicutes* consist mostly of gram-positive bacteria, while *Bacteroidetes* belong to the category of gram-negative bacteria [334]. Gram-positive bacteria are known to produce the short-chain fatty acid butyrate, which can prevent apoptosis of colonocytes [335,336]. Therefore, a shift to increased abundance of butyrate-producing bacteria as a result of obesity could contribute to reduced colonocyte apoptosis after genetic damage, independently of IR (Figure 5.4). Butyrate levels in the feces could be measured to determine if they differ between lean and obese animals. If a shift towards butyrate-producing bacteria correlated with decreased apoptosis during obesity, the microbiota could be manipulated in obese mice so that the *Firmicutes*-to-*Bacteroidetes* ratio is restored to values observed in lean mice prior to radiation. Monitoring butyrate in the feces would provide information on the time point at which butyrate levels are comparable between the two groups, and animals would then be irradiated to evaluate effects on apoptosis. We would predict that normalization of the *Firmicutes*-to-*Bacteroidetes* ratios would increase levels of radiation-induced apoptosis in obese mice to levels observed in lean mice. An alternate approach would be to colonize germ-free mice with microbiota from lean or obese mice before or after irradiation and test if the microbiota from obese mice is sufficient to confer reduced apoptosis.

Linking the anti-tumorigenic and anti-apoptotic roles of IR in the colon

The work described in our AOM-DSS study in Chapter 3 provided novel evidence that IR protects against colon tumorigenesis by enhancing the oncogenic actions of IGF1R. In line with this evidence, it would be reasonable to hypothesize that IGF1R and not IR is the main mediator of CEC survival after genetic damage, which may favor initiation of colonic pre-cancerous lesions. Surprisingly, IR but *not* IGF1R deletion was found to result in higher apoptosis, as shown in Chapter 4, suggesting

anti-apoptotic roles of IR at least in the context of early radiation-induced apoptosis. The counterintuitive results that IR loss in CECs leads to enhanced tumorigenesis in the AOM-DSS model but enhanced apoptosis in a genetic damage model raise two important questions for future consideration: (i) what is the significance of apoptosis in the colon epithelium after radiation-induced DNA damage with respect to tumor development and (ii) what is the role of colon epithelial IGF1R during DNA damage and tumorigenesis and does this depend on whether IR expression or signaling is intact? These questions will be addressed below.

Apoptosis: A beneficial or detrimental response to radiation-induced DNA damage?

One interpretation of enhanced apoptosis of genetically damaged CECs due to IR loss is that IR has anti-apoptotic roles that may favor tumorigenesis by allowing genetically damaged cells to escape apoptosis. This interpretation assumes that apoptosis is a beneficial response to DNA damage that reduces tumor risk. This is based on the well-established concept that apoptosis occurs to eradicate genetically damaged cells that accumulate mutations or chromosomal aberrations and could survive and expand to initiate cancer [288,289]. However, our observations indicate that IR normally limits radiation-induced apoptosis and limits tumor development at least in the AOM-DSS model. Therefore, these findings suggest that apoptosis may have no role in the AOM-DSS model or, alternatively, increased apoptosis in the absence of IR might have unfavorable consequences for tumorigenesis which may be dependent on inflammation. Massive DNA damage and apoptosis can have adverse effects on the regenerative capacity of stem cells or progenitors, thus compromising epithelial renewal [289]. Increased apoptosis can disrupt the integrity of the epithelial barrier, allowing penetration of luminal bacteria into the mucosa which triggers an inflammatory response (Figure 5.6) [337-339]. It is well known that inflammation causes DNA damage, but emerging evidence has led to a new concept that DNA damage can in fact trigger inflammation [340]. This process, which is reviewed in detail by McCool and Miyamoto, 2012, appears to involve activation of NF-κB by ATM-induced phosphorylation of NEMO (NF-κB essential modulator) [341-343]. Therefore, IR loss could favor tumor initiation by increasing apoptosis and secondarily exacerbating inflammation after

DNA damage (Figure 5.6). We have in hand NF-κB^{EGFP} mice, which express EGFP upon NF-κB activation [67]. Generating NF-κB^{EGFP} mice with IEC-specific IR deletion (NF-κB^{EGFP}/VC-IR$^{\Delta/\Delta}$) would allow us to test if loss of IR favors enhanced NF-κB activation as a biomarker of inflammation in the colon epithelium after DNA damage. Evaluating inflammatory markers in VC-IR$^{\Delta/\Delta}$ CECs would provide information on the extent of inflammation resulting from DNA damage in the absence of IR. Moreover, additional time points and higher radiation doses would allow us to assess if IR loss affects later waves of apoptosis that follow the initial wave at ~4 hours after radiation or impairs epithelial regeneration. Whether loss of IR in the colon epithelium affects barrier function or permeability is unknown, but our previous *in vitro* findings provided evidence that IR-B promotes barrier function in differentiated Caco2 cells [97]. Assessing colon epithelial permeability in WT-IR$^{fl/fl}$ *versus* VC-IR$^{\Delta/\Delta}$ mice would be possible by rectal administration of the fluorescent probe FITC-dextran [344]. We would predict that VC-IR$^{\Delta/\Delta}$ animals would have elevated levels of FITC-dextran in serum and increased FITC immunofluorescence within the colonic mucosa, reflecting increased epithelial permeability. In summary, our work supports a model where increased apoptosis caused by IR loss may contribute to barrier dysfunction and increased permeability that enhances inflammation after DNA damage, which could explain the increased inflammation-associated colon tumorigenesis observed in mice lacking IR (Figure 5.6).

Contributions of IGF1R to apoptosis and tumorigenesis in the absence of IR

In order to understand the consequences of IR loss on radiation-induced apoptosis and tumor initiation, it is necessary to understand what roles IGF1R plays during these events. IGF1R has previously been linked to resistance to apoptosis induced by DNA damage. Inhibition of IGF1R was shown to sensitize multiple cancer cells lines to apoptosis induced by radiation and chemotherapeutic agents [345-348]. *In vivo* work using breast, gastric, and pancreatic xenografts provided further evidence that combining radiation and IGF1R inhibition more effectively reduced tumor growth [349-351]. Nevertheless, the role of IGF1R in modulating apoptosis of non-cancerous cells in animal models has not been well explored. In the normal intestine, the concept that IGF1R may exert anti-apoptotic actions is supported by findings

that apoptosis was decreased in mice overexpressing IGF1 and increased in mice lacking IRS-1 [112,113,235]. However, these studies did not specifically address whether these effects were mediated by IGF1R or IR. Our work demonstrated that in colon, IR loss rather than IGF1R loss increased radiation-induced apoptosis of CECs. It is therefore possible that in the normal colon, preventing apoptosis is a physiological function of IR and not IGF1R. Since previous work by our group showed that local production of IGF1 is increased in the colon of DSS-treated mice [352], it could be speculated that colon epithelial IGF1R may, instead, play a larger role in mediating the regenerative and proliferative actions of IGF1 following mucosal injury [131]. We have in hand colon histology samples from WT-IGF1R$^{fl/fl}$ and VC-IGF1R$^{\Delta/\Delta}$ treated with exogenous IGF1 for 5 days after 14-Gy radiation. Evaluating the degree of IGF1-induced crypt regeneration in the presence or absence of IGF1R should provide insight into the role of IGF1R in mediating colon mucosal repair. These experiments could also be performed on WT-IR$^{fl/fl}$ and VC-IR$^{\Delta/\Delta}$ mice to define whether IGF1 signals primarily through IGF1R or IR to promote colon epithelial regeneration. If IGF1R is found to be essential for repair after injury caused by high dose radiation, the same could be true for injury caused by inflammation. Therefore, in the context of inflammation-induced mucosal damage, IGF1R hyperactivation in VC-IR$^{\Delta/\Delta}$ mice may occur in an effort to heal the damaged tissue and compensate for loss of the anti-apoptotic IR. Hyperactive IGF1R combined with exposure to a carcinogen, like AOM, would lead to enhanced proliferation of aberrant cells, which will eventually favor colon tumorigenesis (Figure 5.7).

Additional future studies addressing the roles of colon epithelial IR *in vivo*

Generation of IEC-specific IR-B knockout mice

IR has been understudied in the intestine and our previous and current studies indicate that further research on the roles of IR in the gut are critical to understanding mechanisms of intestinal physiology and disease. To confirm our findings that deletion of IR in IEC enhances colon tumorigenesis, future studies will use WT-IR$^{fl/fl}$ and VC-IR$^{\Delta/\Delta}$ mice crossed with Apc$^{Min/+}$ mice to test if IR loss also promotes formation of spontaneous colon tumors driven by a mutation that increases CRC risk in humans.

IR isoforms have attracted significant interest in recent years because of their potential roles in metabolism and cancer. To date, the availability of resources to study the individual isoforms is limited due to the very small difference in their mRNA and protein sequences (36 base pairs and 12 aminoacids, respectively). Our mouse studies on colon cancer and apoptosis suggested novel roles for IR, but the individual contributions of IR-A and IR-B remain unknown. *In vivo* or *in vitro* models targeting exclusive expression or deletion of IR-B or IR-A will be very useful to specifically delineate their roles. Transgene-mediated expression of IR-B is one approach, potentially using the villin promoter. Disruption of only IR-B by deleting exon 11 would directly test if expression of IR-B protects against tumors. Re-introduction of IR-A or IR-B in cultured VC-IR$^{\Delta/\Delta}$ tumor cells is another approach, assuming we can transduce these cells with sufficient efficiency.

Modeling obesity-associated colorectal cancer

Because of the current obesity epidemic and its link to increased CRC risk, the need for a good model of obesity-associated CRC is critical. To date, there are no animal models that successfully combine obesity and colon cancer. Since obesity is associated with intestinal inflammation [66], we used the DSS-AOM approach in an effort to mimic tumorigenesis that occurs in a setting of chronic inflammation, as occurs in obesity. However, this model is not optimal.

Researchers have used multiple AOM injections in combination with HFD feeding, but a major problem with this model is that in the standard 20-week time point after the last AOM injection, animals do not become obese [327,353]. Other groups used AOM treatment in the *db/db* genetic model of obesity and type 2 diabetes but chose earlier time points to assess the impact of obesity on pre-cancerous ACF and did not report on tumor development [331,332]. In order to achieve obesity and significant weight gain, Tuominen et al. maintained mice on HFD for 9-10 months, allowing them to recover the weight they had lost between the start of the AOM treatment and the fourth week after the end of AOM [327]. However, the authors did not measure fasting blood glucose or plasma insulin, so the insulin resistance status in these

mice was not clear. Furthermore, a concern about such long-term experiments is the confounding factors that may result from ageing.

Genetic models of intestinal adenomas and obesity/diabetes have previously been generated by crossbreeding *db/db* mice with $Apc^{Min/+}$ mice [354,355]. Although animals with *db/db*-$Apc^{Min/+}$ mutations develop significantly more small intestinal adenomas than those with $Apc^{Min/+}$ alone, the number of colon adenomas ranged between 0 and 2 per mouse [354,355]. In a report by Algire et al., animals were fed a "high-energy diet" and given a subcutaneous injection with a mouse colon carcinoma cell line, in order to study the impact of diet-induced hyperinsulinemia on tumorigenesis and, also, the beneficial effects of metformin [279]. However, an important limitation of xenograft models is that tumor growth does not occur in the colon and the tumor microenvironment is therefore different [356]. To overcome this issue, "orthotopic" models of CRC have been described, where cancer cell lines or cells from previously grown tumors are implanted in the submucosa of the cecum or rectum [356-359]. Although technically challenging, the authors of these studies argued that these models permit rapid cancer formation and mimic human CRC better than subcutaneous implantation [356-359]. Although the orthotopic approach has never been implemented in models of obesity, it may represent a possible option for studying obesity-associated CRC. A less invasive and more feasible alternative would be to colonize germ-free $Apc^{Min/+}$ mice with gut microbiota from obese mice or humans and test whether microbes from obese donors increase tumors. Transfer of gut microbes from an obese donor to a lean host was previously shown to induce metabolic changes characteristic of an obese phenotype within 2-6 weeks [360,361]. This would provide an experimental design that is time effective and would overcome the problem of body weight loss resulting from the AOM treatment. However, the problem this colonization model may face is the low tumor incidence in the colon.

Overall, development of a reliable animal model of obesity-associated CRC combined with IEC-specific deletion of IR will be a critical step in our future studies. These models would significantly contribute to improving our ability to study mechanisms of increased CRC risk in the growing obese population and should therefore be a priority in the field of gastroenterology research.

Conclusions and overall significance

The work presented in this dissertation highlights the importance of IR or IR isoforms in CRC risk, which have been under-emphasized as major focus has been centered on IGF1R. As described above, the larger attention given to IGF1R resulted from findings that this receptor is overexpressed in colon tumors and provides resistance to cancer treatments. However, our studies suggest that more attention should be focused on the roles of these receptors in *earlier* stages of colorectal neoplasia to better understand their contributions to CRC risk. This would ultimately lead to improved diagnosis and prevent progression to malignant lesions.

A major concept emerging from the current research is that decreased IR function, particularly IR-B, may promote colon tumors by enhancing oncogenic actions of IGF1R, and this may be a particular problem during elevated insulin as occurs in obesity. The reduced IR-B expression in mouse colon tumors and in normal rectal mucosa of patients with adenomas and elevated plasma insulin suggest that strategies to maintain IR-B signaling may be beneficial preventive measures. Therefore, insulin-sensitizing drugs may represent good candidates for CRC prevention in obesity even before type 2 diabetes occurs. Loss of IR-B signaling during insulin resistance may promote compensatory IGF1R activation and predispose to cancer. Generation of animal models lacking IR-B will provide information on whether IR-B loss promotes tumorigenesis by enhancing both IGF1R and IR-A action or if the remaining IR-A is sufficient to attenuate IGF1R signaling. Based on our *in vivo* evidence that IR favors decreased apoptosis in normal colon epithelium, we speculate that this effect results from IR-A action, which may normally protect cells from apoptosis induced by environmental factors that cause DNA damage. We therefore hypothesize that, during colon homeostasis, IGF1R may be primarily proliferative and IR-A anti-apoptotic, while IR-B may act as a rheostat to attenuate the growth and anti-apoptotic actions of IGF1R and IR-A, respectively, and favor differentiation (Figure 5.8). During carcinogenesis in the context of obesity or insulin resistance, IR-B signaling may be impaired and therefore cannot limit the proliferative and anti-apoptotic actions of IGF1R and IR-A (Figure 5.8). This would lead to the conclusion that both IR-A and IGF1R are likely to contribute to tumor growth, and inhibitors targeting specifically these receptors without affecting

IR-B may represent an effective therapy for CRC [151]. If IR-B is dysfunctional as a result of insulin resistance, combined IGF1R/IR-A inhibitors and insulin-sensitizing medications such as metformin would successfully block IGF1R/IR-A activity and promote IR-B signaling [151].

To date, most of our knowledge of the impact of the insulin/IGF pathway on intestinal growth come from studies focused on the small intestine [113,129,137,138,235,290]. Our research strongly suggests that more studies on colon are critical in order to understand the mechanisms of colon responses to genotoxic stimuli, regeneration after injury, or cancer. Findings from such studies are relevant to improving prevention strategies or therapeutic interventions for the increased risk of CRC associated with obesity and type 2 diabetes. Further studies validating reduced IR-B expression in normal mucosa as a predictive biomarker of CRC risk could have important implications in CRC prevention and early diagnosis. Our epidemiological and pre-clinical work supports development of new approaches to maintain IR-B expression and signaling in the colonic epithelium to prevent or treat CRC, especially in obese and diabetic individuals who are at increased risk of CRC.

Figures

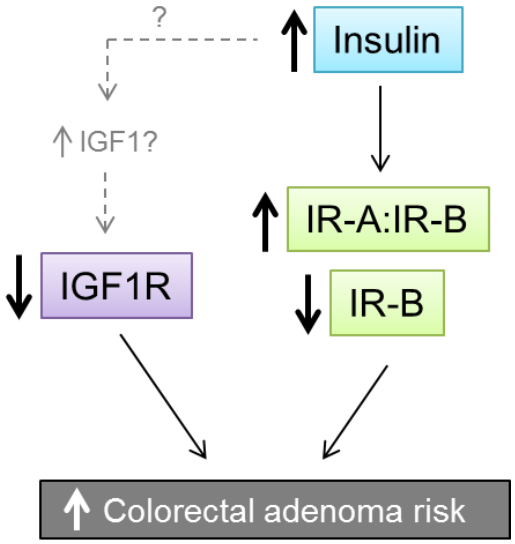

Figure 5.1: Summary of key findings in the human study described in Chapter 2.
Decreased levels of *IGF1R* mRNA predicted increased risk of colorectal adenomas. This may be due to increased endocrine or paracrine effects of IGF1, which would down-regulate *IGF1R* by negative feedback. Increased IR-A:IR-B ratio was associated with increased likelihood of having adenomas in patients with high plasma insulin.

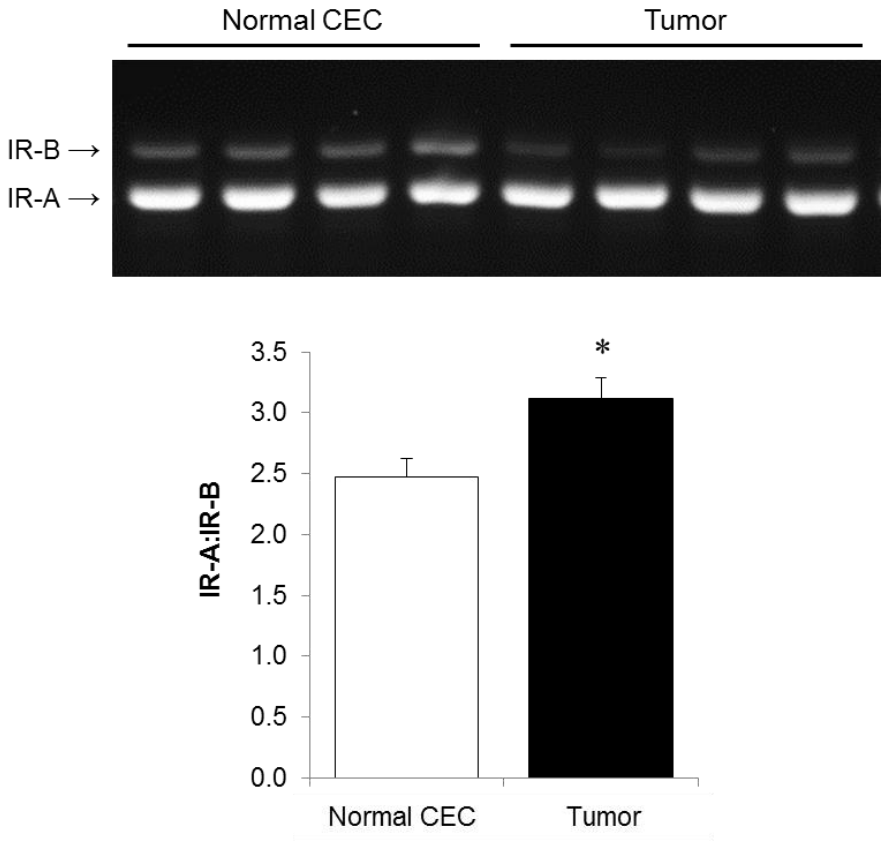

Figure 5.2: IR-A:IR-B ratio is increased in colon tumors versus normal colon epithelium, and this is due to reduced IR-B mRNA.

IR-A and IR-B mRNA levels were assessed as described in Andres et al., 2013, in normal colon epithelial cells (CEC) from untreated and tumors from AOM-DSS treated WT-IR$^{fl/fl}$ mice. IR-A:IR-B ratio was significantly increased in tumors relative to normal CECs (26 ± 7%, $P = 0.03$). Qualitative analyses show a reduction in IR-B and maintained IR-A expression. Data are expressed as mean ± SEM (n = 4), *$P <$ 0.05, unpaired t-test.

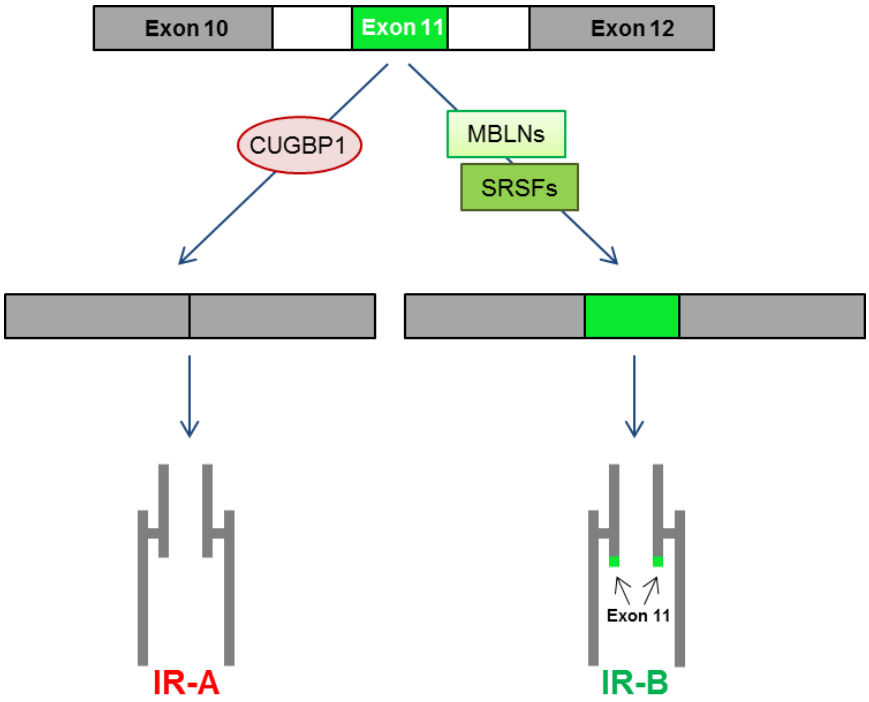

Figure 5.3: Regulation of IR isoform pre-mRNA splicing.
IR isoforms result from alternative pre-mRNA splicing. CUG binding protein 1 (CUGBP1) promotes exclusion of exon 11 and expression of IR-A. Muscleblind proteins (MBLNs) and serine/arginine-rich splicing factors (SRSFs) favor inclusion of exon 11 and expression of IR-B. (References: Savkur et al., 2001, Dansithong et al., 2005, Sen et al., 2009 and 2010, Andres et al., 2013).

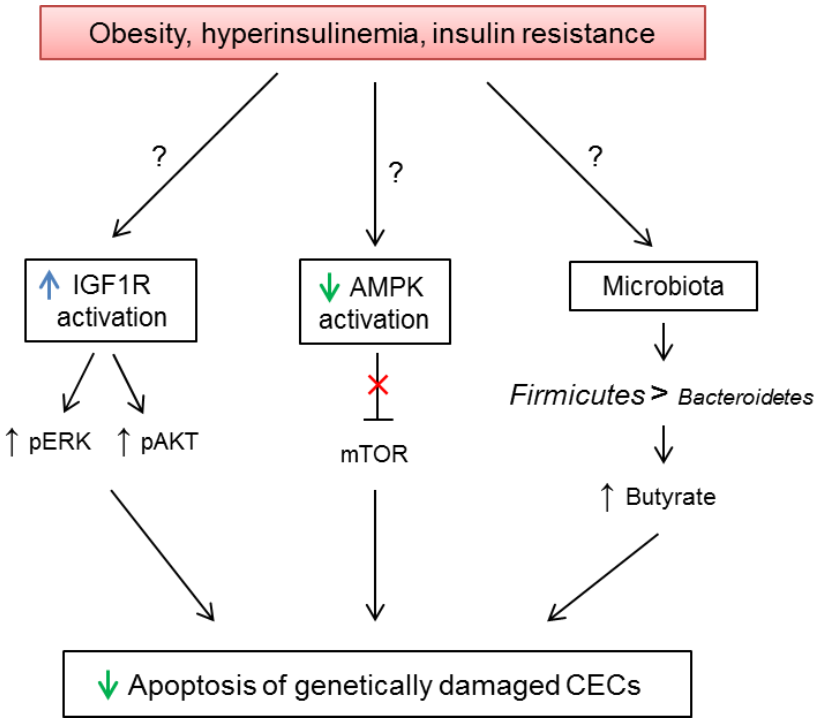

Figure 5.4: Possible mechanisms of decreased radiation-induced apoptosis during obesity, hyperinsulinemia, and insulin resistance.
IGF1R activation may be enhanced in the absence of IR and mediate anti-apoptotic signals associated with hyperinsulinemia via increased phosphorylation of ERK or AKT. During the DNA damage response, AMP-activated protein kinase (AMPK) suppresses the anti-apoptotic actions of mammalian target of rapamycin (mTOR). However, AMPK action is decreased during insulin resistance and the resulting lack of mTOR inhibition may play a role in decreasing apoptosis of genetically damaged colon epithelial cells (CECs). It is well-established that during obesity there is a switch in the microbiota composition towards increased *Firmicutes* relative to *Bacteroidetes*. *Firmicutes* produce butyrate, which can decrease apoptosis in the colonic mucosa. An increase in butyrate-producer bacteria could therefore contribute to reduced apoptosis in obese mice. (References: Ruderman et al., 2013, Sanli et al., 2014, Pernicova et al., 2014, Ley et al. 2005 and 2006, Louis et al., 2009, Hass et al., 1997, Claus et al., 2003).

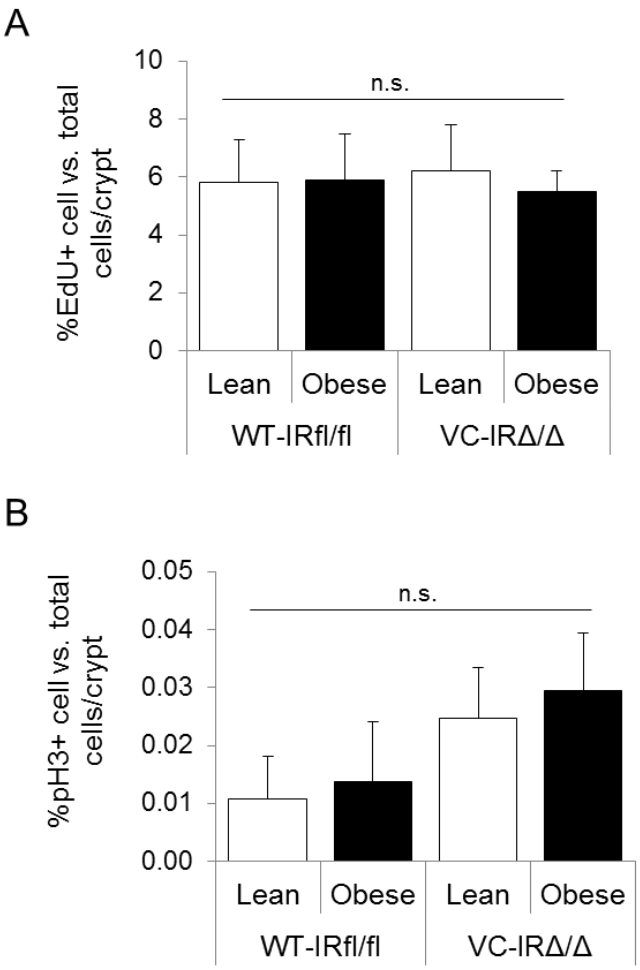

Figure 5.5: Markers of S- and M-phase are not altered by diet or IR loss 4 hours after radiation-induced DNA damage.

The number of cells in S-phase or M-phase in lean and obese WT-IR$^{fl/fl}$ and VC-IR$^{\Delta/\Delta}$ mice was assessed by histology using EdU (5-ethynyl-2'-deoxyuridine) or pH3 (phospho-histone 3) markers, respectively. No significant changes in the number of (A) EdU or (B) pH3 positive cells were found across groups. Data represent mean ± SEM ($n \geq 3$ for EdU and $n \geq 4$ for pH3), n.s.: no significance, two-way ANOVA.

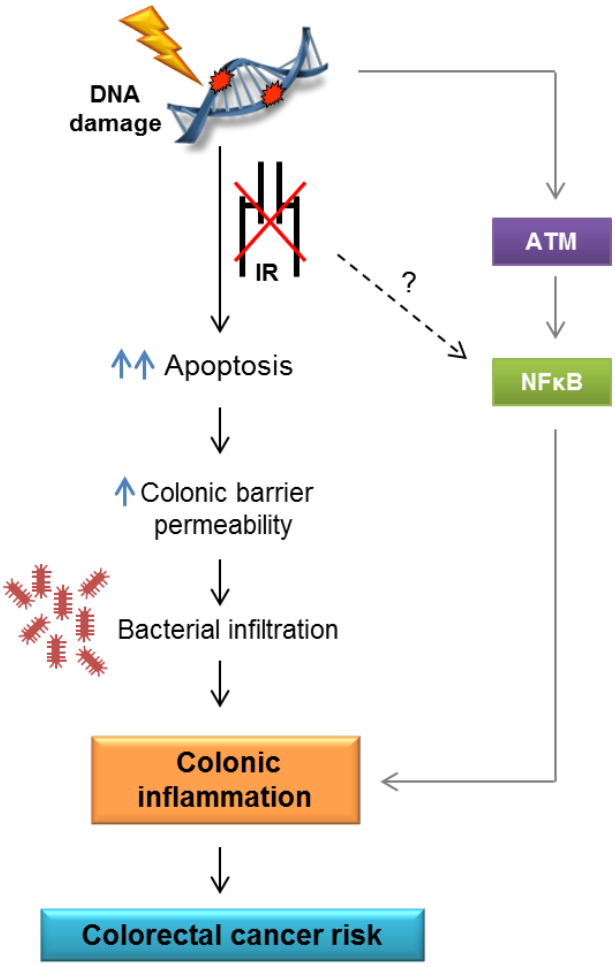

Figure 5.6: Proposed model of enhanced colon tumor susceptibility resulting from increased apoptosis after DNA damage.
Loss of IR leading to increased apoptosis could compromise the epithelial integrity of the colon, making it more permeable. This would allow components of the microbiota to penetrate the mucosa and cause inflammation. DNA damage in itself has been suggested to lead to inflammation via NFκB, which is activated by ATM (ataxia telangiectasia mutated) in response to DNA damage. Thus, inflammation as a result of DNA damage could be further exacerbated by IR loss. Whether IR plays a role in activation of NFκB is unknown. (References: Bojarski et al., 2001, Schulzke et al., 2006, Nenci et al., 2007, Kidane et al., 2014, Piret et al., 1999, Wu et al., 2006).

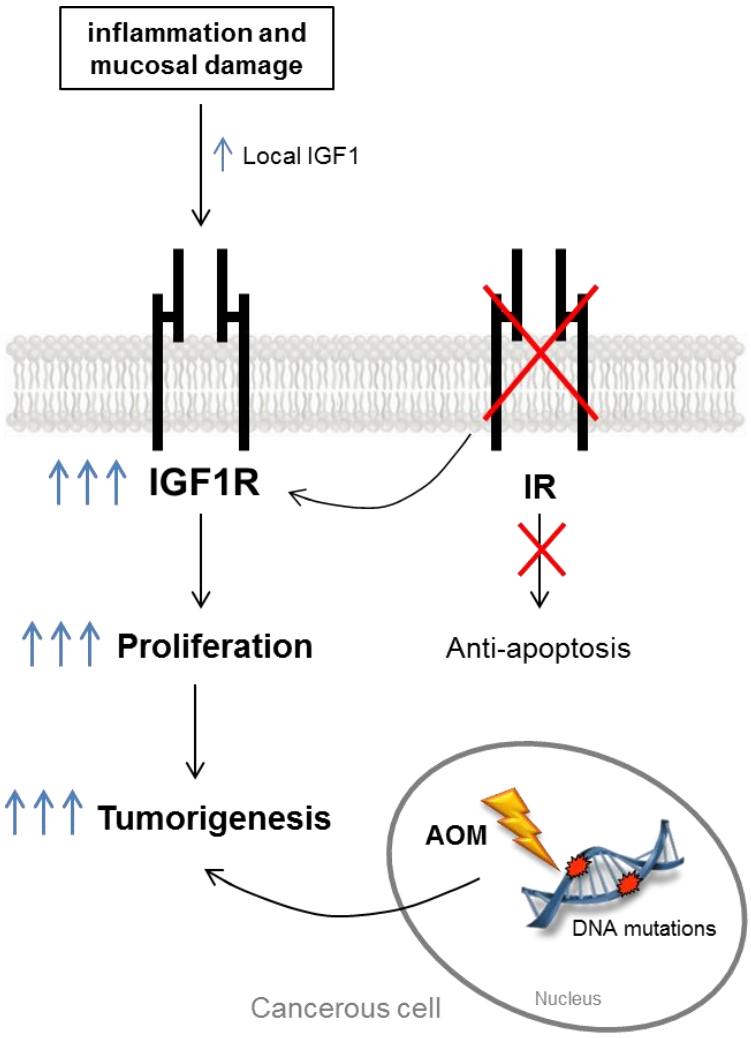

Figure 5.7: Proposed roles of IGF1R during inflammation-induced CRC in the absence of IR. In response to inflammation-induced damage to the colonic mucosa, levels of local of IGF1 are increased and IGF1R may be activated to initiate proliferation that leads to repair. In the absence of IR signaling, IGF1R becomes further activated to compensate for the loss of the anti-apoptotic actions of IR. As a result, colon epithelial cells acquire a hyperproliferative phenotype which, in the presence of a mutagen such as AOM, will enhance formation of tumors.

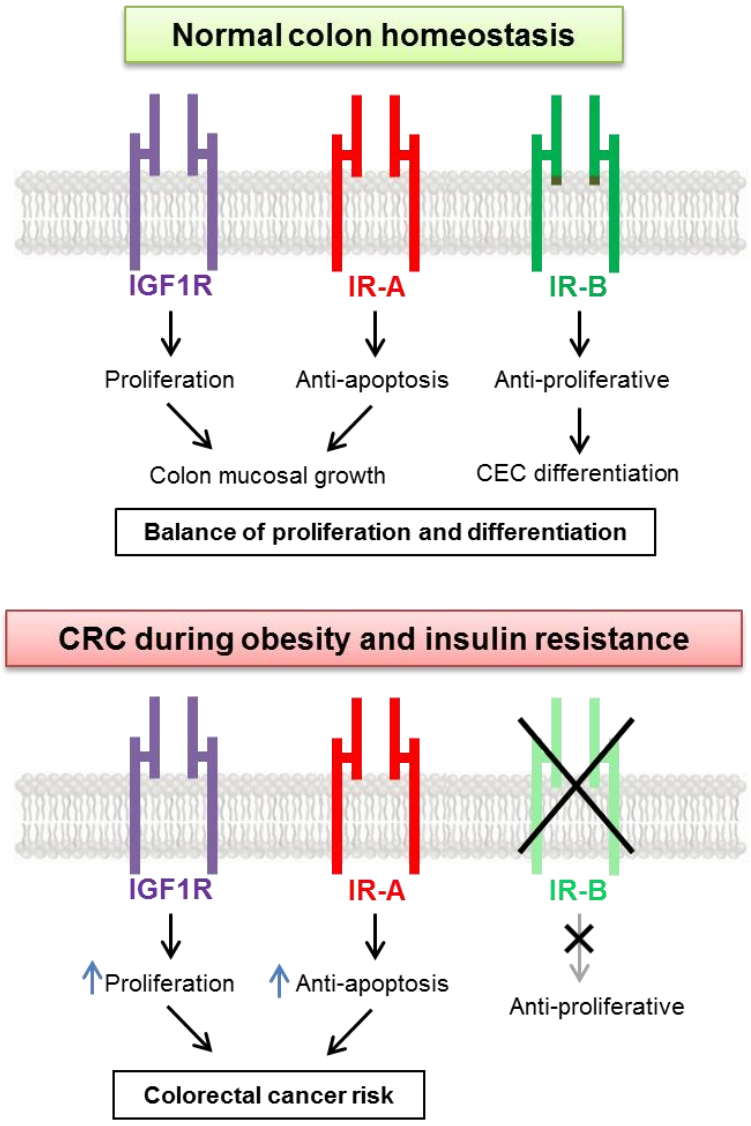

Figure 5.8: Proposed model of the roles of IGF1R, IR-A, and IR-B in normal colon physiology and CRC risk associated with obesity and insulin resistance.
In order to maintain a homeostatic balance between proliferation and differentiation in the normal colon epithelium, IGF1R may exert primarily proliferative actions and IR-A may primarily prevent apoptosis, thereby favoring normal mucosal growth. On the other hand, IR-B may limit the growth-promoting and anti-apoptotic actions of IGF1R and IR-A to promote differentiation. During CRC risk associated with obesity or insulin resistance, impaired IR-B signaling may lead to a compensatory increase in proliferation and decrease in apoptosis through IGF1R and IR-A, respectively.

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