The role of TGIFs in colorectal cancers

Anant Ajitkumar Shah
Eden Prairie, Minnesota

B.A., Northwestern University, 2012
M.S., University of Virginia, 2018

A Dissertation presented to the Graduate Faculty
of the University of Virginia in Candidacy for the Degree of
Doctor of Philosophy

Department of Biochemistry and Molecular Genetics

University of Virginia
November, 2018

_____

_____

_____

_____

Abstract

Tgif1 and Tgif2 repress gene expression by binding directly to DNA, or interacting with Transforming Growth Factor (TGF) β-responsive SMADs. Tgifs are essential for embryogenesis and may function in tumor progression. By analyzing both gain and loss of Tgif function in a well-established mouse model of intestinal cancer, we show that Tgifs promote adenoma growth in the context of mutant *Apc* (*Adenomatous Polyposis Coli*). Despite the tumor suppressive role of TGFβ signaling, transcriptome profiling of colon tumors suggests minimal effect of Tgifs on the TGFβ pathway. Instead, it appears that Tgifs, which are up-regulated in *Apc* mutant colon tumors, contribute to reprogramming metabolic gene expression. Integrating gene expression data from colon tumors with other gene expression and chromatin binding data identifies a set of direct Tgif target genes encoding proteins involved in acetyl CoA and pyruvate metabolism. Analysis of both tumor and non-tumor tissues indicates that these genes are targets of Tgif repression in multiple settings, suggesting this is a core Tgif function. We propose that Tgifs play an important role in regulating basic energy metabolism in normal cells, and that this function of Tgifs is amplified in some cancers.

Acknowledgements

I would like to my deepest gratitude to Dr. David Wotton, my Ph.D. mentor. Without his guidance and suggestions, this project would simply not have been possible. Under his mentorship, I have learned to think critically and creatively about scientifc questions, and I will take all of these acquired skills with me to my future endeavors.

I would also like to thank members of my thesis committee, Dr. Marty Mayo, Dr. Stefan Bekiranov, and Dr. Dan Gioeli. My path from the qualifying exam to the dissertation defense was…less than what was ideally expected of a graduate student, and they went along with it while also asking thoughtful and critical questions about my work to increase its quality.

There are many members of the Wotton Lab which deserve my thanks for helping develop my technical laboratory skills. First and foremost, I must thank Tiffany Melhuish-Baxter without whose technical expertise and institutional knowledge would have made a lot of the experiments presented in this thesis poorly done or impossible. Additionally, Anoush Anderson helped get me oriented in the lab when I first joined, and taught me many basic lab skills. Yi Hao and Arkadi Manukyan helped develop and troubleshoot some of the tissue culture experiments presented in this thesis and helped me streamline those protocols. I want to thank other former members of the Wotton Lab, including Annie Carlton, Agata Lemiesz, Iza Kowalczyk, and Kasia Kedzierska for providing support and friendship, especially during times when experiments were failing. It was much easier to work in lab with you all around.

Lastly, I need to thank my friends and family for their support the past 5+ years I have spent chasing after my Ph.D. One of the best things about the Ph.D. experience for me was the opportunity to make strong and lasting friendships with my classmates. It certainly made life a lot easier to have this community. In particular I want to thank Michael Schappe, my roommate for three years, and Gabriel Falcao Alencar for all the stupid things we watched and talked about – I'm sad it's rapidly coming to an end. To my parents – your enthusiasm for my Ph.D. pursuit was often greater than mine, and that kept me productive and moving forward. Thanks to my brother, Soham, for telling me how to clarify my scientific explanations and for putting up with me as we lived together.

Finally, to my girlfriend, Anupama Dathan, thanks for putting up with this for so long. I appreciate it greatly. But now I'm done! Get ready.

Table of Contents

ABSTRACT ..... **II**

ACKNOWLEDGEMENTS ..... **III**

TABLE OF CONTENTS ..... **V**

LIST OF FIGURES ..... **VIII**

LIST OF ABBREVIATIONS ..... **X**

CHAPTER 1 – GENERAL INTRODUCTION **1**

1.1 Colorectal Cancer (CRC) **1**
1.1.1 Background 1
1.1.2 WNT signaling and the role of APC 5
WNT signaling 5
Cannonical WNT signaling pathway 6
APC, a tumor suppressor 9
Roles of APC outside Canonical WNT signaling 10
APC involvement in cytoskeletal integrity, cellular adhesion, and migration 10
APC involvement in Chromosomal Instability (CIN) 11
1.1.3 Treatments for CRCs 12
1.1.4 Mouse models of CRC 13
Genetically modified mice 16

1.2 TGFβ Signaling Pathway **18**
1.2.1 Canonical TGFβ signaling pathway 19
1.2.2 Tumor-suppressive role of TGFβ 22
1.2.3 Tumor-promoting role of TGFβ 24

1.3 Thymine-Guanine Interacting Factors **25**
1.3.1 Transcriptional Regulation by TGIFs 25
Protein interactions with TGIF1 and TGIF2 facilitate its repressor activity 28
TGIF-mediated repression 29
Direct transcriptional repression by TGIFs 29
Regulation of the TGFβ signaling pathway 30
Regulation of Nuclear Hormone Regulated Responses 30
1.3.2 Mouse Models of TGIFs 31
1.3.3 TGIFs in Cancer 33

1.4 Cancer Metabolism **35**
1.4.1 The Warburg effect and Hypoxia 36
1.4.2 Glycolytic metabolites as intermediates for additional pathways 41
Pentose Phosphate Pathway (PPP) 41
Hexosamine biosynthesis 42

Amino acid biosynthesis and the one-carbon cycle 42
1.4.3 Tricarboxylic acid cycle 43
1.4.4 Aceyl-CoA regulation 44
Fatty acid biosynthesis 45
Epigenetic Regulation 46

CHAPTER 2 – TGIF1 EXPRESSION PROMOTES INTESTINAL TUMORIGENESIS 49

2.1 Introduction 49

2.2 Materials and Methods 52
2.2.1 Cell culture 52
2.2.2 CRISPR/Cas9 mediated knockout and verification 52
2.2.3 Cell Proliferation Assay 53
2.2.4 Mice 53
2.2.5 Tumor analysis, IF, and histology 54
2.2.6 RNA isolation and qRT-PCR 54
2.2.7 Tissue western blot 54
2.2.8 Statistical methods 55

2.3 Results 56
2.3.1 Increased TGIF expression in human colorectal tumors 56
2.3.2 Modulation of levels of Tgifs in the intestinal epithelium 61
2.3.3 Increased Tgif expression in colorectal tumors 69
2.3.4 Altered tumor burden in the small intestine 72

2.4 Discussion 80

CHAPTER 3 – TGIFS REGULATE ACETYL-COA METABOLISM 82

3.1 Introduction 82

3.2 Materials and Methods 86
3.3.1 Mice 86
3.3.2 Tumor analysis, IF and histology 86
3.3.3 Cell culture 87
3.3.4 RNA isolation and qRT-PCR 87
3.3.5 RNA-sequencing and analysis 87
3.3.6 Western blot 88
3.3.7 Chromatin immunoprecipitation (ChIP) 88
3.3.8 Statistical methods 89

3.3 Results 90
3.3.1 Transcriptional changes in Tgif mutant tumors 90
3.3.2 Altered metabolic gene expression in tumors lacking Tgifs 96
3.3.3 Tgifs repress expression of genes involved in Acetyl-CoA metabolism 102
3.3.4 TGIF repression of genes involved of Acetyl-CoA in cancer 113

3.4 Discussion 122

CHAPTER 4 – GENERAL DISCUSSION 126

4.1 TGIF connection to proliferation 127

4.2 Tgif disconnect with TGFβ and Wnt signaling 131
4.2.1 Tgifs do not appear to regulate TGFβ signaling in our CRC model 131
4.2.2 Tgifs do not appear to regulate WNT signaling in our CRC model.....132

4.3 Tgif regulation of metabolism..... 134

4.4 Potential Medium & Long Term Projects..... 141
4.4.1 Metabolomics experiments 141
4.4.2 Identification of additional primary effects of Tgif knockout in our mouse model of CRC.....143
4.4.3 Identification of secondary effects of Tgif knockout in our mouse model of CRC 143
4.4.4 Generation of a new mouse model of CRC.....145
Problems with our mouse model 145
A new model.....145

REFERENCES 149

List of Figures

Figure 1.1 – Cannonical progression of sporadic CRC. 3

Figure 1.2 – The WNT signaling pathway..... 8

Figure 1.3 – The TGFβ signaling pathway. 21

Figure 1.4 – TGIF1 and TGIF2 proteins..... 27

Figure 1.5 – Changes in metabolism between a normal cell and a cancer cell..... 38

Figure 2.1 – Increased expressinon of TGIFs in CRC..... 58

Figure 2.2 – CRISPR-mediated TGIF1 knockout in HCT116 cells leads to decreased proliferation. 60

Figure 2.3 – Mice with intestine-specific knockout of Tgif1 and Tgif2 are viable. 63

Figure 2.4 – *Villin*-T7-TGIF1 expression is limited to the intestines. 65

Figure 2.5 – cdKO mice have fewer proliferating cells in the intestinal epithelium. 68

Figure 2.6 – Tgif expression in mouse intestinal tumors. 71

Figure 2.7 – Tgif expression promotes small intestinal tumorigenesis. 74

Figure 2.8 – Tgif expression promotes tumorigenesis of large tumors in the small intestine..... 77

Figure 2.9 – Tgif expression promotes colon tumorigenesis. 79

Figure 3.1 – Gene expression changes in Tgif mutant colon tumors..... 92

Figure 3.2 – Wnt and TGFβ target gene activity in colon tumors. 95

Figure 3.3 – Altered metabolic gene expression in Tgif mutant colon tumors..... 98

Figure 3.4 – Glut1 expression in mornal and tumor tissue. 101

Figure 3.5 – Identification of putative Tgif target genes. 104

Figure 3.6 – Acetyl-CoA and pyruvate metabolism pathway map. 107

Figure 3.7 – Increased Acss2 expression oin Tgif mutant tumors and crypts. 110

Figure 3.8 – Increased expression of acetyl-CoA and pyruvate metabolism genes in Tgif null small intestine and primary MEFs..... 112

Figure 3.9 – Tgif regulation of acetyl-CoA metabolic gene expression..... 115

Figure 3.10 – Increased expression of acetyl-CoA and pyruvate metabolism genes in HCT116 cells with TGIF knockdown. 117

Figure 3.11 – TGIF1 expression negatively correlates with genes involved in acetyl-CoA and pyruvate metabolism in different cancery types. 120

List of Abbreviations

ACAT1	Acetyl-CoA acetyltransferase 1
ACC	Acetyl-CoA carboxylase
ACLY	ATP-citrate lyase
ACSS1	Acetyl-CoA synthetase short-chain family, member 1
ACSS2	Acetyl-CoA synthetase short-chain family, member 2
AKT	Protein kinase B
AML	acute myeloid leukemia
AOM	azoxymethane
APC	Adenomatous Polyposis Coli
ApcTT	Apc;Tgif1;Tgif2
ATP	adenosine triphosphate
BMP	bone morphogenitic protein
CCLE	cancer cell line encyclopedia
CDKi	cell cycle dependent kinase inhibitor
cdKO	conditional double knockout
ChIP-seq	chromatin immunoprecipitation sequencing
CIN	chromosomal instability
CRC	colorectal cancer
CRISPR	clustered regularly interspaced short palindromic repeats
CtBP	C-terminal binding protein
EGFR	estrogen growth factor receptor
EMT	epithelial-to-mesenchymal transition
ES	embryonic stem (cells)
f	floxed
FAP	familial adenomatous polyposis
FASN	Fatty acid synthase
G6P	glucose-6-phosphate
G6PD	Gucose-6-phosphate dehydrogenase
GSK3β	Glycogen synthase kinase 3β
GTP	guanosine triphosphate
H&E	hematoxylin and eosin
HAT	Histone Acetyltransferace
HD	homeodomain
HDAC	Histone Deacetylase
HIF	Hypoxia-Inducible Factor
HK	Hexokinase
HPE	holoprosencephaly
HRE	hormone response element
ID	Inhibitor of Differentiation
IF	immunofluorescence
ISC	intestinal stem cell
KD	knockdown
KO	knockout
LDHA	Lactate Dehydrogenase A

LRP5/6	Low-density lipoprotein receptor-related protein 5/6
MCR	mutation cluster region
MEF	mouse embryonic fibroblast
MLL	mixed lineage leukemia
MLYCD	Malonyl-CoA decarboxylase
MPC1	Mitochondrial pyruvate carrier 1
NR	nuclear receptor
PC	Pyruvate carboxylase (human homolog)
PCX	Pyruvate carboxylase (mouse homolog)
PET	positron emission tomography
PFK	Phosphofructokinase
PHGDH	Phosphoglycerate dehydrogenase
Pi3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PPP	pentose phosphate pathway
r	recombined
R5P	ribose 5 phosphate
RD	repression domain
RNA-seq	ribonucleic acid sequencing
ROS	reactive oxygen species
RT-qPCR	real time quantitative polymerase chain reaction
RXR	retinoid X receptor
SARA	Smad anchor for receptor activation
SHMT2	Serine hydroxylmethyltransferase 2
SI	small intestine
siRNA	small interfering RNA
TAs	transit amplifying cells
TALE	three amino acid loop extension
TCA	tricarboxylic acid cycle
TGFBRI	TGFβ receptor I
TGFBRII	TGFβ receptor II
TGFβ	Transforming Growth Factor β
TGIF	Thymine/Guanine-interacting factor
THF	tetrahydrofolate
VEGFR	Vascular endothelial growth factor receptor
WT	wild type

Chapter 1 – General Introduction

1.1 Colorectal Cancer (CRC)

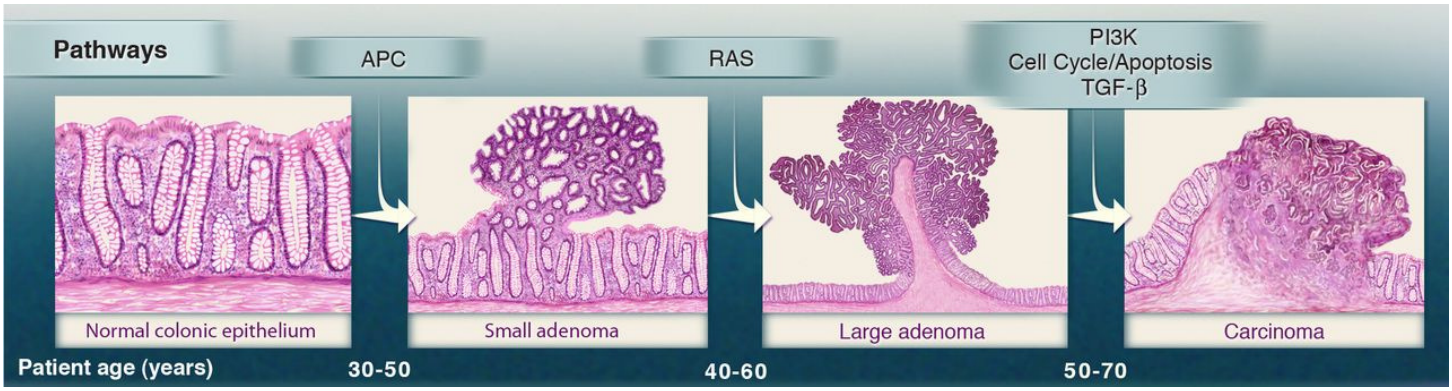
1.1.1 Background

Colorectal cancers (CRCs) are the third most prevalent cancers and are one of the leading causes of cancer-related deaths in the United States. Over 140,000 new cases are estimated to be diagnosed in 2018 in the United States alone (1), and worldwide, it is estimated that 700,000 people die from CRCs yearly (2). There are two forms of CRCs, sporadically obtained and genetically inherited, and the vast majority of cases of CRC are sporadic in nature, accounting for nearly 75% of newly diagnosed cases every year (3). While the majority of this thesis work focuses on sporadically obtained CRC, understanding how both of these types of cancer arise yields a complete and overlapping insight into the molecular mechanisms of tumorigenesis, disease progression, and treatment. As such, is not surprising to find that CRCs have been heavily studied, and there is a well-established order in which mutations arise to cause a colorectal growth to become a carcinoma (4) (Figure 1.1).

Colon cancer is a disease of the epithelial cells in the colon (5). The colonic epithelial cells form invaginations called crypts, the key structural feature of the intestines. The life span of a colonic epithelial cell is very short, just 3-5 days. As such, the intestinal stem cells (ISCs), found at the bottom of colonic and small intestinal crypts (henceforth referred to as ‘crypts’), are constantly dividing to replace the cells that are lost, and these stem cells give rise to the rest of the differentiated cell types found in the epithelium. ISC progeny, transit amplifying (TA) cells, are highly proliferative. These

Figure 1.1 – Canonical progression of sporadic CRC (6)**.**

Transitions from normal tissue to invasive carcinoma are partitioned by major pathways mutated within the progression of CRC, and patient age indicates the approximate age at which mutations occur in the appropriate pathways.


Pathways
APC
RAS
PI3K
Cell Cycle/Apoptosis
TGF-β
Normal colonic epithelium
Small adenoma
Large adenoma
Carcinoma
Patient age (years)
30-50
40-60
50-70

cells divide and differentiate into the various cell types populating the crypt including goblet cells, enterocytes, and enteroendocrine cells (7), and this all of this rapid division is thought to force differentiated cells to migrate upwards along the crypt axis (8,9). As mature epithelial cells die, they are released from the epithelial surface, and ISCs and TAs constantly are required to replace them (5). The one key difference between small intestinal and colonic crypts is the presence of Paneth cells in the small intestinal crypts but not in colonic ones. These cells protect the small intestine ISCs from potential enteric pathogens and additionally secrete various pro-growth ligands, including WNT, facilitating the growth and division of ISCs (10,11).

There are currently two theories on the cell of origin in CRC – a bottom-up theory proposing the ISC is the cell of origin and the top-down theory in which either an ISC or differentiated cell can be the cell of origin. Evidence for the top-down theory has mostly come from histological studies which were unable to directly identify the direct cell of origin due to the experimental method used (5). Additional support for the top-down theory comes from the many murine models of CRCs which utilized Cre-mediated deletion of *Adenomatous polyposis coli* (*Apc*) (more on *Apc* below) in all cells of the intestinal epithelium, not just the stem cells, in order to generate tumors (12–14). However, recently, there has been some strong evidence for the bottom-up theory through genetic manipulations in mouse models. Mice with ISC specific deletions for *Apc* rapidly developed colonic adenomas, while *Apc* deletions in TAs and differentiated cells only sporadically resulted in adenomas (15). Additional evidence for the bottom-up theory has been found in mouse models of prostate cancer (16) and glioblastoma (17). The sum of

the evidence suggests ISCs are most likely the cell of origin in CRCs, and stem cells are most likely the cells of origin for many types of cancer (18).

Canonically, tumor initiation begins with a mutation in the tumor suppressor APC, a key negative regulator of the WNT pathway, in ISCs, causing changes in colonic crypt morphology (5,19). The crypts have more proliferative cells which are less differentiated, and crypt fission, the process by which a crypt splits in two, is observed. This generates the colonic polyp, an abnormal growth from the surface of the mucosal membrane (Figure 1.1). Then, additional mutations in the RAS/MAPK pathway (mutated in 66% of CRCs) help accelerate the growth of the polyp in to an adenoma. Over time, pathways involved in cellular proliferation/survival (PI3K/AKT) (mutated in 36% of CRCs), cell cycle/apoptosis (p53) (mutated in 60% of CRCs), and TGFβ signaling (mutated in 28% of CRCs) can become mutated, turning an adenoma into an invasive carcinoma (3,6,12,19) .

1.1.2 WNT signaling and the role of APC

WNT signaling

Wingless or WNT signaling was initially described in *Drosophila melanogaster* and then was subsequently described in other model organisms, including but not limited to *Xenopus* and *Mus musculus*. WNT signaling plays a critical role in embryonic development, cell proliferation, cell migration, and cell fate (20–22). Aberrant WNT signaling has been implicated in the carcinogenesis of many cancers, including, but not limited to, breast, prostate, and CRCs. WNT signaling can be broken down into two groups, canonical and non-canonical. The key difference between the canonical and non-canonical branches of WNT signaling depends on the involvement of β-catenin –

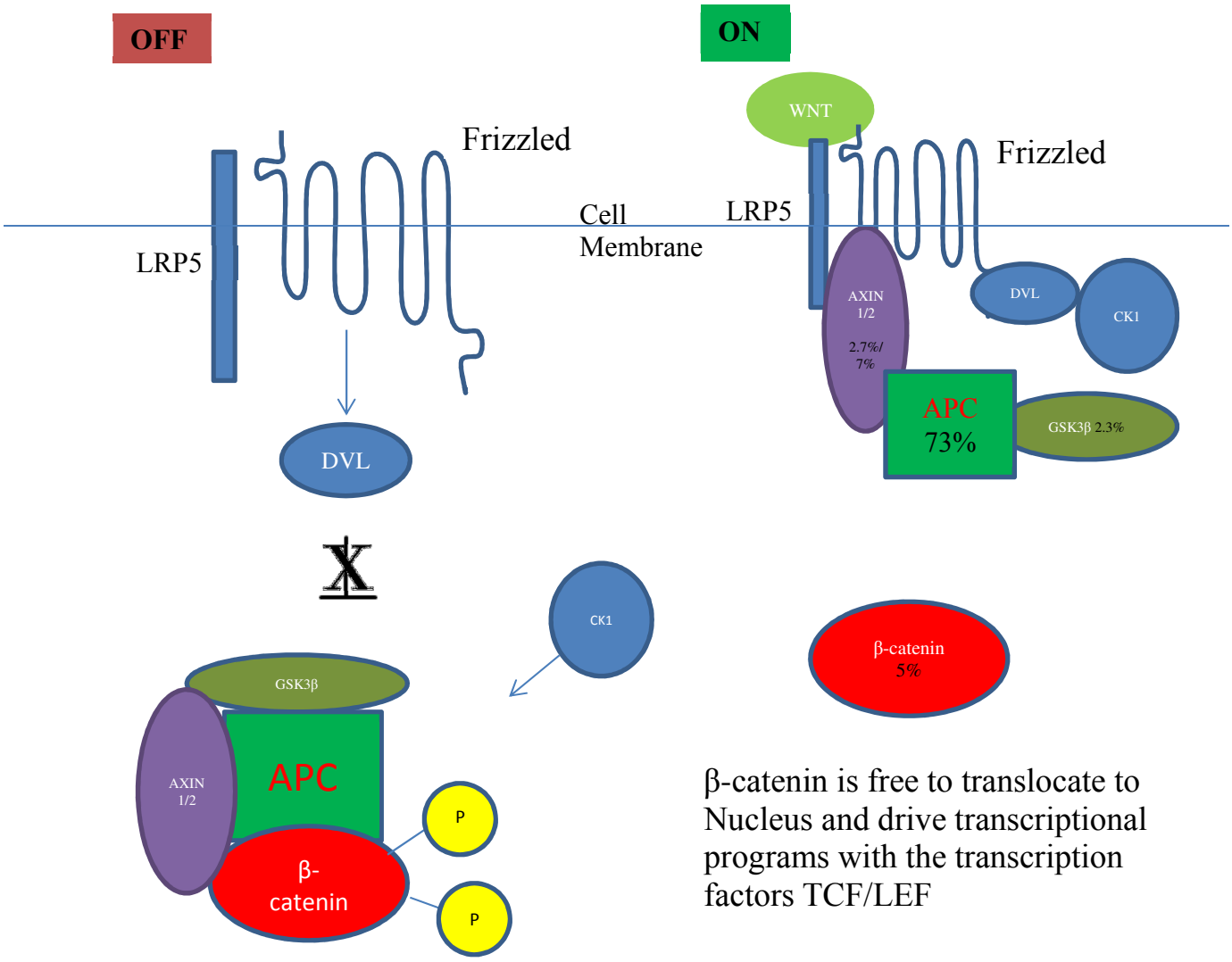
canonical WNT signaling uses β-catenin to drive transcriptional programs while non-canonical WNT signaling occurs independently of β-catenin involvement and regulates planar cell polarity and the WNT/calcium pathways (22). Canonical WNT signaling is the best studied and understood within the context of CRCs and will be in focus moving forward.

Cannonical WNT signaling pathway

In the absence of WNT pathway activation, APC forms the β-catenin destruction complex by scaffolding and binding AXIN1/2, GSK3β, and β-catenin (Figure 1.2). This destruction complex sequesters β-catenin within the cytoplasm. Cytoplasmic β-catenin is subsequently phosphorylated by casein kinase 1/2 (CK1/2), targeted for ubiquitination, and degraded. When canonical WNT signaling is activated, the WNT ligand binds to the extracellular domain of the FRIZZLED receptor at the amino terminus, which then causes FRIZZLED to couple with a co-receptor, e.g. LRP5/6, to disrupt the β-catenin destruction complex. When bound to WNT, the FRIZZLED/ LRP5/6 complex binds AXINs, part of the β-catenin destruction complex, to the cellular plasma membrane, causing the dephosphorylation if AXINs. This sequestration of AXINs, in turn, causes another protein, DISHEVELED, to become activated and inhibit the activity of GSK3β, a kinase able to phosphorylate β-catenin and facilitate its eventual proteolysis. As the destruction complex is sequestered at the plasma membrane and is unable to facilitate the phosphorylation of β-catenin, β-catenin accumulates in the cytoplasm. β-catenin then is able to enter the nucleus and drive transcriptional programs by binding to the TCF/LEF transcription factors (20–22).

Figure 1.2 – The WNT signaling pathway.

The WNT signaling pathway is shown here in both the OFF and ON states. Percentages underneath proteins indicate how often they are mutated in CRC.



β-catenin is free to translocate to Nucleus and drive transcriptional programs with the transcription factors TCF/LEF

β-catenin is targeted for degradation by proteasome

APC has a key role in the negative regulation of the WNT pathway within multiple different tissue types and specifically within the epithelial cells of the colon, the typical progenitors of CRCs (15,18,22,23). The vast majority of sporadic cases of CRCs involve dysregulation of the WNT signaling pathway, with the largest proportion being mutations in APC. Other, less common mutations, in β-catenin and AXIN1/2 also inhibit the destruction of β-catenin (Figure 1.2). Taken together, it is very clear that dysregulation of WNT signaling has a critical role in the initiation and propagation of CRCs.

APC, a tumor suppressor

Sporadic CRCs make up the majority of cases diagnosed every year, and up to 80% are characterized by a mutation or deletion in the tumor suppressor *Adenomatous polyposis coli* (*APC*) gene (12,19). *APC* encodes a 2843 amino acid single helix protein that serves as a scaffold for the β-catenin destruction complex which includes β-catenin, AXIN1/2, and GSK3β. Most mutations in APC in CRCs occur in the mutation cluster region (MCR), a region spanning amino acids 1286-1514, and these mutations are almost exclusively create truncated versions of APC (12). Cancers with mutations in APC in the MCR typically have more severe phenotypes than cancers with mutations in APC outside the MCR. One study even found that expression of a truncated version of APC (APC^{1638T}), a truncated version of Apc with the MCR intact, did not generate tumors in mice, suggesting a tumor selection bias for APC mutations to occur in the MCR (24). Normally, the MCR contains β-catenin binding regions and facilitate the appropriate scaffolding configuration of the β-catenin destruction complex. Mutations in the MCR affect the β-catenin binding and downregulation domains in APC. Additionally, as these

mutated APC proteins are also truncated, they lack AXIN binding sites and the microtubule binding Basic domain. These mutations in APC render it unable to serve as the scaffolding protein in the β-catenin destruction complex, resulting in the dysregulation of β-catenin signaling and eventual cancer (19,25). Familial adenomatous polyposis (FAP), a heritable form of CRC with germ-line mutations in APC rendering it less able to bind and form the β-catenin destruction complex, results in a virtual 100% lifetime risk of development of CRCs, highlighting the critical role of APC function in CRC pathogenesis (26). Less frequently, CRCs can be caused by mutations in other members of the WNT pathway including β-catenin activating mutations, mutations in regulatory phosphorylation sites marking β-catenin for degradation, and loss-of-function mutations in *AXIN1/2* (Figure 1.2).

Roles of APC outside Canonical WNT signaling

APC has been found to have multiple other roles outside of canonical WNT signaling. These roles include maintenance of the actin cytoskeleton, cell-cell adhesion, and cellular migration. Additionally, mutations in APC are linked in with a phenomenon known as chromosomal instability (CIN), leading to cellular aneuploidy in up to 85% of all CRCs (19).

APC involvement in cytoskeletal integrity, cellular adhesion, and migration

APC function has been implicated in the maintenance of normal cellular cytoskeleton and adhesion at both the single cell and tissue levels in the colonic epithelium, and mutations in APC have been connected to the aberrations in these two processes in CRCs. APC has been linked to cytoskeletal maintenance by its interaction with β-catenin and γ-catenin, which promotes actin stabilization (27). Additionally,

mutations in APC result in a disorganization of adherens junctions by breaking up the E-cadherin, β-catenin, γ-catenin, and actin complex because of the inability of APC to bind to β-catenin properly (25,28). This dysregulation of adherence junctions can lead to loss of cellular polarity and abnormal cellular migration in the colonic epithelial crypts. Cells, instead of moving upward from the bottom of the crypt towards the top, may migrate abnormally or not at all, remaining at or near the bottom of the crypt as a result of mutations in APC (29). As these cells accumulate and form a polyp, they eventually can become tumorigenic through the activation of β-catenin target genes (e.g. c-MYC, CyclinD) and aneuploid via CIN.

APC involvement in Chromosomal Instability (CIN)

CRCs are often found to be aneuploid, having an abnormal number of chromosomes. This aneuploidy can be found in approximately 85% of CRCs, from early stage adenomas through carcinomas, perhaps suggesting it plays an important role in the progression of cancer. Aneuploidy in CRCs can lead to further defects in chromosome separation, or CIN. How CIN is initiated in tumorigenesis is unclear, but APC has been implicated with CIN and may play an integral role. APC has a microtubule binding domain, the basic domain, which is thought to connect microtubules to chromosomes during mitosis to facilitate proper chromosome segregation (30). APC binds the plus end of a microtubule through an adaptor protein EB1 and attaches it to the kinetochore by binding and forming a complex with BUB1 and BUB3 (31). The two BUB proteins are mitotic checkpoint proteins; therefore, wild-type APC helps facilitate normal mitotic spindle formation, maintaining cellular diploidy. Most mutant versions of APC are truncated proteins which have lost the microtubule binding domain. They, therefore, may

not be able to bind to BUB1 (32), thus disrupting the microtubule-to-kinetochore attachment, leading to a defect improper chromosome segregation and resulting aneuploidy.

Taken together, APC plays a major role in CRC tumorigenesis and propagation, mainly through the regulation of the WNT/β-catenin pathway. It has additional roles in maintenance of cellular architecture, cellular adhesion, and migration and may play a role in the chromosomal instability seen in the vast majority of CRCs.

1.1.3 Treatments for CRCs

With the current public awareness of the necessity to screen for CRCs, many CRCs are caught at early stages of tumorigenesis, either as polyps or at Stages I or II. Treatment for Stage I and II cancers, early stage tumors which have not expanded through the colonic mucosa or submucosa, is surgical resection of the tumor(s), with adjuvant chemotherapy utilizing DNA-damaging agents (leucovorin/irinotecan) and DNA replication inhibitors (5-fluorouracil/capcitabine) (33,34). Stage I and II treatments for rectal cancer include neoadjuvant chemotherapies previously mentioned followed by radiation treatment. Radiation treatment is typically not a feasible option for colon cancers. For patients with Stage III tumors, tumors that have grown through the muscle layer around the colon but have not broken through the outer layer of the colon, platinum-based DNA damaging compounds, e.g. oxaloplatin/carboplatin, are added to the adjuvent regiment for Stages I and II (34). For Stage IV cancers, tumors that have broken through the wall of the colon and potentially metastasized to other organs, the primary treatment typically is chemotherapy. Treatment includes the drugs used to treat tumors in Stages I-

III in addition to more targeted therapies such as monoclonal antibodies against epidermal growth factor receptor (EGFR) and vascular endothelial growth factor (VEGFR) and pan-receptor tyrosine kinase inhibitors (35,36). At the moment, few targeted treatments are available for CRCs, though that number is slowly growing.

1.1.4 Mouse models of CRC

While no animal model of CRC is able to fully capture the full extent of the human disease, development of murine models of CRC has been critical in understanding its pathogenesis. Mouse models need to have three important characteristics in order to have translational potential to human disease. First, the disease needs to present in the appropriate tissue. Second, the murine disease analog needs to share similar molecular and histological features to the human disease. Finally, the murine disease model must mimic both the underlying molecular mechanisms of pathogenesis and the complexity in human disease (12).

There are three ways to induce tumor formation in mice – spontaneously, using carcinogens or other environmental factors, or via genetic manipulation (12). Mice rarely develop CRCs spontaneously, with an incidence rate of 4% in the small intestine and 1% in the colon at 24 months of age (37). As a result, the spontaneous model of CRC is both an inefficient and expensive way to study the human disease even though most human CRC arises spontaneously. Additionally, given how infrequently the CRCs arise spontaneously, it is difficult to adequately characterize the molecular mechanisms underlying the disease.

One murine model of CRC with potential human relevance is the western diet model. The western diet, characterized by consumption of large amounts of processed

and red meats, fats, and sugars, has been shown to modulate the risk of CRC in humans, especially in western countries (38). This model of an exogenous factor (diet) influencing the spontaneous CRCs is an appealing one because it is potentially able to represent the underlying complexity of the development of human CRCs. Murine studies have focused on this diet in order to recapitulate a potential exogenous promotor of human CRCs. Mice are typically fed a diet containing four times as much fat and 90% less calcium (39). These models result in hyperplasia/dysplasia in the intestine, appearing to prime the intestine for the incidence of colon cancer as opposed to directly causing it (39,40). In one study, mice fed a diet high in fat and low in fiber developed intestinal tumors with characteristics of human invasive adenocarcinomas. However, only 25% of the cohort studied developed intestinal tumors, and the mice were fed the Western Diet for two years (37). In addition to the time, cost, and inefficiency in the number of mice developing intestinal tumors, there is a major problem with the Western Diet model of CRC - the molecular mechanisms by which tumors arise in mice as a result of the Western Diet are unclear whereas how human CRCs arise is well understood and characterized (Figure 1.1). As a result, it is possible that the physiological changes induced by the Western Diet model of CRC may not accurately mimic the human disease pathogenesis.

A second exogenously induced model of murine CRC utilizes chemicals with mutagenic potential. Few chemicals are commonly used. One of the most often used chemicals is azoxymethane (AOM), a strong alkylating agent (41). Intraperitoneal or subcutaneous injections of AOM induce tumors in murine colons, most often by inducing mutations in β-catenin, allowing the protein to resist regulatory degradation (42,43).

However, in order for AOM to become carcinogenic, it needs to be metabolized into its active form. This process occurs in the liver, after which the active form of AOM is excreted into the intestines through the bile duct (44). However, there are studies in rats which provide evidence suggesting AOM metabolism to its active form can also take place in epithelial cells, including the cells in colonic crypts (45,46). Through both of these routes, AOM is highly carcinogenic, and the induction of tumors is highly dose-dependent (47–49). While the chemically induced murine models of CRC follow similar molecular mechanisms of pathogenesis, humans typically are not exposed to such large amounts of alkylating agents. Additionally, the tumors that do develop typically do not follow the metastatic patterns of the human disease. AOM induced tumors rarely metastasize to the liver or lung, two organs to which human CRCs commonly metastasize (50). With these two drawbacks, this model may not represent the typical sporadic nature of the human disease.

The vast majority of work in a mutagenesis induced murine model has been done in the Apc^{Min} mouse. This mouse has a truncating mutation in the APC gene at amino acid 850 and causes tumors in both the small intestine and colon (51). Due to this mutation being present through the entirety of the lifetime of the mouse, these mice become anemic 60 days post birth and die at 120 days post birth (52). Apc^{Min} mice develop about ten times more tumors in the small intestine than in the colon unlike the human disease where no tumors are found in the small intestine. However, this model of CRC accurately captures the molecular and pathological traits seen in the human heritable CRC, FAP, and these traits are comparable to spontaneous CRCs as well (53). As a result, much work has been done within this model to not only characterize the

development of CRCs but also to study preventative measures and treatments for the human disease. This model has also been useful in elucidating modifiers of CRC risk, but these modifiers are very dependent on the genetic background of the mouse lines used (54–57). Overall, the Apc^{Min} model has been very important in understanding the pathogenesis of human CRC but has major drawbacks including the inability to control the timing disease initiation and the impact of the genetic background of the mice used in study on potential modifiers of the disease.

Genetically modified mice

The advent of genetically modified mice has allowed researchers to more faithfully recreate human CRCs at the molecular and pathological level compared to the previous models discussed. There are three main advantages of the genetically modified mice models of CRCs. Firstly, genetic models allow researchers to identify and characterize the roles of specific genes in the pathogenesis of CRCs. Secondly, the timing of tumor initiation can be controlled. Finally, the disease can be contained to the intestines (12).

The Cre/loxP system is typically used to create inducible mouse models of CRCs. The Cre is commonly linked to the promoter of the intestinal epithelial cell specific gene *Villin* (14), although other genes have also been used (13,23,58–61). *Villin* is a gene expressed throughout the epithelial cells in both the small and large intestines of mice, making it a suitable candidate for intestinal-specific transgenic activation. However, *Villin* expression is not limited to the epithelial cells of the intestines. A *Villin*-LacZ transgene showed expression in the intestines and in the kidney starting at embryonic day (e) 9 (14). While this additional expression is a potential weakness of the *Villin* transgene,

subsequent transgenes purportedly specific to the murine intestines also have been shown to have extra intestinal expression, with some causing more severe unintended developmental consequences than others. It appears that all of the published intestinal-specific genes used for the Cre/LoxP system have their specific drawbacks, and no one promoter or method can specifically target and confine transgenic expression to the intestines (12).

The *Villin* promoter has been used to generate two versions of Cre able to create intestinal-specific deletions of floxed alleles, one that is activated when the gene is activated during normal embryonic development and one that is tamoxifen-inducible (62). The key difference between these two constructs is the time at which they are activated. The normal *Villin*-Cre is activated at e9, while the tamoxifen-inducible Cre is only activated when the mouse is injected with tamoxifen. The advantage of this tamoxifen-inducible Cre is that it more faithfully resembles the nature of the human CRCs in that the mutations required to generate the disease are generated after birth, but both versions of the *Villin*-Cre use a version of a mutated *Apc* to generate intestinal tumors mimicking the molecular mechanisms and pathology of human CRCs.

There are many mouse models of CRC which use a transgenic Cre, e.g. *Villin*-Cre, in combination with floxed alleles of oncogenes, e.g. *Kras* (63) and *Ctnnb1* (encoding β-catenin) (64), and tumor suppressors, e.g. *Msh2* (65) and *TgfbrII* (66), but floxed alleles of *Apc* are used in conjunction with an intestinal specific Cre to induce tumorigenesis. Typically, loxP sites flanking either exon 14 (67–69) or 15 (70) are used in animal studies, with Cre-mediated deletion of exon 14 being the more commonly used mouse model of the two (Apc^{CKO}) (69). Apc^{CKO} mice, in the presence of Cre, have a

frameshift causing a stop codon in the gene, resulting in a protein which only contains the first 580 amino acids of the 2843 amino acid wild type protein. Apc^{CKO} mice, in conjunction with a Cre-mediated (e.g. *Villin*-Cre) deletion to instigate tumorigenesis, is a mouse model also able to identify the role of specific genes in the pathogenesis of CRCs. By allowing the researcher to knockout or increase the expression of a specific target gene potentially implicated in CRC pathogenesis, researchers have been able to leverage this model effectively in order to understand the critical roles of genes involved in CRCs.

1.2 TGFβ Signaling Pathway

TGFβ signaling regulates a wide variety of molecular processes from cell migration, adhesion, and differentiation to embryogenesis and organ development in various different tissue types and contexts (71–75). This pathway is often misregulated in diseases, including cancers, and its effects are heavily context dependent. In cancer, the role of TGFβ signaling can vary depending on the state of the tumor. In pre-malignant states, TGFβ signaling typically is considered to have a tumor-suppressive role by limiting cellular proliferation and promoting cellular cytostasis and differentiation. In contrast, malignant tumors are able to hijack TGFβ signaling to promote metastasis by inducing epithelial-to-mesenchymal transition (EMT), increasing cellular motility, and, at times, evading immune regulation. Mutations in different pathway components of the TGFβ signaling pathway are commonly seen in CRCs, often in the later stages of tumor progression, after the tumor has already developed (Figure 1.1). TGFβ signaling, along with its differing roles within tumors, will be discussed in further detail moving forward.

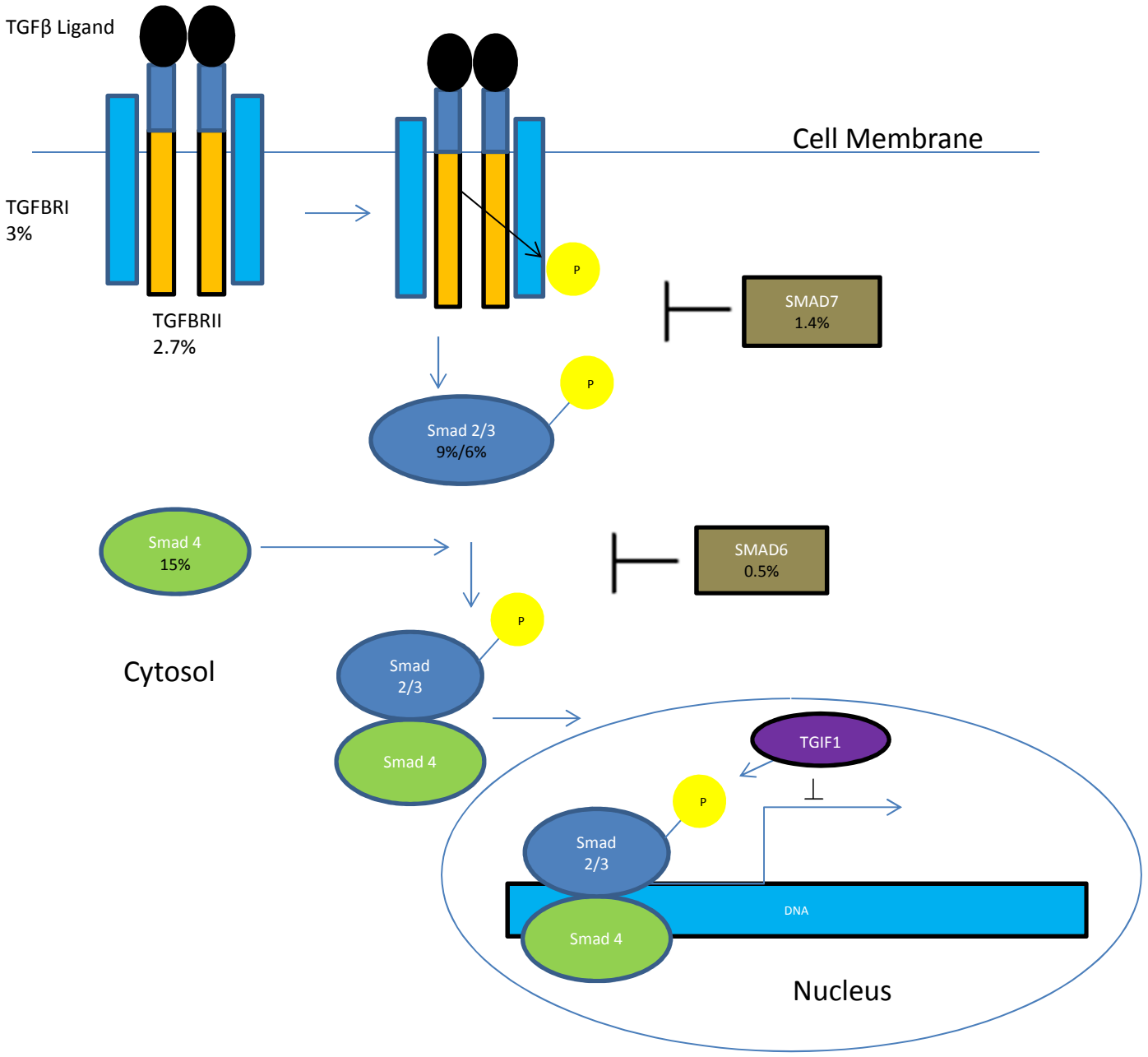
1.2.1 Canonical TGFβ signaling pathway

There are two branches of the TGFβ signaling superfamily, the TGFβ /Nodal/Activin subfamily and the bone morphogenetic proteins (BMP) subfamily, and both subfamilies use a similar mechanism to regulate transcriptional programs (76). TGFβ signaling is activated when a ligand, e.g. TGFβ, activin, BMP, binds to a TGFβ type II receptor (e.g. TGFBRII) homodimer which subsequently forms a heterotetramer with a TGFβ type I receptor (e.g. TGFBRI) homodimer (Figure 1.3). This complex functions as a serine/threonine kinase as TGFBRII phosphorylates TGFBRI which then phosphorylates receptor-activated effector SMADS (R-SMADS), SMADs 2/3 for TGFβ and SMADs 1/5/8 for BMP. These phosphorylated SMADs associate with the co-Smad, SMAD4, translocate to the nucleus, and, in conjunction with other transcriptional activators and repressors, modulate gene expression (71–76). Each protein complex consisting of SMAD4-RSMAD-cofactor regulates a different set of genes depending on the cell type and molecular context. Through this interchangeability, the TGFβ superfamily is able to affect gene programs in both many tissue types and in different contexts.

TGFβ signaling is regulated at many levels within the cell, from the cell membrane to the nucleus. R-SMADS, when not acting as downstream effectors for TGFβ signaling, can be regulated in the cytoplasm by SARA (Smad anchor for receptor activation) (77). SARA acts as an anchor and holds R-SMADS near the cellular surface, which primes R-SMADS for activation through phosphorylation. Once TGFBRI phosphorylates an R-SMAD, the R-SMAD loses its affinity for SARA and exposes the region of the protein responsible for nuclear import. Once the R-SMAD binds to

Figure 1.3 – The TGFβ signaling pathway.

The TGFβ signaling pathway is shown. Percentages underneath proteins indicate how often they are mutated in CRC.


TGFβ Ligand
Cell Membrane
TGFBRI
3%
TGFBRII
2.7%
P
SMAD7
1.4%
P
Smad 2/3
9%/6%
Smad 4
15%
SMAD6
0.5%
Cytosol
P
Smad
2/3
Smad 4
TGIF1
P
Smad
2/3
DNA
Smad 4
Nucleus

SMAD4, it is able to move into the nucleus.

Inhibitory SMADs, SMAD6 and SMAD7, block TGFβ signaling at the protein level at both the TGFβ receptors and R-SMADS (78). SMAD6 competes with R-SMADs to bind the co-SMAD, SMAD4. SMAD7 competes with R-SMADS to interact with activated TGFBRI and TGFBRII, targeting them for degradation by recruitment of the E3 ubiquitin ligases, SMURFs (Smad ubiquitin regulatory factors). Additionally, SMAD-regulated transcription can be regulated at the by the repressors SKI and SnoN, both of which function independently from each other (79,80). TGIFs (Thymine-Guanine Interacting Factors) also regulate TGFβ signaling by acting as a corepressor and binding the SMAD complex already bound to DNA and inhibiting transcription of TGFβ target genes (81–83). In sum, there are multiple mechanisms that can regulate TGFβ signaling to maintain cellular and tissue function.

1.2.2 Tumor-suppressive role of TGFβ

TGFβ signaling has a key role in tumor suppression by inhibiting cellular proliferation and promoting cellular cytostasis and differentiation (71–73). TGFβ signaling has been found to inhibit cell cycle progression through G1 by two mechanisms – inhibition of *c-MYC* and upregulation of cyclin-dependent kinase inhibitors (CDKis). *c-MYC* transcription is inhibited by a protein complex consisting of SMAD3/4, E2F4/5, p107, and C/EBPβ; SMAD2/3 and E2F4/5 bind to the *c-MYC* promoter, and transcription is repressed by the repressor p107, which recruits other co-repressors (84,85). CDKi regulation via TGFβ signaling is direct through SMAD-mediated transcription and is cell type specific, with different cell types requiring different CDKis to arrest growth. For example, in epithelial cells, TGFβ signaling can induce expression of the CDKis,

p21CIP1 and p15INK4b, which inhibit Cyclin E and Cyclin D, respectively (84). However, in hematopoietic progenitors, TGFβ induces the CDKi, p57KIP2, able to inhibit CYCLINs A, D, and E (86). However, the sum total of these transcriptional activities, the inhibition of *c-MYC* and upregulation of CDKis, limits progression through the cell cycle and promotes cytostasis in various cell types (73).

TGFβ signaling promotes cellular differentiation to less proliferative cell types by negatively regulating transcription ID proteins. ID proteins (*I*nhibitor of *D*ifferentiation) act as antagonists to prodifferentiation transcription factors by directly binding to a class of transcription factors characterized by a basic helix-loop-helix (bHLH) motif (87). Proteins with a bHLH motif, such as MYOD and NEUROD, factors that cause cells to differentiate into muscle or neuronal cells, are able to bind DNA and regulate transcription once they dimerize. ID proteins have a helix-loop-helix motif but are unable to bind DNA. Thus, when ID proteins bind to proteins with a bHLH motif, they negatively regulate the ability of bHLH-containing proteins to bind DNA and regulate transcription (87). Published studies on ID proteins have shown ID proteins promote murine embryonic stem cell self-renewal through Bmp-mediated Smad activation (88). TGFβ signaling has been found to suppress tumor formation and proliferation in murine endothelial and epithelial cells through downregulation of ID protein expression mediated by Smad3-mediated recruitment of the repressor ATF3 to the *Id1* promoter (89). Thus, by downregulating ID proteins in epithelial and endometrial cell lines and xenografts of Ras-driven epithelial breast cancer cell line (90), TGFβ signaling is able to promote cellular differentiation and inhibit cellular proliferation.

1.2.3 Tumor-promoting role of TGFβ

The TGFβ signaling pathway can also have a tumorigenic role in cancers. This tumorigenic role typically occurs in later-stage epithelial cancers when the tumor suppressive function of TGFβ signaling has been lost, and it instead promotes tumor growth and invasion. TGFβ signaling achieves this through both SMAD-dependent and independent pathways.

Epithelial-to-mesenchymal transition (EMT) is a process by which cells lose components of cell junctions and become motile and invasive. This process is essential in embryonic gastrulation to create, among other things, the neural crest and somites (73). This highly motile process is often hijacked by solid carcinomas in the process of metastasis. Within the context of cancers, TGFβ signaling has been found to be sufficient in order to induce EMT-like behavior in transformed epithelial cells primed to become tumors (91). Additionally, there is enrichment for TGFβ ligands in the stroma of the leading edge of invasive cancer. TGFβ is able to induce EMT through SMAD-regulated transcription, by inducing the transcription factors SNAIL, TWIST, and SLUG (92). This SMAD-dependent signaling is enhanced by RAS activity (93), especially as RAS is mutated in ~40% of human CRCs (12). Inhibition of TGFBRI by a kinase inhibitor (LY2109761) has been shown to change the fate of breast cancer CD44+ of cells undergoing EMT from a mesenchymal-like state to a more epithelial like state (94).

TGFβ has also been reported to facilitate EMT through a SMAD-independent mechanism, though this pathway has been less studied. Once activated, TGFBRII phosphorylates PAR6 which then recruits its effector, SMURF1, an ubiquitin ligase that targets RHOA for degradation (95,96). RHOA is a protein that stabilizes and maintains

the cell-cell tight junctions. Destabilization and breakdown of these tight junctions, in addition to the other pro-EMT TGFβ /SMAD-dependent signaling events, facilitates EMT seen at the leading edge of cancers in a TGFβ-dependent manner.

1.3 Thymine-Guanine Interacting Factors

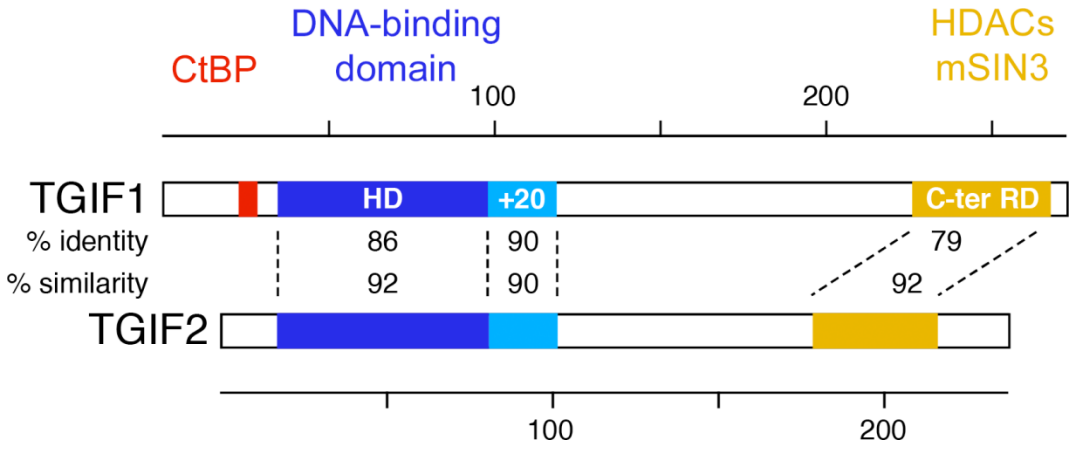
Thymine/Guanine-Interacting Factors (TGIFs) include the proteins TGIF1 and TGIF2 which function as transcriptional (co)repressors (81,82). TGIF1 was first identified by its ability to bind a DNA sequence (5' CTGTCAA 3') within the promoter of the rat *CrbpII* gene (97). When TGIF1 binds to this site, it blocks the binding of retinoid X receptor (RXR), repressing the expression of the *CrbpII* gene. TGIF2 was subsequently discovered by its similarity to TGIF1 through a search for human expressed sequence tags (ESTs). Both TGIF1 and TGIF2 share highly conserved homeodomain and carboxyl-terminal repression domains, but the rest of their respective sequences have minimal overlap (Figure 1.4), indicating that while their core functions may be similar, they may have different mechanisms of regulation (as expounded upon later).

1.3.1 Transcriptional Regulation by TGIFs

TGIF1 was initially discovered by its ability to bind and inhibit transcription of a retinoid response element (97). This repression was further shown to be a result of direct competition with RXR to bind to RXR responsive elements. TGIFs are members of the atypical TALE (three amino acid loop extension) superfamily of proteins (97–99). The

Figure 1.4 – TGIF1 and TGIF2 proteins.

Human TGIF1 and TGIF2 protein schematics with percent identity and similarity for conserved domains are shown. Amino acid scale is shown above and below for each protein. Major domains are represented: the homeodomain (HD), the 20 amino acid region carboxyl-terminal to it (+20), and the carboxyl-terminal repression domain (C-ter RD) are present in both TGIFs. The red region amino terminal to the HD represents the five amino acid PLDLS CtBP binding motif which is only present in TGIF1 and not in TGIF2. DNA-binding by the homeodomain and carboxyl-terminal repression domain interaction with HDACs and mSIN3 is indicated. Adapted from Wotton and Taniguchi, 2018 (100).


DNA-binding
domain
CtBP
HDACs
mSIN3
100
200
TGIF1
HD
+20
C-ter RD
% identity
86
90
79
% similarity
92
90
92
TGIF2
100
200

TGIF homeodomain of consists of three helices, comprised of sixty amino acids (101,102), and the TALE. Located between the first two helices and having minimal effect on DNA binding, the TALE may facilitate interactions with other proteins, including other homeodomain proteins (98,103). For example, PBX-HOX interactions are TALE-dependent and help facilitate animal development (104,105). However, it is currently unknown if the TALE facilitates any protein-proteins interactions between TGIF1/2 and other proteins (106).

Protein interactions with TGIF1 and TGIF2 facilitate its repressor activity

TGIFs are able to recruit and interact with other transcriptional corepressors, including mSIN3 and histone deacetylases (HDACs), to facilitate repression (81–83,107,108). TGIF1 interaction with mSIN3 (Figure 1.4) was shown to be through the repression domain closest to the carboxyl terminus, and HDACs can bind mSIN3, as corepressors such as mSIN3 are required to facilitate HDAC function with certain DNA binding repressors (83). TGIF1/2 interaction with mSIN3 is required for repression of TGFβ signaling. Compounding this, TGIFs, have been shown to interact with HDACs to repress transcription without mSIN3. Thus, the exact TGIF corepressor complex may contain some combination of TGIFs, HDACs, and mSIN3, but the identity of this complex is unclear (108).

TGIF1 is able to recruit and bind to the corepressor, CtBP, through an N-terminal PLDLS motif (107). Crucially, TGIF2 lacks this motif and is therefore unable to bind to CtBP (Figure 1.4). Therefore, TGIF2 cannot repress gene expression through a CtBP interaction and might only function as a HDAC-dependent repressor (108). TGIF1 and TGIF2 have mostly been considered and studied as repressors of the TGFβ pathway and

appear to regulate similar genes, but they are also able to bind directly to DNA to inhibit transcription independent of the TGFβ pathway. Thus, TGIF2, unable to interact with CtBP, may function as a HDAC-dependent repressor version of TGIF1.

TGIF-mediated repression

TGIF-mediated gene repression happens in one of three ways: TGIFs bind directly to DNA at the consensus site, TGIFs bind to active SMAD proteins at TGFβ-responsive genes, or TGIFs compete with or interact indirectly with ligand-bound nuclear hormone receptors. These are discussed below.

Direct transcriptional repression by TGIFs

Analysis of genome wide chromatin immunoprecipitation sequencing (ChIP-seq) for TGIF1 in mouse embryonic stem cells has yielded some interesting results. Much of the genome (~6000 ChIP-seq peaks in gene promoter regions) has peaks for TGIF1 (109), suggesting that the major role of TGIF1is direct transcriptional repression. Once TGIF1 is bound to the genome at its consensus binding site, other corepressors, e.g. HDACs, are recruited in order to further repress transcription (81–83,107,108). This observation from the analysis of ChIP-seq data for TGIF1 is consistent with multiple TGIF1 knockdown or knockout RNA-sequencing (RNA-seq) analyses of various cell and tissue types which suggest the majority of gene expression changes observed following decreased expression of TGIF1 are independent of the TGFβ pathway (109–111). Most of the published data on TGIF transcriptional regulation has been done on TGIF1 with some data about TGIF2. However, given that TGIF1 and TGIF2 share functional domains, it is reasonable to think the two proteins have similar functional, even

redundant, roles with regards to transcriptional regulation, potentially through different mechanisms.

Regulation of the TGFβ signaling pathway

The main body of work on TGIF function thus far has been on its role regulating the TGFβ signaling pathway, and, as a result of this, TGIFs have been misnamed TGFβ-interacting factors. TGFβ signaling modulates gene expression through an R-SMAD/SMAD4 complex. This complex translocates into the nucleus where it is able to activate or repress TGFβ targets with the help of other co-activators or repressors. TGIFs regulate this TGFβ target transcription by binding to SMADs already bound to DNA (81,82,112). TGIF1/2 binding to the SMAD complex results in the inhibition of the TGFβ-regulated gene transcription. This binding event is independent of TGIFs binding to DNA and is in competition with coactivators to bind to the SMAD complex. It should be noted that while regulation of TGFβ signaling via the SMAD complex by TGIFs does not require DNA binding, this potential form of further regulation has not been ruled out. In fact, the TGIF1 homeodomain has been shown to interact with the SMAD MH1 domain and decrease the DNA binding affinity of the whole protein complex (112). There is little evidence to suggest TGFβ signaling regulates TGIFs directly, suggesting that while TGIFs regulate and repress TGFβ signaling at a cellular level, there is no further feedback mechanism due to this interaction.

Regulation of Nuclear Hormone Regulated Responses

A large family of transcription factors, nuclear receptors (NRs) dimerize in response to ligand and bind to hormone response elements (HREs) within DNA to control gene expression programs. Initially, TGIF1 was reported to bind to a retinoid

response element in the rat *Crbp2* gene (97), limiting its transcriptional activity through a proposed mechanism of competing with RXR, a common partner for many different nuclear receptors, including retinoic acid receptors. TGIF1 can be recruited to NR-bound direct repeat HREs and function as repressors through two mechanisms. The first is direct binding to its consensus site. The second is via a direct interaction between TGIF1 and RXR through the RXR ligand binding domain to limit gene transcription via the recruitment of CtBP without the need for a TGIF1 binding site to be present (113). Therefore, this would indicate that TGIFs potentially would be able to regulate a large number of transcriptional programs without the need for a TGIF1 consensus binding site. However, it is unclear if direct DNA binding is a requirement for repression of NR-responsive genes. Furthermore, murine embryos null for *Tgif1* have shown evidence for sensitivity to retinoic acid *in utero*, with teratogenic effects of RXR signaling leading to reduction of forebrain and hindbrain development (113,114), providing further evidence that Tgifs are able to regulate the responses of nuclear hormone regulated transcriptional programs.

1.3.2 Mouse Models of TGIFs

Mutations in TGIF1 have been associated with holoprosencephaly (HPE) (115), a developmental disorder in which the brain fails to divide into two hemispheres. Most work characterizing the function of TGIFs has been done within the context of this disease in loss of function mouse models focusing on early embryogenesis (100). Many groups created mice of different strains with *Tgif1* deletions, and none of these knockout mice recapitulated the HPE phenotype (113,116–118). However, in a relatively pure C57BL6 strain developed by the Wotton lab, *Tgif1* null mice were less viable with

growth delays and placental defects (119,120). *Tgif1* null embryos were also more sensitive to retinoid acid-induced teratogenicity, resulting in an increased proportion of null embryos with exencephaly (113,114). Taken together, *Tgif1* has a developmental role in mice, through the inhibition of TGFβ and retinoic acid pathways, but knockout of *Tgif1* does not appear to cause HPE.

As previously mentioned, TGIF1 and TGIF2 may have similar or redundant functions given both the structural and functional similarities both proteins possess (Figure 1.4) (81–83,107,108). Expression of *Tgif1* and *Tgif2* overlaps during embryonic development, as *Tgif1* is first detected between e6-7.5 (116) and *Tgif2* is first detected between e6-8.5 (118,121). *Tgif2* null mice, also developed by the Wotton lab, were mostly phenotypically normal, and *Tgif2* null embryos did not exhibit any severe developmental defects. Embryos null for both *Tgif1* and *Tgif2* fail to complete gastrulation; however, the majority of embryos with one functional copy of either *Tgif1* or *Tgif2* are normal and viable (121), suggesting *Tgif1* and *Tgif2* have redundant and essential functions in embryogenesis.

To bypass these defects in gastrulation, mice with *Tgif2* null alleles were crossed to mice with conditional *Tgif1* alleles, and a *Sox2*-Cre transgene was used to delete *Tgif1* (121). Embryonic expression of *Sox2*-Cre leads to cre-mediated deletion at e6.5 in the cells of the epiblast (122). Nearly all of these embryos, with a *Tgif2* null background and as conditional deletion of *Tgif1*, survive to e10.5-11 and have both HPE-like phenotypes and left-right asymmetry (106,121). Additionally, these embryos fail to close the midbrain neural tube by e9.25 and have abnormal ventral forebrain morphology with a failure to bisect the midline of the ventral head mesenchyme (106,123). Consistent with

these observations, *in situ* hybridization expression of *Pax7* and *Pax2* shows that nasal (*Pax7*) and eye (*Pax2*) fields do not separate in *Tgif1* and *Tgif2* double knockout embryos (106). All of these observations are consistent with HPE-associated defects, and it is clear *Tgifs* play an important role in early embryonic development.

1.3.3 TGIFs in Cancer

While most work on TGIFs has focused on early development and embryogenesis, and given the role TGIFs play in inhibiting TGFβ signaling, one could reasonably think TGIFs may have an oncogenic role in cancers. Indeed, there have been recent reports indicating overexpression of TGIFs in different cancers, including but not limited to lung (124), esophageal (125) , and ovarian cancers (126), and this overexpression of TGIFs leads to both a worse prognosis and decreased survival for patients with these cancers compared to those with lower expression of TGIFs. However, the mechanisms by which TGIF overexpression leads to worse patient outcomes have not been fully elucidated as of yet, but an obvious candidate model would be one in which TGIFs functioned as repressors of a repressor, i.e. TGIFs inhibit TGFβ signaling-mediated inhibition of proliferation.

Contradictory to this expected result, patients with acute myeloid leukemia (AML) have better survival with higher levels of TGIF1 (127). Patients with mixed lineage leukemia (MLL) rearranged AML have demonstrably reduced TGIF1 expression, and when TGIF1 is re-expressed in cells *in vitro*, the cells exited the cell cycle and differentiated. Additionally, when MLL-AF9 cells expressing TGIF1 were injected into irradiated mice, onset of leukemia was delayed; however, all the mice in this experiment did die before day 30 post injection. Mechanistically, this changes were shown to be as a

result of TGIF1 competing with MEIS1, another TALE family member acting as an activator, to bind competitively MEIS1-bound regions to inhibit activation transcriptional programs regulated by MEIS1 (127).

In a completely a different mechanism of TGIF1 regulation in cancers, TGIF1 was shown to be pro-tumorigenic and participate in a feed-forward network with WNT signaling in breast cancer, in a manner independent of TGFβ signaling (128). B-catenin and its coactivator TCF were found to ChIP to the TGIF1 promoter, indicating direct WNT regulation of TGIF1, though the consensus sequence for this binding was not published, making it difficult to recapitulate this finding. Additionally, TGIF1 was shown to interact with AXIN1 and AXIN2 in the nucleus and sequester them there, thereby preventing these proteins from shuttling back into the cytoplasm and form the β-catenin destruction complex (128). This nuclear localization allows for β-catenin to accumulate in the cytoplasm, translocate to the nucleus, and drive WNT target genes and TGIF1 expression through the β-catenin/TCF complex regulation, thus completing the feed-forward loop.

This interaction between TGIF1 and WNT signaling was also identified in human colorectal cancer cell lines (129). This study demonstrated TGIF1 is able to promote tumorigenesis *in vivo* through a xenograft model and tumor proliferation *in vitro* via cell culture. TGIF1 knockdown was found to decrease transcription of WNT-responsive genes, including *WNT5a*. Additionally TGIF1 was found to be able to promote the interaction between β-catenin and TCF4. However, critically, this study was did not see any effects on β-catenin or AXIN1/2 levels within the nucleus with differing levels of TGIF1. Instead, this study proposed that increased levels of TGIF1 are able to modulate

the output of WNT signaling through two mechanisms - modulating direct transcriptional outputs of WNT signaling and promoting the interaction between β-catenin and TCF4, potentially by the formation of a trimeric complex (129). Additional mechanisms are proposed, including TGIF1 regulating chromatin accessibility through its interactions with HDACs, but these mechanisms were only hypothesized and never tested. Overall, it appears TGIFs promote tumorigenesis, but the mechanisms of regulation and potential overlapping roles of TGIF1 and TGIF2 remain to be completely elucidated.

In summary, TGIF1 and TGIF2 function as repressors, either by inhibiting TGFβ (81,82) or RXR signaling (97,113) or directly binding to DNA (82,108,109), and TGIFs have an important role in proper embryonic development and prevention of HPE (100). Recently, the role of TGIFs in cancer has begun to be elucidated in various different cancers, with evidence suggesting TGIF1 can promote CRC tumorigenesis and proliferation through an interaction with the WNT signaling pathway (128,129). However, the role of TGIFs in cancer has not been fully elucidated to this point.

1.4 Cancer Metabolism

Altered metabolism in cancer was first reported nearly one hundred years ago, and this field of study continues to not only yield a new and deeper understanding of metabolic reprogramming in tumorigenesis but also has led to the development of new therapeutics. The many metabolic changes that happen during tumorigenesis cause changes in glucose and amino acid uptake which in turn have a cascading effect on

multiple different pathways (Figure 1.5). In this section, I will enumerate and describe some of the most relevant metabolic shifts seen in cancers.

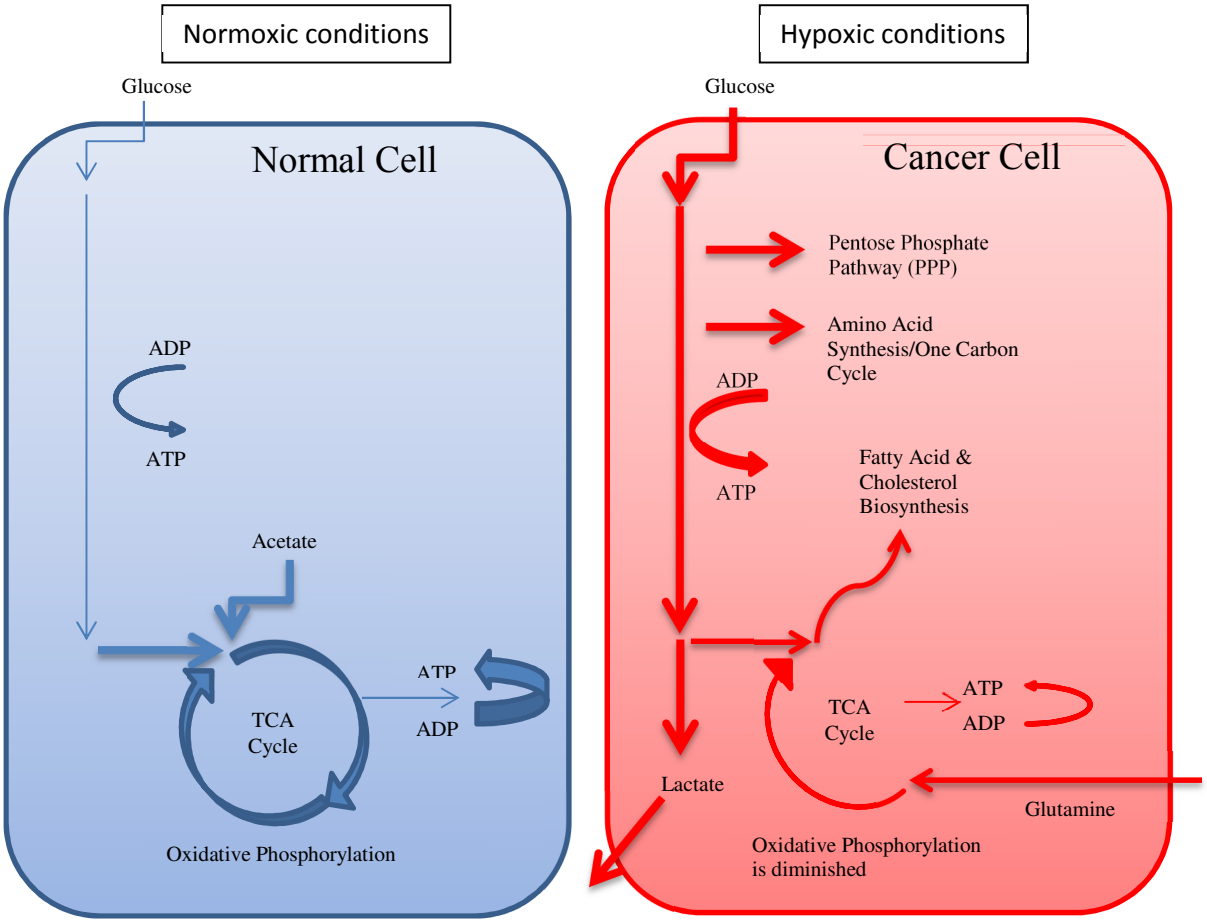
1.4.1 The Warburg effect and Hypoxia

Glycolysis is the metabolic pathway cells use to convert glucose to pyruvate in an oxygen-independent manner, and the released free energy is captured in the form of adenosine triphosphate (ATP) and NADH. Otto Warburg made one of the most famous observations in the field of cancer metabolism when he described the increased consumption of glucose in tumors compared to non-tumors even in normoxic conditions (130,131). This observation has led to the diagnostic method of using positron emission tomography (PET) imaging, with the use of a radioactively labeled glucose dye, to identify, diagnose, and stage tumors (132). Normal cells, when cultured in nutrient-rich media, do not constantly uptake the nutrients, e.g. glucose, due to tightly regulated intake of nutrients (133). Cellular nutrient uptake, especially glucose uptake, has been found to be regulated by growth factor signaling *in vitro*, and cells cultured without growth factors are unable to uptake glucose to maintain even basic cellular bioenergetics (134,135).

However, this requirement for growth factor regulation of glucose uptake can be avoided by cancer cells/tumors by overexpression of GLUT1, a plasma membrane glucose transporter, and hexokinase (HK), the first enzyme in the glycolytic pathway which irreversibly phosphorylates glucose (136). Thus, by increasing the uptake of glucose into the cell and irreversibly phosphorylating it, the rate of glucose import into the cell increases. The Warburg effect, the increase in glycolysis, even under aerobic conditions, is beneficial for tumors overall because, not only does it generate fewer

Figure 1.5 – Changes in metabolism between a normal cell and a cancer cell.

Changes in metabolism due to tumorigenesis are shown. On the left is a normal cell, and on the right is a cancer cell. Normal cells import glucose, turn it into acetyl-CoA, and use it to generate ATP through the TCA cycle and oxidative phosphorylation. Tumor cells import much more glucose and generate ATP through aerobic respiration. Width of arrows represents the amount of each process taking place. Adapted from Pavlova and Thompson, 2016 (137).


Normoxic conditions
Hypoxic conditions
Glucose
Glucose
Normal Cell
Cancer Cell
ADP
ATP
Pentose Phosphate Pathway (PPP)
Amino Acid Synthesis/One Carbon Cycle
ADP
ATP
Fatty Acid & Cholesterol Biosynthesis
Acetate
ATP
ADP
TCA Cycle
TCA Cycle
ATP
ADP
Lactate
Glutamine
Oxidative Phosphorylation
Oxidative Phosphorylation is diminished

reactive oxygen species (ROS), but also allows tumors to adapt to hypoxic conditions which occur in rapidly proliferating tumors with poor vascularization (138) (more on this below).

In addition to increased glucose uptake due to the Warburg effect, tumor tissues must proliferate in the hypoxic environment created when a rapidly proliferating tumor consumes more oxygen than is available. Hypoxia has been implicated in a number of pro-tumorigenic pathways including angiogenesis, metastasis, and proliferation and is a predictor of patient mortality in numerous cancers including breast, colon, brain, and ovarian (139). Hypoxia leads to increased cellular activity of the appropriately named transcription factor family, hypoxia-inducible factors (HIFs). HIF transcription factors are heterodimeric, consisting of oxygen-regulated α subunits and constitutively expressed β subunits. In normoxic conditions, HIF-α (HIF1α, HIF2α, HIF3α) is oxygenated and subsequently targeted for degradation (140,141). However, in hypoxic conditions, HIF-α subunits are no longer oxygenated, accumulate, and dimerize with Hif1β to drive transcription of target genes (140). It is important to note that HIF-α subunits have been shown to be stabilized by both loss of function mutations in known tumor suppressors (e.g. PTEN and p53) and gain of function in known tumor suppressors (e.g. RAS, MYC, and mTOR) (142–144). RAS/MAPK and PI3K/AKT pathways both are commonly mutated in human CRCs (12). Critically, mutations in either tumor suppressors or oncogenes in these pathways are able to stabilize HIF-α subunits independent of hypoxic conditions (144–146), highlighting the dependence of CRCs on alterations in these pathways.

As tumors are typically hypoxic environments, one of the biggest consequences of HIF-mediated signaling is angiogenesis which drives new blood vessel formation to supply more oxygen for rapidly growing tumors (147,148). However, these newly created blood vessels create a two-fold problem. First, these vessels are often quite leaky and abnormal. Secondly, increased oxygenation of tumor tissue promotes even more proliferation, and these new cells create a hypoxic environment (149–151). This creates a perverse feedforward cycle of dysfunctional vasculature in hypoxic tissues.

Hypoxic conditions also cause adaptive metabolic shifts in cancers with an increase in glycolysis and a decrease in oxidative respiration in order to limit the number of ROS generated as a byproduct (152). HIF signaling increases the expression of GLUT1 (153), promoting influx of glucose into the cell and increased glycolysis. Additionally, hypoxia drives a further increase in glucose metabolism by converting pyruvate into lactate through increased expression of lactate dehydrogenase A (LDHA) (153). This upregulation of LDHA helps generate more NAD^+ to prevent the accumulation of NADH and ATP in the cytosol (148). With decreased levels of cytosolic NADH and ATP, the tumor cell is able to maintain the intracellular signal to continue importing glucose via the overexpressed GLUT1 (137). Increased levels of ATP inhibit the action of phosphofructokinase (PFK) in the glycolytic pathway while increased levels of NAD^+ help alleviate any ROS. Through this mechanism, hypoxia drives the glycolytic pathway and production of its intermediates for additional pro-proliferative pathways.

It is not advantageous for cancers to proliferate in a nutrient/metabolite deficient state. To avoid this, mutated genes in cancer often facilitate increases in glucose/nutrient uptake to the cancer cell. The glycolytic pathway is very versatile for the cancer cell

because it provides intermediates, which become precursors, for multiple biosynthetic pathways. The pentose phosphate pathway, hexosamine biosynthesis, phospholipid biosynthesis, and one-carbon cycle all begin with glycolytic intermediates (137,138). This upregulation of glycolysis yields a positive effect on branching pro-proliferative pathways to the benefit of tumor growth and survival. Given that the Warburg effect describes how tumors use aerobic glycolysis instead of oxidative phosphorylation for energy production (130,131), the fact that glycolysis is upregulated in tumors suggests the Warburg effect is a well-regulated metabolic state imperative in meeting the increased biosynthetic demands of the tumor.

1.4.2 Glycolytic metabolites as intermediates for additional pathways

Pentose Phosphate Pathway (PPP)

The metabolic pathway first enriched in cancers through the glycolytic pathway is the pentose phosphate pathway (PPP). Glucose-6-phosphate (G6P) is oxidized to create NADPH and ribose-5-phosphate (R5P), a sugar that is a critical component in the generation of nucleotides (138). As tumor cells are rapidly dividing, the generation of nucleotides is needed to sustain this replication, and the enzymes involved in the key steps of nucleotide synthesis from R5P are often overexpressed in cancers. This pathway can be regulated by more than just irregular glucose import into the tumor cell. Tumors with RAS mutations exhibit upregulation of enzymes involved in the production of R5P as RAS can upregulate mRNA expression of *GLUT1*, *HK1*, *HK2*, and *PFK1*, all genes involved in glycolysis (154). Wild type p53, a famous tumor suppressor often mutated in CRCs (see previous section on mutated pathways in CRCs), has been shown to inactivate

nucleotide synthesis via PPP through direct binding the rate-limiting enzyme in PPP, glucose-6-phosphate dehydrogenase (G6PD) (155).

Hexosamine biosynthesis

Fructose-6-phosphate is the next molecule generated in the glycolytic pathway after glucose-6-phosphate, and it is an important precursor for hexosamine production. Hexosamines, sugars with an attached amine group, are important precursors for glycosylation reactions. Additionally, they are crucial for the synthesis of heparin sulfate and hyaluronic acid, two molecules important for cellular growth and also as potentiators for receptor mediated signaling for tumor metastasis and angiogenesis (156–158). Hexosamine production also results in the production of glycolipids and proteoglycans, thus regulating stability a subset of proteins, especially c-MYC (158), and leading to increased proliferation.

Amino acid biosynthesis and the one-carbon cycle

One well characterized glycolytic metabolite used outside glycolysis is 3-phosphoglycerate, a precursor molecule for the synthesis of the amino acids serine and glycine. Additionally, 3-phosphoglycerate can be used to generate methyl donor groups for subsequent methylation reactions. 3-phosopoglycerate dehydrogenase (PHGDH), the rate-limiting enzyme in serine biosynthesis, is amplified with copy number alterations in epithelial cancers, specifically in breast cancer and melanomas (159,160). Interestingly, this study used metabolic flux experiments to suggest melanoma and breast cancer cell lines may use up to 50% of the carbon in imported glucose for serine production and catabolism rather than glycolysis (159).

Occupying a key role in the one-carbon cycle (or folate cycle), serine production is has a unique metabolic impact within cancer cells. Briefly, the gamma carbon on serine can be transferred to the carrier protein tetrahydrofolate (THF) by serine hydroxylmethyltransferase 2 (SHMT2), generating glycine and 5, 10-methylene-THF. 5, 10-methylene-THF is an important precursor molecule that undergoes many oxidative-reductive reactions and produces a number of one-carbon THF species (161). These one-carbon-THF molecules are then utilized for the synthesis of purines, thymidine, and S-adenosylmethionine, a key substrate for methylation reactions (137). Critically, one-carbon-THF is a substrate that can used to generate NADPH which can neutralize ROS generated under hypoxic conditions commonly found in tumors (162), and hypoxic conditions induce SMHT2 expression to protect tumors from hypoxia-generated oxidative stress (163).

Overall, the glycolytic pathway provides the tumor with many intermediate molecules to use for branching pathways, and is very often upregulated in malignancies. The tumor reaps the metabolic benefits of this set of branching pathways while simultaneously repressing the potentially toxic side effects of glycolysis, e.g. excess pyruvate production and ROS generation.

1.4.3 Tricarboxylic acid cycle

The tricarboxylic acid cycle (TCA) is utilized by the cell for aerobic respiration and ATP generation and takes place in the mitochondria. Similar to glycolysis, this pathway can be used to generate metabolic intermediates as biosynthetic precursors for other pathways. Briefly, the TCA cycle uses pyruvate imported from the cytosol to the

mitochondria and converts it acetyl-CoA as an initial substrate. Acetyl-CoA is then converted to numerous different substrates, generating both NADH and FADH2 and also generating GTP. NADH and FADH2 are then used for oxidative phosphorylation, generating ATP, the key molecule for energy storage and consumption within the cell (164). The TCA cycle is the key pathway used by the cell for energy generation and is considered to be one of the key metabolic pathways conserved in many organisms. However, in tumors, this pathway is often downregulated, and the metabolic intermediates are directed toward other metabolic pathways including synthesis of nonessential amino acids, such as asparagine and aspartate (137,165,166), and fatty acid synthesis (167,168) .

1.4.4 Aceyl-CoA regulation

Acetyl-CoA is a molecule occupying a vital role in multiple metabolic pathways (137,169). It can be used as both a metabolic precursor and a source for protein and histone acetylation reactions (170). Cytosolic acetyl-CoA levels in the tumor cell are increased compared to normal cells (171). Normal cytosolic generation of acetyl-CoA is controlled by two pathways, one involving the reduction of glutamine and another using ethanol or acetate (169). In hypoxic conditions commonly seen in tumors, cytosolic acetyl-CoA generation from acetate is driven by acetyl-CoA synthetase short-chain family, member 2 (ACSS2) (172), and ACSS2 activity in the nucleus generates acetyl-CoA. This promotes increased histone acetylation directly mediated by histone acetyltransferases (HATs) (173), thereby changing the epigenetic programs to increase cellular growth and proliferation (169). ACSS2 is upregulated in both hypoxic conditions (174) and in epithelial carcinoma cell lines from many tissue types, including breast,

lung, colon, skin, and liver (175), with elevated levels corresponding with higher tumor grade and negatively correlating with patient survival (172,176,177). However, it is important to note that while ACSS2 activity may be beneficial for tumor cells, it is not considered to be an oncogene as there is little evidence to suggest overexpression of this protein initiates tumorigenesis (169).

Fatty acid biosynthesis

Tumors strikingly upregulate lipid and fatty acid production compared to most adult tissues, with the exceptions being lipogenic tissues such as the liver and adipose tissue (167,168). The upreguation of fatty acid synthesis has a two-fold benefit for the proliferating tumor cell – it provides more lipids for the cellular membrane, a phospholipid bilayer, and it helps the cell relieve oxidative stress generated by hypoxic conditions (178). Oxidative stress is generated when rapidly dividing cells, e.g. tumor cells, produce a large amount of ROS which can damage DNA and induce senescence or apoptosis (179,180). In order propagate, tumor cells need to bypass this negative regulation of growth. The generation of fatty acids partially addresses this issue as the tumor cell can alter membrane lipid composition to one more capable of adapting to oxidative stress (178).

Fatty acid synthesis upregulation in tumors begins with cytosolic citrate, exported from the mitochondria, being converted to acetyl-CoA by Akt-mediated upregulation of ATP-citrate lyase (ACLY) (181,182). Acetyl-CoA converted by acetyl-CoA carboxylase (ACC) to malyonyl-CoA. This molecule is a branch point for the production of fatty acids and cholesterol biosynthesis. Fatty acids are made when malyonyl-CoA is converted by fatty acid synthase (FASN) into fatty acid chains, e.g. palmitate (183). Key

enzymes in this pathway, ACLY, ACC, and FASN are all upregulated in epithelial cancer cell lines and cancers (e.g. breast, lung, and colon), and inhibition of these proteins has been shown experimentally to inhibit cancer growth, both *in vitro* in cell lines and *in vivo* in murine xenograft models (184–187).

Epigenetic Regulation

As previously mentioned, ACSS2 nuclear localization can provide acetyl-CoA to facilitate histone acetylation (188,189). HATs are sensitive to acetyl- CoA levels as they require acetyl-CoA as a cofactor for activity (173). Also, increased levels of histone acetylation have been shown to be present in tumors. This global increase in the epigenetic acetylation profile can stimulate cell growth and proliferation (171,190,191), increase glycolysis (192), and an increase in resistance to oxidative stress (193), especially in hypoxic conditions.

Embryonic stems cells have been shown to lose their pluripotency as acetyl-CoA levels are depleted, and this is accompanied by decreased global histone acetylation profiles, glycolysis, and proliferation (194). However this loss of pluripotency was rescued by the addition of exogenous acetate which was sufficient to maintain histone acetylation profiles (194). This suggests high acetyl-CoA levels can facilitate maintenance of a stem-like state. Similarly, cancer cells have been shown to ectopically synthesize acetyl-CoA from pyruvate during S phase of the cell cycle, and this ectopic acetyl-CoA can be used to increase levels of histone acetylation (188,189,192,195). Analysis of multiple cancer cell lines, SF188 (breast), PC-3 (prostate), LN229 (glioblastoma) showed increased histone acetylation with increased concentrations of glucose, and increases in tumor glucose uptake directly lead to increases in acetyl-CoA

generation (171). The acetylated genes in response increased acetyl-CoA levels were involved in cell cycle progression, growth, and DNA replication in LN229 cells. More importantly, this increased expression was dependent on oncogenic RAS and AKT signaling, two signaling pathways often mutated in cancers, including CRCs (171). Thus, the changes in epigenetic regulation due to increased levels of acetyl-CoA can have a profound proliferative and growth impact in tumorigenesis.

Rapidly proliferating tumor cells upregulate the pentose phosphate pathway, amino acid, and fatty acid synthesis using metabolic intermediates from both glycolysis and the TCA cycle. Overall, in cancers, the Warburg effect, the marked upregulation of aerobic respiration, is observed, and hypoxic conditions can also increase glycolysis, leaving to metabolic shifts and changes in epigenetic profiles to benefit rapidly proliferating cells.

For my thesis work, I further characterized the role of TGIFs in colorectal cancer. It is clear TGIFs have some oncogenic role in CRC as levels of TGIFs are increased, promote tumor proliferation, and are associated with poor patient prognosis, but we understand little of its function. Understanding how TGIFs interact with both the TGFβ and WNT pathways in human disease would play a critical role in both CRC pathogenesis and treatment. Additionally, though TGIFs have context-dependent and tissue-dependent roles, understanding how and what genes are regulated by TGIFs across a variety of tissues would help gain insight to the core functions of TGIFs. To that end, we used genetically engineered mouse models to study both the function of Tgif1 and

Tgif2 in intestinal/CRC and identify downstream Tgif targets. Tgif1 and Tgif2 deletion in an APC mouse model of CRC cancer reduced tumor size and number in the small intestine and tumor size in the colon. TGIF1 overexpression increased the overall number and size of tumors in the small intestine. To elucidate the mechanisms TGIFs use to result in these tumor size and number of differences observed, transcriptome profiling was performed on colon tumors from these mice. These results demonstrated deletion of Tgifs had little effect on both WNT and TGFβ signaling. Instead, we see a novel result – Tgifs appear to regulate gene expression in multiple metabolic pathways. This result would suggest Tgifs participate in the metabolic reprogramming occurring in CRC and may be applicable to other cancers.

Chapter 2 – TGIF1 expression promotes intestinal tumorigenesis[1,2]

2.1 Introduction

Colorectal cancer (CRC) is among the most frequently diagnosed cancers in the United States and is the cause of over 140,000 deaths every year (1). Most cases of CRC are sporadic in nature, and *Adenomatous Polyposis Coli* (*APC*), a gene encoding a scaffolding protein which assembles the β-catenin destruction complex consisting of GSK3β, AXINs, and β-catenin, is mutated in >70% of these sporadic cases of CRC. In the absence of WNT ligand, β-catenin is phosphorylated and targeted for degradation. WNT signaling inhibits the phosphorylation and eventual degradation of β-catenin, allowing it to accumulate in the nucleus. Then, β-catenin can translocate into the nucleus and drive transcriptional programming by interacting with the LEF/TCF family of transcription factors. Mutations or deletions in *APC* have the functional consequence of constitutively active β-catenin, leading to aberrant WNT signaling. Additionally, Apc^{MIN} mice, mice with germline inactivation of one allele of *Apc*, and Apc^{CKO} mice, mice with Cre-mediated excision of a loxP flanked exon (exon 14) develop many adenomas, due to stochastic inactivation of the intact allele.

Presence of a TGFβ superfamily ligand, e.g. Activin, Nodal, TGFβ, induces TGFβ receptor type 1 and type 2 to form a heterotetrameric complex, resulting in the

[1] This work is a part of a manuscript currently under revision at *Genes and Development*. Shah, A., Melhuish, T.A., Frierson Jr., H.F., Wotton, D. (2018) TGIF transcription factors repress acetyl-CoA metabolic gene expression and promote intestinal tumor growth.

[2] Tiffany Melhuish helped with counting the murine tumors and performed the initial western blots for the *Villin*-T7-hTGIF1 construct. Dr. Henry Frierson analyzed histological sections from the murine tumors.

phosphorylation and activation of SMAD2 and SMAD3. These SMADs associate with the co-SMAD, SMAD4, and translocate to the nucleus to affect gene expression. TGFβ signaling is often tumor suppressive in cancers due to its anti-proliferative effects, and different mutations in this pathway are found in patients. *TGFBRII*, encoding TGFβ receptor type II, is mutated in 25% of CRCs (196,197), and loss of heterozygosity in a region of chromosome 18 including SMAD2 and SMAD4 occur in 70% of CRCs (though it is important to point out this is less common in colon adenomas) (198).

Thymine-Guanine Interacting Factor 1 (TGIF1) and the paralogous TGIF2 are homeodomain transcription factors which are part of the TALE (three amino acid loop extension) superfamily (97,108,199). Other proteins in the TALE family of homeodomain proteins include MEIS and PBX which activate gene expression (103). In contrast, however, TGIF1 and TGIF2 are transcriptional repressors that interact with other general corepressors, including mSIN3 and histone deacetylases (81,108). TGIF1, but not TGIF2, can additionally recruit CtBP1/2 through a conserved interaction motif (107). TGIFs inhibit TGFβ-mediated gene responses by binding to the SMAD complex on DNA and recruiting other corepressors to inhibit SMAD mediated transcription (81,108). Loss of function of *TGIF1* has been associated with holoprosencephaly (HPE), a severe genetic disease affecting forebrain development (100). Additionally, TGIFs have been found to be upregulated in a variety of epithelial cancers, including ovarian (126), esophageal (125), and lung (124) among others. One murine study of breast cancer reported TGIF1 promoted tumorigenesis independent of the TGFβ pathway, and *Tgif1* was a direct β-catenin/TCF transcriptional target (128,200). Taken together, TGIFs may have an oncogenic role.

As TGIF functions may overlap with the WNT and TGFβ signaling pathways, both of which are important in CRC, we first analyzed different human CRC datasets to identify if TGIFs were indeed upregulated. We then used a human CRC cell line, HCT116, to assay the role of TGIF1 *in vitro*. We subsequently used genetically engineered mouse models to address the function of Tgifs in intestinal cancer tumorigenesis. Knockout of *Tgifs* in SI crypts resulted both in fewer cells and fewer proliferating cells while overexpression of TGIF1 had the opposite effect. In a mouse model of CRC, over-expression of TGIF1 in intestinal epithelial cells increased the size and number of adenomas in the small intestine (SI), and deletion of *Tgif1* and *Tgif2* reduced tumor size in both the SI and colon. These results taken together suggest Tgifs have a role in intestinal tumorigenesis, and it may be due to differences in proliferative potential of intestinal crypts.

2.2 Materials and Methods

2.2.1 Cell culture

HCT116 cell line were from the Wotton Lab at the University of Virginia. HCT116 cells were cultured in RPMI-1640 (Gibco 11875-093) supplemented with 10% fetal bovine serum (FBS) (HyClone SH30396.03), 1% Anti-Anti (Gibco15240-062), and 100µg/mL Normocin (InvivoGen ant-nr-1). Cells were grown in a humidified 37°C incubator supplemented with 5% CO_2. Cell line identity was verified by STR profiling.

2.2.2 CRISPR/Cas9 mediated knockout and verification

Guide RNAs targeting the second exon of *TGIF1* were cloned into pX330 (Addgene #42230). The guide RNA used for *TGIF1* was: 5' – CTGTGCAGATTCTTCGGGAT – 3'. HCT116 cells were plated in 6-well plates (200,000/well). Cells were transfected the next day with 1µg of pX330 with a sgRNA against TGIF1 and 400ng of a puro-resistance knock in cassette. The puro-resistance knock in cassette was generated by PCR from pBabe-puro with overhangs homologous to TGIF1, at the site of the Cas9-mediated cut. Media on the transfected cells was changed after 24 hours to regular culture media. 72 hours after transfection, cells were split into 60mm plates, and puromycin, at 0.5µg/mL, was added. Cells were allowed to grow and form colonies for approx. two weeks. During this time, culture media was changed every third day. Once colonies had sufficiently formed, individual colonies were picked and placed into single wells of a 12-well plate, eventually sequentially expanded for protein samples and DNA. Protein samples from individual colonies were prepared in MSLD for DNA pulldown followed by western blot to verify TGIF1 absence. *Evi5l* primers were used for pulldown as described in (111).

PCRs for endogenous *TGIF1* and 3'/5' puromycin cassette integration were sent for sequencing to verify *TGIF1* knockout.

2.2.3 Cell Proliferation Assay

Wild type and two TGIF1 mutant HCT116 cell lines were plated in triplicate at 300,000 cells/plate. Three days later, cells were trypsinized, and live cells were counted via trypan blue exclusion. 300,000 cells were replated to count at the next passage. This was repeated six times in total. Fold change was calculated after each passage, and total fold change was calculated by multiplying the fold changes after each passage together.

2.2.4 Mice

All animal procedures were approved by the Animal Care and Use Committee of the University of Virginia, which is fully accredited by the AAALAC. Conditional alleles with loxP flanked exons are referred to here as 'f' for loxP flanked, or 'r' for recombined (null). Mice were maintained on a predominantly C57BL/6J background. Conditional *Apc* mice were from the NCI, and the *Villin*-Cre line was from Jax (B6.Cg-Tg(Vil1-cre)1000Gum/J; #021504; (14)). Conditional Tgif2 mice were generated from targeted ES cells obtained from EUCOMM ($Tgif2^{tm1a(EUCOMM)Wtsi}$; IKMC project 24492) and crossed to conditional a Tgif1line (121). *Villin*-TGIF1 transgenic mice were generated at the UVA GEMM Core. The human TGIF1 cDNA with an amino-terminal T7 epitope tag was inserted into the *Villin* promoter plasmid (12.4kb*Villin*-ΔATG), which was a gift from Deborah Gumucio (Addgene plasmid # 19358; (14)). Germ line transmission was verified by PCR and expression by western blot.

2.2.5 Tumor analysis, IF, and histology

Tissues were fixed in zinc-formalin, paraffin-embedded, sectioned at 5 microns, and stained with Hematoxylin and Eosin (H&E) or prepared for immunostaining as described (201). Images were captured with 10, 20, or 40x objectives, using a Nikon Eclipse NI-U with a DS-QI1 or DS-Ri1 camera and NIS Elements software, and adjusted in Adobe Photoshop. For IF, antibodies were as follows: Rabbit anti-Acss2 (Abcam 66038), rabbit anti-Slc2a1 (Millipore 07-1401), mouse anti-β-catenin (BD Transduction Labs 610153).

2.2.6 RNA isolation and qRT-PCR

RNA from snap-frozen tissue was isolated and purified using Absolutely RNA kit (Agilent) and quality checked by Bioanalyzer. cDNA was generated using Superscript III (Invitrogen) and analyzed by real time PCR using a BioRad MyIQ cycler and Sensimix Plus SYBRgreen plus FITC mix (Bioline), with intron-spanning primer pairs selected using Primer3 (http://frodo.wi.mit.edu/). Expression was normalized to Rpl4 and Cyclophilin using the delta Ct method.

2.2.7 Tissue western blot

Tissues were ground in PBS, followed by addition of NP-40 to 1%, then lysates were separated by SDS-PAGE, transferred to Immobilon-P (Millipore) and proteins visualized using ECL (Pierce). Primary antibodies were against Acss2 (Abcam 66038), TGIF1(82), γ-tubulin (Sigma T6557), and HSP90 (Cell Signaling #4874). Pcx was detected using Neutravidin conjugated HRP (ThermoFisher).

2.2.8 Statistical methods

Experiments in cell lines were completed three independent times with n=3 technical replicates, and data is shown as mean +/- SD of one experiment, unless otherwise specified. Cell line and mouse data was analyzed using one- way ANOVA followed by pairwise t-tests with Holm post-hoc tests. p-values are denoted in figure legends. Data was analyzed using Microsoft Excel and RStudio.

2.3 Results

2.3.1 Increased TGIF expression in human colorectal tumors

Analysis of CRC data sets showed elevated *TGIF1* and *TGIF2* in CRCs (Figure 2.1A-D) and increased expression in both adenomas and carcinomas (Figure 2.1C). Comparison of *TGIF1* expression in paired tumor and normal patient samples also showed increased expression in tumors in all cases (Figure 2.1E). Similarly, analysis of TCGA colorectal data showed elevated *TGIF1* and *TGIF2* in adenocarcinomas, with additionally elevated levels of *TGIF1* in mucinous CRC and cecum adenocarcinomas (Figure 2.1F).

To test what role elevated *TGIF1* might have in CRCs, we used CRISPR/Cas9 to knockout *TGIF1* in HCT116 cells, an immortalized human adenocarcinoma cell line. HCT116 cells are in the top 15% of CRC cell lines expressing TGIF1 according to CCLE (Cancer Cell Line Encyclopedia), making it a suitable cell line in which to test the role of TGIF1. Multiple knockout clones were generated (Figure 2.2A) and sequenced (Figure 2.2B). TGIF1 expression is robust in this cell line (Figure 2.2C), and CRISPR/Cas9 mediated knockout completely abrogates TGIF1 expression in the mutants. With two of the mutant clones, we tested if TGIF1 knockout affected growth in HCT116 cells using a 3T3 assay with serial replating. At the second passage, HCT116 cells with TGIF1 knockout grew significantly slower (Figure 2.2C), and this remained the case at the fourth passage. Cells were passaged six times, and, at the second passage (and every subsequent passage), both TGIF1 knockout HCT116 cell lines grew slower than the HCT116 controls (Figure 2.2D), suggesting TGIF1 function is important for cellular proliferation.

Figure 2.1 – Increased expression of TGIFs in CRC.

A-D) Expression of TGIF1 and TGIF2 was analyzed from publicly available CRC gene expression array data-sets obtained from GEO. Data is plotted as relative expression (median, with upper and lower quartiles [box] and 5^{th} and 95^{th} percentiles [whiskers]) for normal (N) and tumor (T) (panels A, B, D), or normal (N), adenoma (Ad), and carcinoma (Ca) in panel C. E) Relative expression of TGIF1 in paired normal and tumor samples from panel D is shown. p-values for comparisons to normal are shown. F) Log2 median centered expression data for *TGIF1* and *TGIF1* in the TCGA colorectal dataset (analysis from Oncomine, with upper and lower quartiles and 10^{th} and 90^{th} percentiles), for normal and the indicated tumor types. p-values for comparisons to normal are shown.

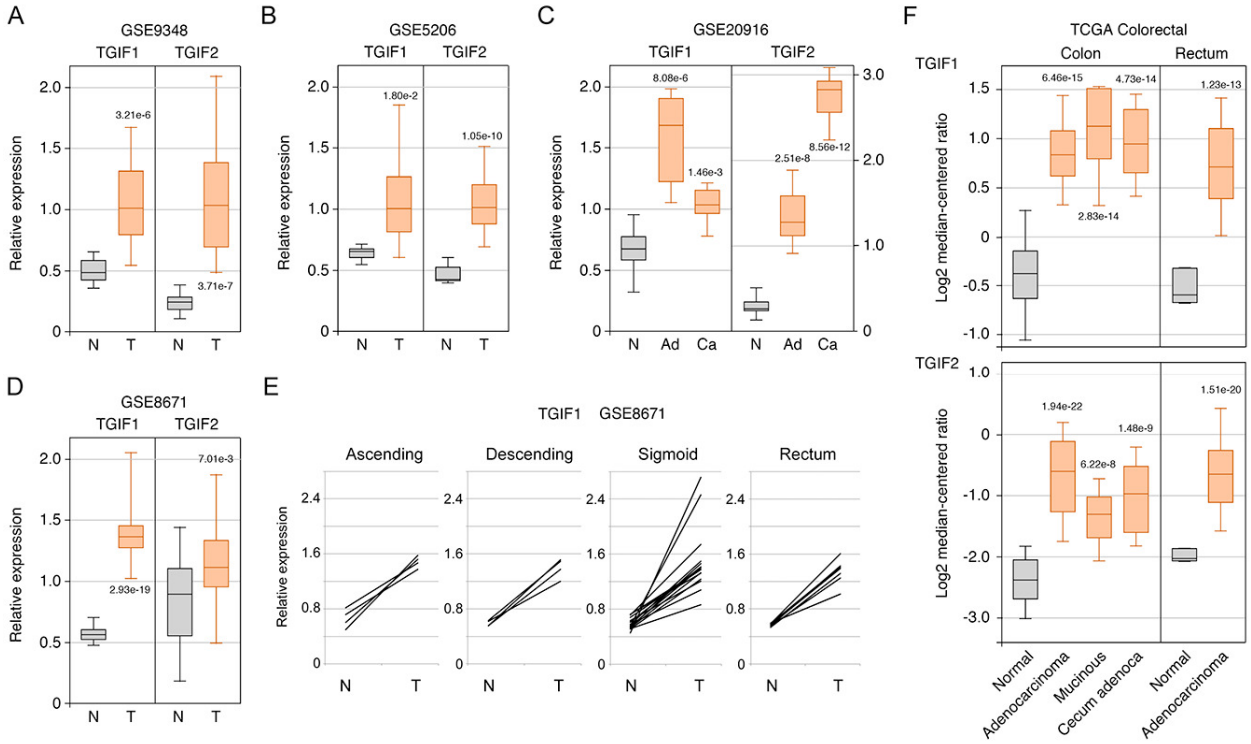
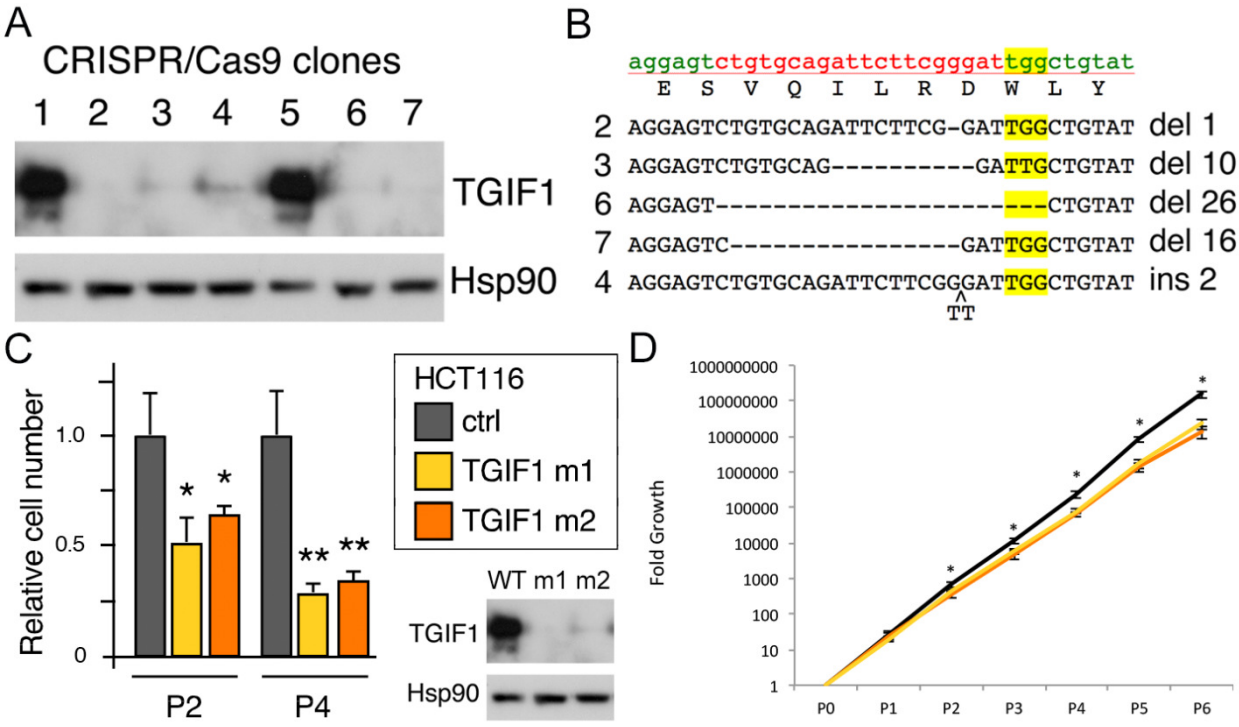

A
GSE9348
TGIF1
TGIF2
Relative expression
3.21e-6
3.71e-7
N
T
B
GSE5206
1.80e-2
1.05e-10
C
GSE20916
8.08e-6
1.46e-3
2.51e-8
8.56e-12
Ad
Ca
D
GSE8671
7.01e-3
2.93e-19
E
TGIF1 GSE8671
Ascending
Descending
Sigmoid
Rectum
F
TCGA Colorectal
Colon
Rectum
Log2 median-centered ratio
6.46e-15
4.73e-14
2.83e-14
1.23e-13
1.94e-22
1.48e-9
6.22e-8
1.51e-20
Normal
Adenocarcinoma
Mucinous
Cecum adenoca

Figure 2.2 – CRISPR-mediated TGIF1 knockout in HCT116 cells leads to decreased proliferation.

A) Expression of TGIF1 was analyzed by western blot (with Hsp90 as a loading control) from seven HCT116 TGIF1 colonies. Note that colonies 1 and 5 appear do not appear to have any TGIF1 mutations. B) A schematic of the sgRNA (red) used as a guide for Cas9 and the sequences of the five colonies from A with abolished TGIF1 expression. Note the generation of clones with both deletions and insertions. C) Relative cell number of HCT116 control or two mutant colonies shown after passage 2 (P2) or P3. * $p < 0.05$, ** $p < 0.01$. Western blot showing TGIF1 expression in wild type (WT), mutant 1 (m1), and mutant 2 (m2) shown. D) Fold growth of HCT116 WT, m1, and m2 cells shown over six passages with serial replating displayed. Note the logarithmic scale. * $p < 0.05$.


A
CRISPR/Cas9 clones
1 2 3 4 5 6 7
TGIF1
Hsp90
B
aggagtctgtgcagattcttcgggattggctgtat
E S V Q I L R D W L Y
2 AGGAGTCTGTGCAGATTCTTCG-GATTGGCTGTAT del 1
3 AGGAGTCTGTGCAG----------GATTGCTGTAT del 10
6 AGGAGT----------CTGTAT del 26
7 AGGAGTC----------GATTGGCTGTAT del 16
4 AGGAGTCTGTGCAGATTCTTCGGGATTGGCTGTAT ins 2
TT
C
Relative cell number
1.0
0.5
0
P2
P4
HCT116
ctrl
TGIF1 m1
TGIF1 m2
WT m1 m2
TGIF1
Hsp90
D
Fold Growth
1000000000
100000000
10000000
1000000
100000
10000
1000
100
10
1
P0 P1 P2 P3 P4 P5 P6

We attempted to create *TGIF1* and *TGIF2* double knockout HCT116 cells using CRISPR/Cas9. We attempted to knockout *TGIF2* within two of the *TGIF1* KO HCT116 cloes we had initially generated, but we were unable to generate any colonies from this transfection. We then created *TGIF2* HCT116 KO cells and then attempted to knockout *TGIF1* within these *TGIF2* KO cells. However, of the 29 colonies with puromycin knockin in one *TGIF1* allele that we sequenced for the *TGIF1* allele from this transfection, all of the colonies had a second wild type, unaltered *TGIF1* sequence. Taken together, this further suggested that TGIFs may have an important role in CRCs.

2.3.2 Modulation of levels of Tgifs in the intestinal epithelium

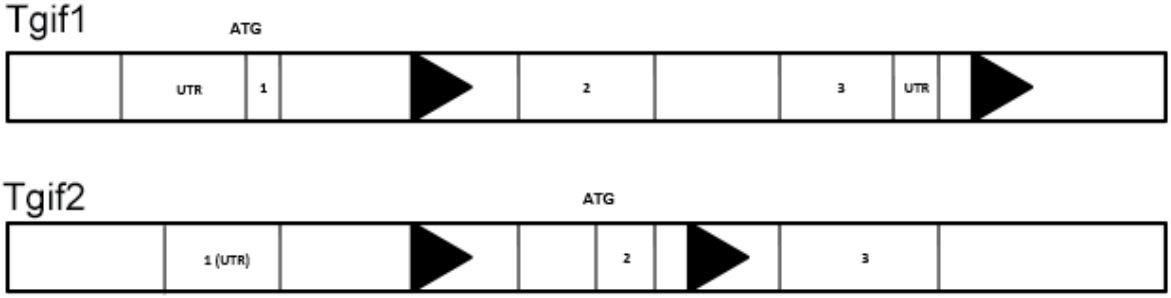
To test effects of decreased Tgif1 and Tgif2 expression in the intestine, mice lacking both Tgif1 and Tgif2 in the intestinal epithelium were generated. We used *Villin*-Cre to delete loxP flanked *Tgif1* (118) and loxP flanked *Tgif2*, derived from a knockout first allele from EUCOMM (Figure 2.3A). Male mice lacking both Tgifs were approximately 10% lighter by 42 days, and maintained this difference (Figure 2.3B); however, female mice lacking both Tgifs did not show any differences in weight (Figure 2.3C). Additionally, there was no change in the length of the small intestine (Figure 2.3D), though the lengths were somewhat variable. Overall, mice lacking both Tgifs (conditional double knockouts or "cdKOs") from the intestinal epithelium were viable, grossly normal, and fertile.

To test effects of increased TGIF1 expression in intestine, we generated a transgene in which an amino terminal T7-epitope tagged human *TGIF1* was expressed from the *Villin* promoter (Figure 2.4A). Analysis of expression in a panel of tissues by western blot showed robust expression in the intestine, with no detectable expression in

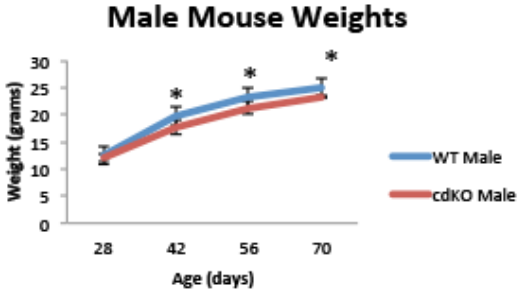
Figure 2.3 – Mice with intestine-specific knockout of Tgif1 and Tgif2 are viable.

A) Schematic of loxP flanked alleles of *Tgif1* and *Tgif2*. Exons, ATG transcription start sites, and UTRs are denoted. Black arrows represent loxP sites. Cre-mediated deletion of *Tgif1* results in loss of exons 2 and 3 of *Tgif1* and loss of exon 2 of *Tgif2*. B-C) Wild type or *Tgif1;Tgif2* (cdKO) male/female mice were weighed from 4 weeks to 10 weeks of age. p-values and relative weight shown below. D) Small intestine length (cm) was measured in WT and cdKO mice.

A

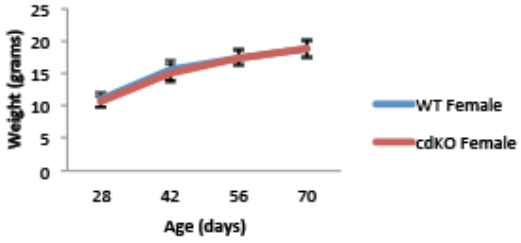


B



	28 Days	42 Days	56 Days	70 days
T-test	0.441	0.0271	0.0332	0.0337
Relative weight	0.95	0.9	0.92	0.93

C



	28 Days	42 Days	56 Days	70 days
T-test	0.435	0.582	0.988	0.991
Relative weight	0.96	0.97	1	1

D

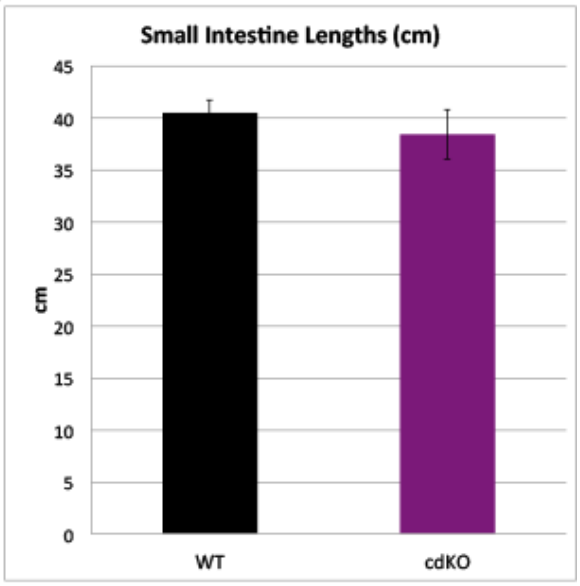
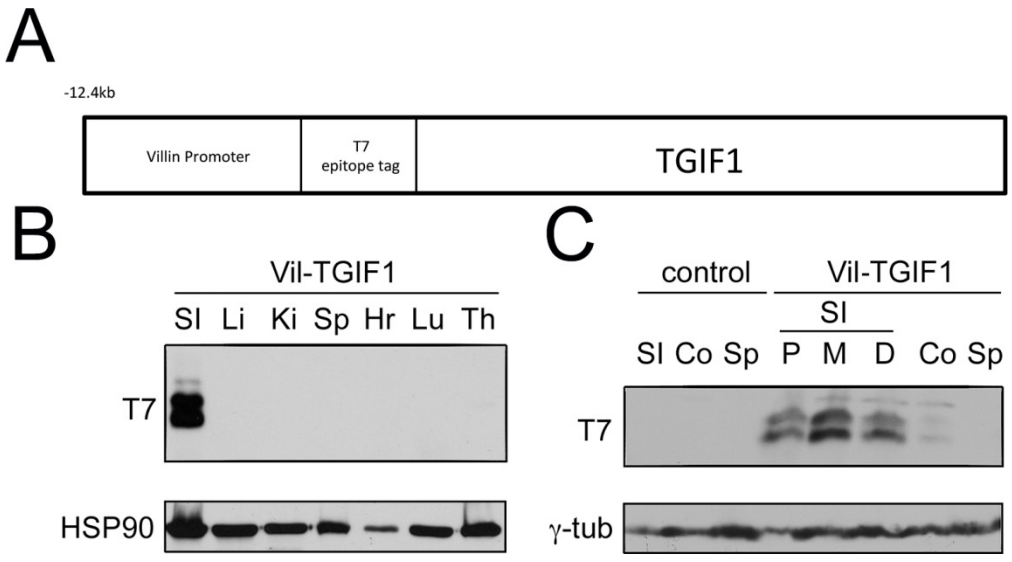


Figure 2.4 – *Villin*-T7-TGIF1 expression is limited to the intestines.

A) A schematic of the *Villin*-T7-TGIF1 transgene is shown. B) Expression of a *Villin* promoter-T7-TGIF1 transgene (Vil-TGIF1) was analyzed by western blot for the T7-epitope tag in a series of tissues (SI: small intestine, Li: liver, Ki: kidney, Sp: spleen, Hr: heart, Lu: lung, Th: thymus). C) Expression of the Vil-TGIF1 transgene in a non-transgenic (control) and transgenic animal (SI: small intestine [P: proximal, M: middle, D: distal thirds], Co: colon, Sp: spleen). Hsp90 or γ-tubulin loading controls are shown.

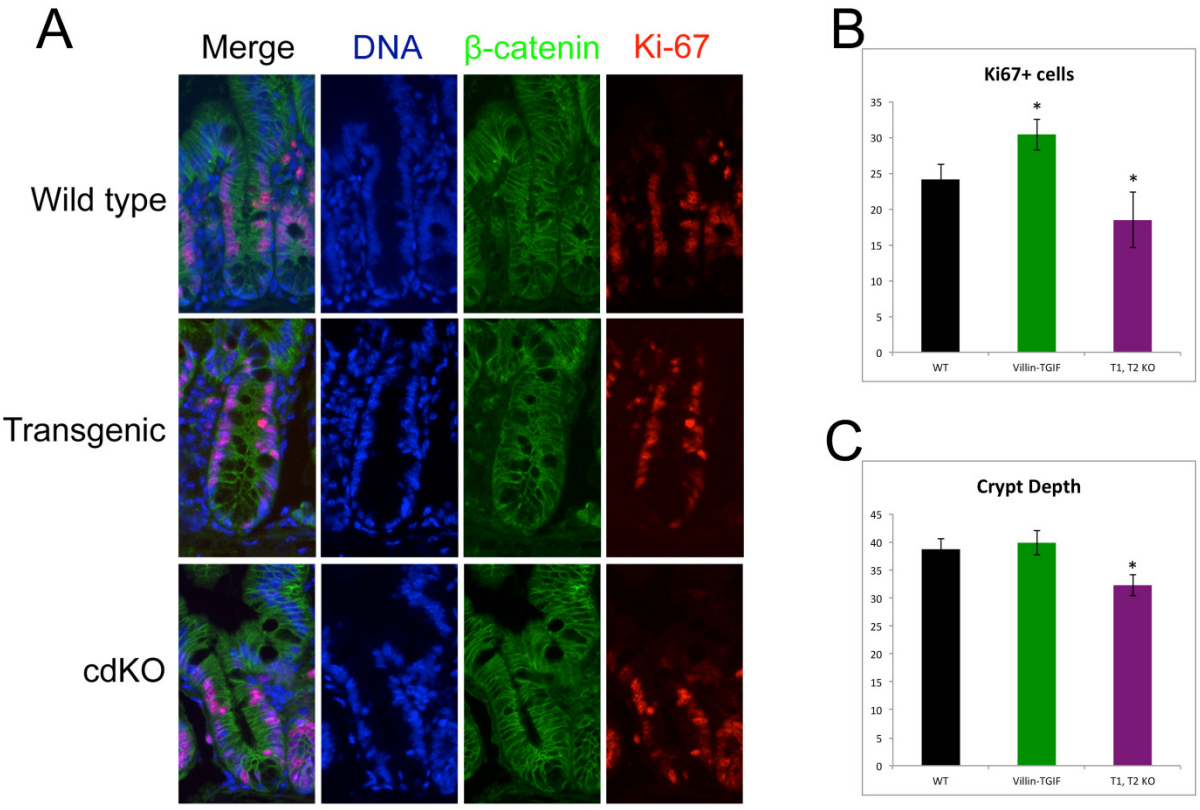

A
-12.4kb
Villin Promoter
T7
epitope tag
TGIF1
B
Vil-TGIF1
SI Li Ki Sp Hr Lu Th
T7
HSP90
C
control
Vil-TGIF1
SI
SI Co Sp P M D Co Sp
T7
γ-tub

any other tissue examined (Figure 2.4B). Within the small intestine, we observed readily detectable expression in the proximal, middle, and distal thirds, with much lower expression in the colon and none in non-transgenic tissue (Figure 2.4C and (202)). Similarly to mice lacking both Tgifs, Vil-TGIF1 transgenic mice were normal and viable to at least 150 days.

As HCT116 cells lacking TGIF1 showed decreased proliferative potential, we decided to compare intestinal cellular proliferation in wild type, cdKO, and transgenic mice. We analyzed the SI crypts of these three genotypes of mice as the crypts are where the majority of the proliferative potential of the intestinal epithelium lies. We stained intestinal crypts for Ki67 to assay proliferating cells (Figure 2.5A). Strikingly, we observed cdKOs had significantly fewer proliferating cells in the crypts while transgenic mice had significantly more proliferating cells (Figure 2.5B). This observation supports the data obtained from the HCT116 TGIF1 knockout cells further suggesting Tgif knockout causes an anti-proliferative effect. Additionally, as TGIF1 overexpression increases the number of proliferative cells in the intestinal epithelium (Figure 2.5B), this observation even suggests Tgifs promote proliferation. While counting Ki67+ cells, we noted that the cdKO mice had fewer cells per crypt (crypt depth) than the wild type, but there was no difference between wild type and transgenic mice (Figure 2.5C). However, even with differences in proliferative potential in SI crypts, we did not observe any differences in villi length between these three genotypes of mice (data not shown), suggesting that the number of proliferating cells in the SI crypt may not have any effect on villi length.

Figure 2.5 – cdKO mice have fewer proliferating cells in the intestinal epithelium.

A) WT, transgenic, and cdKO SI tissue were fixed, sectioned at 5µm, and stained with β-catenin, DAPI, and Ki67 to measure proliferation in intestinal crypts with representative images shown. B) Quantification of Ki67+ cells from stained crypts. C) Quantification of crypt depth as measured by the number of total cells in each crypt.


A
Merge
DNA
β-catenin
Ki-67
Wild type
Transgenic
cdKO
B
Ki67+ cells
35
30
25
20
15
10
5
0
*
*
WT
Villin-TGIF
T1, T2 KO
C
Crypt Depth
45
40
35
30
25
20
15
10
5
0
*
WT
Villin-TGIF
T1, T2 KO

2.3.3 Increased Tgif expression in colorectal tumors

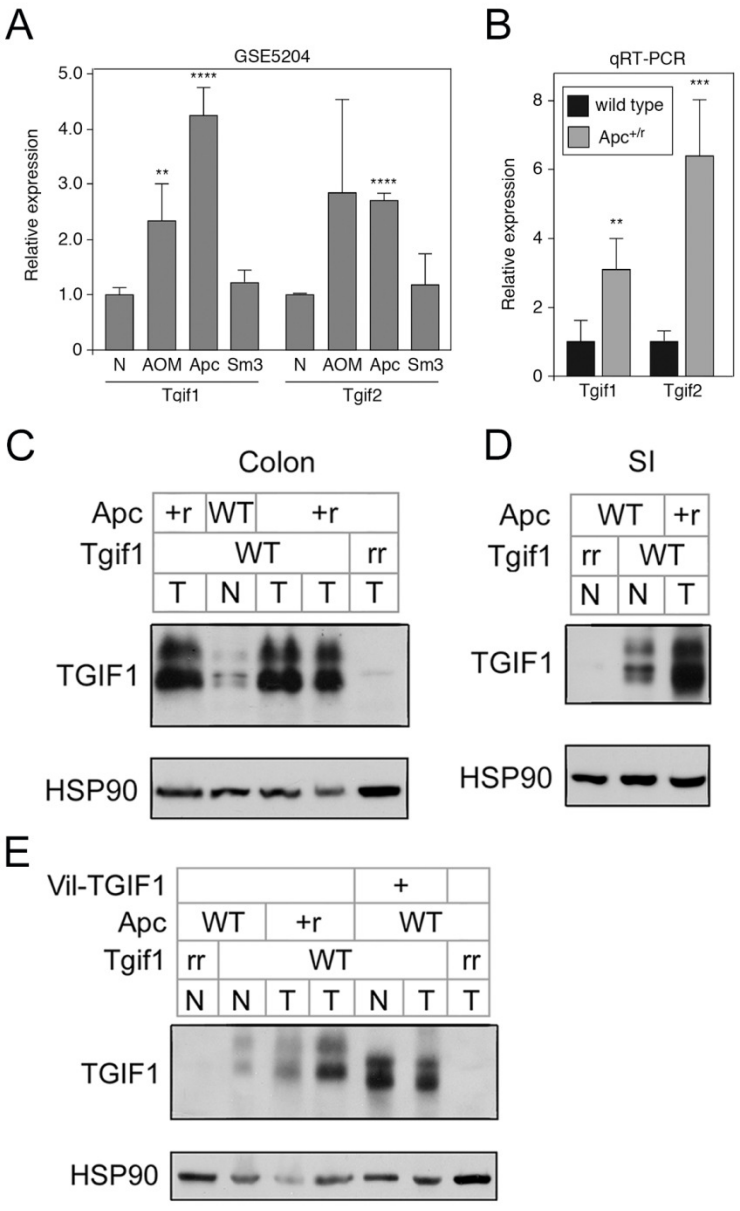
Mouse models of intestinal cancer, based on genetic alterations found in human cancers or treatment with chemical carcinogens, have been analyzed by gene expression array (203). In these analyses, *Tgif1* expression was significantly higher in the azoxymethane (AOM) and *Apc* mutant models but not in one based on inactivation of Smad3, a component of the TGFβ signaling pathway (Figure 2.6A). Similar results were found with *Tgif2* expression in this dataset, although the signal in the AOM samples was too variable to reach statistical significance.

To test expression of *Tgifs* in *Apc* mutant mouse colon tumors, we combined a *Villin*-Cre transgene with a loxP flanked allele of *Apc*, isolated normal colon and colon tumors at 12 weeks of age, and analyzed gene expression by qRT-PCR. We observed a significant increase in expression of both *Tgif1* and *Tgif2* in colon tumors compared to normal tissue (Figure 2.6B). Western blot analysis of similar 12 week tumors showed increased Tgif1 protein expression in tumor compared to normal (Figure 2.6C). We also analyzed tumors in which *Tgif1* was deleted specifically from epithelial cells. Little or no Tgif1 signal was detectable in these samples, suggesting the majority of Tgif1 present in colon and its increase in tumors were due to expression in the intestinal epithelium (Figure 2.6C). An increase in Tgif1 expression in small intestine tumors compared to normal tissue was also observed (Figure 2.6D). Thus expression of both Tgif1 and Tgif2 is higher in *Apc* mutant intestinal tumors in mice, recapitulating observations seen in the human disease.

To compare expression of the Vil-TGIF1 transgene to the endogenous Tgif1 in *Apc* mutant tumors, we performed western blots with a TGIF1 antiserum that recognizes

Figure 2.6 – Tgif expression in mouse intestinal tumors.

A) Relative expression of Tgif1 and Tgif2 (mean + sd) from the GSE5204 dataset, for normal colon (N), tumors from and AOM/DSS model, *Apc* mutant tumors, or those from a Smad3 mutant model. B) Relative Tgif1 and Tgif2 expression (mean + sd of quadruplicate samples), determined by qRT-PCR, from wild type (normal) colon or from *Apc* mutant colon tumors. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. C) Expression of Tgif1 was analyzed by western blot (with Hsp90 as a loading control) from normal colon (N) and tumor (T) from tissue of the indicated genotypes (+: wild type, r: recombined allele). D) Tgif1 expression from small intestine (normal or tumor, as in panel C). E) Expression of Tgif1 in normal (N) and tumor (T) tissue from mice of the indicated genotypes is shown by western blot with a TGIF1-specific antiserum and HSP90 as a loading control. Note the transgenic TGIF1 migrates slightly faster than the endogenous mouse Tgif1.


A
GSE5204
Relative expression
5.0
4.0
3.0
2.0
1.0
0
**
****
****
N AOM Apc Sm3 N AOM Apc Sm3
Tgif1
Tgif2
B
qRT-PCR
wild type
Apc+/r
Relative expression
8
6
4
2
0
**
***
Tgif1
Tgif2
C
Colon
Apc +r WT +r
Tgif1 WT rr
T N T T T
TGIF1
HSP90
D
SI
Apc WT +r
Tgif1 rr WT
N N T
TGIF1
HSP90
E
Vil-TGIF1 +
Apc WT +r WT
Tgif1 rr WT rr
N N T T N T T
TGIF1
HSP90

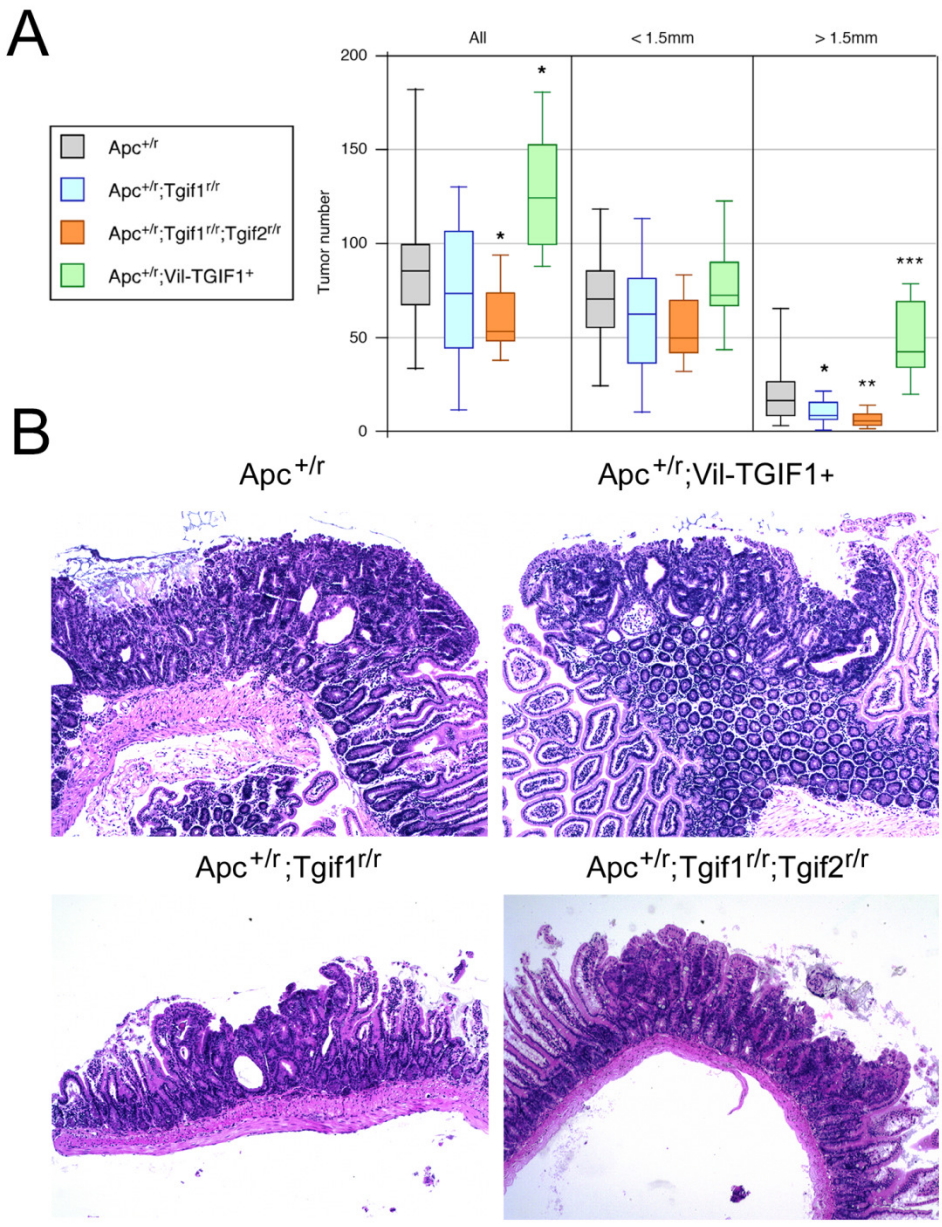
both human and mouse Tgif1. There was an increase in endogenous Tgif1 expression in regions of the SI with tumors, compared to wild type tissue (Figure 2.6E). The levels of expression of transgenic TGIF1 was similar in both tumor and normal and, while higher than the expression of mouse Tgif1 in normal tissue, were quite similar to the increased level of endogenous Tgif1 in tumors (Figure 2.6E). The transgenic TGIF1 migrates more rapidly on SDS-PAGE than mouse Tgif1, and it appears that expression of the transgene effectively reduces expression of endogenous Tgif1, as evidenced by the almost complete absence of the slower migrating Tgif1 band in the transgenic samples (Figure 2.6E). Thus, Vil-TGIF1 is over-expressed to a level similar to that of the elevated endogenous Tgif1 expression seen in tumors.

2.3.4 Altered tumor burden in the small intestine

To test effects of Tgifs on tumorigenesis, we combined conditional alleles of *Tgif1*, both *Tgif1* and *Tgif2*, or the *Vil-TGIF1* transgene with *Villin*-Cre and a heterozygous loxP flanked *Apc* allele. At 12 weeks of age, small intestines were separated into proximal, middle, and distal thirds and opened along the length to identify tumors. Although the number of tumors per animal was quite variable, there was a significant reduction in tumor numbers in mice lacking both *Tgif1* and *Tgif2* and an increase in the Vil-TGIF1 mice (Figure 2.7A). The number of tumors larger than 1.5mm in diameter was significantly lower in both the *Tgif1* and *Tgif1;Tgif2* mutants (Figure 2.7A). The increase in larger tumors in the TGIF1 over-expressing mice was highly significant, whereas there were no significant differences in the number of smaller (<1.5mm) tumors. Histological examination of tumors isolated from animals of all four genotypes revealed no clear differences in tumor morphology (Figure 2.7B). All tumors

Figure 2.7 – Tgif expression promotes small intestinal tumorigenesis.

A) The numbers of tumors per animal (at 12 weeks) in the small intestine are shown (median, upper, and lower quartiles, 5^{th} and 95^{th} percentiles) for each genotype. Numbers are shown for all tumors and separately for those <1.5mm, or >1.5mm in diameter. p-values for comparison to the $Apc^{+/r}$ mice are shown. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. B) Representative images of H&E stained tumors from the indicated genotypes are shown. Images captured at 200x magnification.


A
All
< 1.5mm
> 1.5mm
200
150
100
50
0
Tumor number
*
*
*
*
**
***
Apc+/r
Apc+/r;Tgif1r/r
Apc+/r;Tgif1r/r;Tgif2r/r
Apc+/r;Vil-TGIF1+
B
Apc+/r
Apc+/r;Vil-TGIF1+
Apc+/r;Tgif1r/r
Apc+/r;Tgif1r/r;Tgif2r/r

examined were adenomas, and we did not observe invasive carcinomas in these animals. Thus, increasing TGIF1 expression to a level similar to that seen in *Apc* mutant tumors enhances adenoma growth but does not promote transition to invasive adenocarcinoma.

In the middle and distal regions of the SI, we observed increased numbers of larger tumors in Vil-TGIF1 mice and a decrease in total tumor numbers in *Tgif1;Tgif2* mice (Figure 2.8A-B). These differences were primarily driven by changes in the numbers of larger tumors (Figure 2.8A-B). *Apc* mutant mice lacking only *Tgif1* had an intermediate phenotype between that of the *Apc* and *Apc;Tgif1;Tgif2* mice, especially seen in the number of large tumors in the middle and distal portions of the SI (Figure 2.8C). This was particularly evident when analyzing the proportion of tumors in each mouse that were >1.5mm in diameter (Figure 2.8D).

Although the *Vil-Cre;Apc* model primarily generates tumors in the small intestine, there are also colon tumors in these animals. Comparison of tumor number and size in the colon between *Apc* mice and those lacking Tgif1 did not reveal any significant differences (Figure 2.9A-B). However, in the *Apc;Tgif1;Tgif2* mice, average tumor volume was significantly lower, nearly 45% lower in *Apc;Tgif1;Tgif2* mice compared to *Apc* mice, despite the fact that the tumor sizes were quite variable (Figure 2.9B). Thus, it appears that further reducing overall Tgif levels by deleting *Tgif1* and *Tgif2* enhances the relatively mild effect of deletion of *Tgif1* alone, implying redundant function. As with the SI tumors, there were no clear histological differences between the colon tumors from mice of each genotype (Figure 2.9C). Together, these data suggest Tgif1 and Tgif2 contribute to *Apc* mutant intestinal tumorigenesis, and increasing TGIF1 expression drives adenoma growth.

Figure 2.8 – Tgif expression promotes tumorigenesis of large tumors in the small intestine.

A) Tumor numbers per animal in the middle (A) and distal (B) thirds of the small intestine at 12 weeks are shown (median, upper, and lower quartiles, 5^{th} and 95^{th} percentiles) for each of the four genotypes. C) Large tumor numbers (>1.5mm) per animal in proximal, middle, and distal thirds of the small intestine at 12 weeks shown for *Tgif* knockout mice only (median, upper, and lower quartiles, 5^{th} and 95^{th} percentiles). D) Percentage of large tumors compared to all tumors per animal that are over 1.5mm in diameter. p-values for comparison to the $Apc^{+/r}$ mice are shown for each of the four genotypes. p-values for comparison to the $Apc^{+/r}$ mice are shown. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

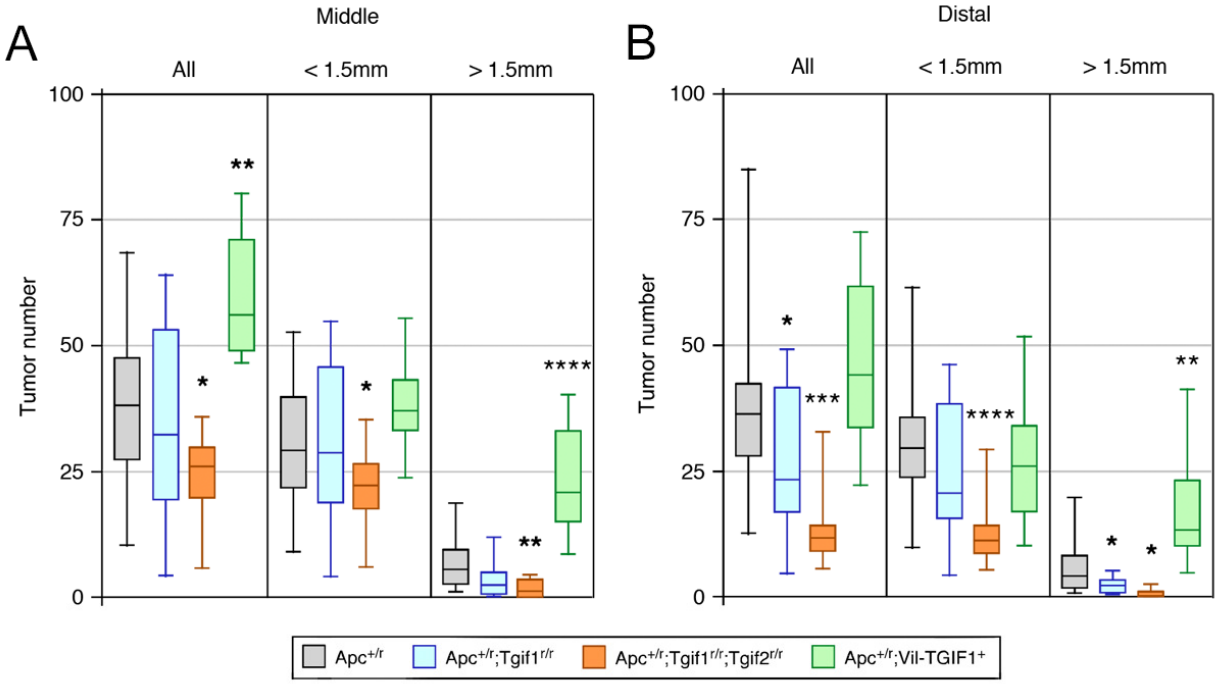
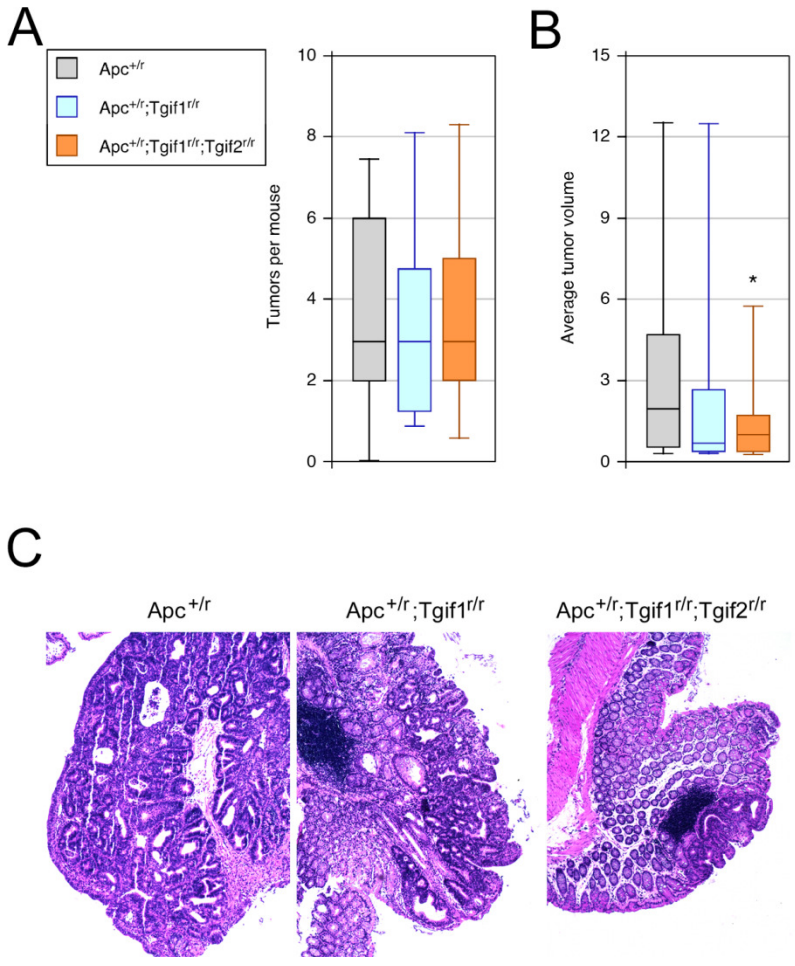

A
Middle
All
< 1.5mm
> 1.5mm
Tumor number
100
75
50
25
0
B
Distal
All
< 1.5mm
> 1.5mm
Tumor number
Apc+/r
Apc+/r;Tgif1r/r
Apc+/r;Tgif1r/r;Tgif2r/r
Apc+/r;Vil-TGIF1+
C
> 1.5mm
Proximal
Middle
Distal
Tumor number
20
10
0
D
Whole SI
Proximal
Middle
Distal
% tumors > 1.5mm

Figure 2.9 – Tgif expression promotes colon tumorigenesis.

A) Average number of colon tumors per mouse (median, upper, and lower quartiles, 5^{th} and 95^{th} percentiles). B) Average tumor volume per mouse (mm^3). p-values for comparison to the $Apc^{+/r}$ mice are shown. * $p < 0.05$. C) Representative images of H&E stained colon tumors from the indicated genotypes are shown. Images captured at 200x magnification.


A
Apc+/r
Apc+/r;Tgif1r/r
Apc+/r;Tgif1r/r;Tgif2r/r
Tumors per mouse
0
2
4
6
8
10
B
Average tumor volume
0
3
6
9
12
15
*
C
Apc+/r
Apc+/r;Tgif1r/r
Apc+/r;Tgif1r/r;Tgif2r/r

2.4 Discussion

Analysis of multiple CRC datasets, including a TCGA dataset, showed elevated levels of *TGIF1* and *TGIF2*. We further show knockout of TGIF1 in HCT116 cells decreases proliferation, and this result was recapitulated by analyzing the proliferative potential of intestinal crypts in *Apc* mice with different levels of Tgifs. Utilizing the *Apc* mouse model of CRC, we provide evidence that expression TGIF transcription factors are upregulated in CRC and impact both intestinal tumor burden and individual tumor size.

TGIF1 is well documented as an inhibitor of TGFβ-activated gene expression. Given the tumor suppressive effects of TGFβ signaling, TGIFs might be expected to be oncogenic by limiting the anti-proliferative effects of TGFβ signaling. Several studies have examined effects of TGIFs on the proliferation of human cancer cell lines or growth in xenograft models, and recent work suggests a pro-tumorigenic role for TGIF1 in colon cancer (129). Knockout of TGIF1 in HCT116 cells reduced proliferation of these cells in culture. In a xenograft model using the human LoVo CRC cell line, reduction of TGIF1 levels resulted in smaller tumors in a xenograft model. Our mouse data support this, in that deleting either *Tgif1* or both *Tgif1* and *Tgif2* together in the background of a heterozygous *Apc* mutation reduced the number of tumors, especially of larger adenomas. Thus, cell culture, xenograft, and mouse genetic models support a role for Tgif1 in intestinal tumor growth.

Deletion of both Tgif1 and Tgif2 had a greater effect on tumor growth than deletion of Tgif1 alone, and this difference was primarily seen in the apparent stepwise decrease in the number of >1.5mm tumors in the middle and distal portions of the small intestine. This difference was also partially seen in the tumor volumes in the colon. Taken

together, these results suggest an overlapping function in the intestine, as in early embryos (106,119,121,123). In contrast to our work, other analyses of Tgif function in cancer have focused on either Tgif1 or Tgif2 alone, without testing potential cooperativity. Our analysis of *Apc* mutant mouse colon and small intestine tumors shows increased expression of both Tgif1 and Tgif2, consistent with increases seen in human gene expression data-sets and for TGIF1 in human CRC samples (129).

Modeling the increase in Tgif1 levels by overexpression of a TGIF1 transgene in intestinal epithelium supported a pro-tumorigenic effect of Tgifs in intestine, with TGIF1 transgenic mice having both more and larger tumors. Additionally, SI crypts in transgenic mice had more proliferative cells, further suggesting the pro-tumorigenic effect of Tgifs. Taken together, this data suggests that increased levels of TGIF1 in the SI crypts may lead to the larger tumors we observed in transgenic mice, perhaps indicating increased TGIF1 levels could prime a tumor to become larger. However, the transgene is poorly expressed in colon, so this analysis is based on tumor numbers in the small intestine. While this is different from the human disease, the difference is dependent on the regulatory elements used to drive the transgene, and analysis of small intestine tumors in mouse models has provided considerable insight into CRC biology. The opposite pattern also held true for the Tgif knockout mice which had both fewer proliferative cells in SI crypts and developed fewer and smaller tumors overall. Overall, our data strongly support a pro-tumorigenic effect of Tgifs in the intestines.

In summary, our data corroborates the increase in Tgif expression seen in human CRC datasets and suggests Tgifs have a pro-tumorigenic role in CRC, potentially through changes in proliferation.

Chapter 3 – Tgifs regulate Acetyl-CoA metabolism[3,4]

3.1 Introduction

Changes in cancer metabolism during tumorigenesis have been observed for nearly a century (131), and tumor cells often are able to obtain nutrients from nutrient-poor environments in order to survive and proliferate. Aerobic glycolysis, one famous metabolic shift observed in cancers better known as the Warburg effect, occurs even in normoxic conditions (130,131). This increase in glycolysis further benefits the tumor by additionally decreasing the generation of reactive oxygen species (ROS) through oxidative phosphorylation (152). The hypoxic tumor environment further drives these metabolic shifts, by reinforcing increased glucose uptake by the tumor cell through the upregulation of *GLUT1*, a glucose transporter (137,153). Hypoxic conditions additionally promote the conversation of pyruvate to lactate, generating NAD^+ as a reducing agent and preventing the accumulation of NADH in the cytosol, further promoting the uptake of glucose (137,148). Thus, both the metabolic shift to aerobic respiration and hypoxia in tumors work together to increase glucose uptake and reduce ROS.

Increased glycolysis and decreased oxidative respiration results in tumor cells using the intermediates of glycolytic pathway as precursors for multiple biosynthetic pathways branching from glycolysis. The pentose phosphate pathway (PPP), an anabolic pathway producing ribose sugars for nucleotide biosynthesis, and one carbon cycle, the

[3] This work is a part of a manuscript currently under revision at *Genes and Development*. Shah, A., Melhuish, T.A., Frierson Jr., H.F., Wotton, D. (2018) TGIF transcription factors repress acetyl-CoA metabolic gene expression and promote intestinal tumor growth.

[4] Tiffany Melhuish performed both the RT-qCPR and ChIP-qPCR in MEFs and small intestinal tissue. Dr. David Wotton created the pathway map (Figure 3.6) and performed RNA-seq mapping to the mouse genome. Additionally, Dr. Wotton assisted me with the subsequent RNA-seq analysis.

metabolism of serine for the biosynthesis of purines, thymidine, and the reducing agent, NADPH, are key examples of biosynthetic pathways which are upregulated in the presence of increased glucose uptake (137,138). The overall upregulation of glycolysis in tumors results in upregulation of downstream pro-proliferative pathways for growth and survival. Taken together, this suggests the Warburg effect is a well-regulated metabolic state required to meet the biosynthetic demands of a rapidly growing tumor.

TGIF1 and TGIF2 (thymine-guanine interacting factors) are homeodomain transcriptional corepressors that are members of the TALE (three amino acid loop extension) superfamily, which interact with the corepressors mSin3 and histone deacetylases (81,108). Additionally, TGIF1 can interact with CtBP1/2 corepressors via a conserved interaction motif (107). Tgifs limit the response to TGFβ signaling by recruiting co-repressors to the SMAD transcription factors (81,108). In addition to SMAD-interaction, other mechanisms for TGFβ pathway inhibition have been suggested, including promoting SMAD2 ubiquitylation and degradation or preventing SMAD2 phosphorylation in response to TGFβ signaling (204,205). Loss of function mutations in *TGIF1* are associated with holoprosencephaly (HPE), a severe developmental disorder adversely affecting forebrain development (100). Mouse models of Tgif1 and Tgif2 loss of function suggest Tgif1 and Tgif2 have a redundant, but essential roles in early embryogenesis (121). Conditional mutants survive to mid-gestation with multiple developmental abnormalities, including HPE (106,123).

Although developmental defects in embryos lacking Tgif1 and Tgif2 can be partly rescued by reducing TGFβ family signaling through mutation of *Nodal* (106,121,123), transcriptome profiling of early embryos or primary mouse embryo fibroblasts (MEFs)

lacking Tgifs suggests that the majority of gene expression changes are unlikely to be due to altered TGFβ family signaling (110,111). TGIF1 was first identified by its ability to bind a retinoid response element of the *Rbp2* gene and reduce activation by RXR nuclear receptors (97). TGIFs can bind directly to DNA and repress transcription via a well-defined consensus site, cTGTCAa, where the central five bases are most important (82,97). Direct repression via this consensus site has been shown for a small number of Tgif target genes (111,123). Recent genome-wide analysis identified a large number of potential Tgif1 binding sites, with enrichment for the known TGIF consensus element (109).

Increased Tgif levels have been implicated in ovarian, esophageal, and lung cancer among others (124–126). Tgif1 promoted breast cancer progression in a mouse model, independent of effects on TGFβ signaling (128). The *TGIF1* gene was shown to be a direct β-catenin/TCF transcriptional target that is activated by Wnt/β-catenin signaling (128), and the possibility that TGIF1 sequesters Axins to activate Wnt/βcatenin signaling was also suggested as a mechanism to explain the its pro-tumorigenic function (128). Recent work with human CRC cell lines suggests a role for TGIF1 in CRC progression and also implicated TGIF1 in controlling the output of the Wnt/β-catenin pathway, although this appeared to be independent of effects on Axins (129). Thus, Tgifs can promote tumorigenesis, but questions regarding mechanisms and overlapping roles of Tgif1 and Tgif2 remain.

We used genetically engineered mouse models to address the function of Tgifs in intestinal cancer and to identify downstream Tgif target genes. Over-expression of TGIF1 in intestinal epithelial cells increased the size and number of adenomas in the small

intestine (SI), and deletion of *Tgif1* and *Tgif2* reduced tumor size in both the SI and colon (from previous chapter). Transcriptional profiling of colon tumors from these mice revealed little effect of Tgifs on either Wnt/β-catenin or TGFβ signaling. Instead, we found that deleting Tgifs from colon tumors caused changes in expression of genes affecting multiple metabolic pathways. Integrating this data with additional gene expression profiling suggests that Tgifs play a fundamental role in regulating energy metabolism, and they may contribute to the reprogramming of metabolic gene expression that occurs in CRC.

3.2 Materials and Methods

3.3.1 Mice

All animal procedures were approved by the Animal Care and Use Committee of the University of Virginia, which is fully accredited by the AAALAC. Conditional alleles with loxP flanked exons are referred to here as 'f' for loxP flanked, or 'r' for recombined (null). Mice were maintained on a predomintly C57BL/6J background. Conditional *Apc* mice were from the NCI, and the *Villin*-Cre line was from Jax (B6.Cg-Tg(Vil1-cre)1000Gum/J; #021504 (14)). Conditional Tgif2 mice were generated from targeted ES cells obtained from EUCOMM ($Tgif2^{tm1a(EUCOMM)Wtsi}$; IKMC project 24492) and crossed to conditional a Tgif1 line (121). *Villin*-TGIF1 transgenic mice were generated at the UVA GEMM Core. The human TGIF1 cDNA with an amino-terminal T7 epitope tag was inserted into the *Villin* promoter plasmid (12.4kb*Villin*-ΔATG), which was a gift from Deborah Gumucio (Addgene plasmid # 19358 (14)). Germ line transmission was verified by PCR and expression by western blot.

3.3.2 Tumor analysis, IF and histology

Tissues were fixed in zinc-formalin, paraffin-embedded, sectioned at 5 microns and prepared for immunostaining as described in (201). Images were captured with 10, 20, or 40x objectives, using a Nikon Eclipse NI-U with a DS-QI1 or DS-Ri1 camera and NIS Elements software, and adjusted in Adobe Photoshop. For IF, antibodies were as follows: Rabbit anti-Acss2 (Abcam 66038), rabbit-anti Slc2a1 (Millipore 07-1401), mouse anti-β-catenin (BD Transduction Labs 610153).

3.3.3 Cell culture

HCT116 and primary MEF cell lines were from the Wotton Lab at the University of Virginia. HCT116 cells were cultured in RPMI-1640 (Gibco 11875-093) supplemented with 10% fetal bovine serum (FBS) (HyClone SH30396.03), 1% Anti-Anti (Gibco15240-062), and 100µg/mLNormocin (InvivoGen ant-nr-1). Primary MEFs were grown in the same conditions as HCT116 cells except with DMEM. Cells were grown in a humidified 37°C incubator supplemented with 5% CO_2. Cell line identity was verified by STR profiling. For siRNA-mediated knockdown, HCT116 cells were seeded at 200,000 cells per well in a six well plate. The next day, cells were transfected with either control or Tgif1 and Tgif2 siRNAs using Turbofect (ThermoFisher R0532) as per the manufacturer's protocol. Knockdown was confirmed by RT-qPCR and western blot.

3.3.4 RNA isolation and qRT-PCR

RNA from snap-frozen tissue and cells was isolated and purified using Absolutely RNA kit (Agilent) and quality checked by Bioanalyzer. cDNA was generated using Superscript III (Invitrogen) and analyzed by real time PCR using a BioRad MyIQ cycler and Sensimix Plus SYBRgreen plus FITC mix (Bioline), with intron-spanning primer pairs selected using Primer3 (http://frodo.wi.mit.edu/). Expression was normalized to Rpl4 and Cyclophilin using the delta Ct method.

3.3.5 RNA-sequencing and analysis

Poly-A RNA-seq libraries generated with Illumina barcodes were sequenced (NextSeq 500 at the UVA GATC) to at least 25M single end 75bp reads per sample. Data was analyzed using the Galaxy server (https://usegalaxy.org/). Transcript quantification was

performed using Salmon (206) to map to the mm10 mouse genome build, and DESeq2 (207) within the Galaxy site was used for normalizing count data, estimating dispersion, fitting a negative binomial model for each gene and comparing expression between groups. A cut-off of +/- 0.5 log2 and an adjusted p-value of <0.01 was considered significant. Enrichment was analyzed with ENRICHR (http://amp.pharm.mssm.edu/Enrichr/) (208,209) and heat maps generated with Heatmapper (http://www2.heatmapper.ca/expression/) (210). Gene set enrichment was by GSEA software from the Broad Institute (211,212). RNA-seq data is deposited at GEO (GSE116578).

3.3.6 Western blot

Tissues were ground in PBS, followed by addition of NP-40 to 1%, then lysates were separated by SDS-PAGE, transferred to Immobilon-P (Millipore) and proteins visualized using ECL (Pierce). Primary antibodies were against Acss2 (Abcam 66038), γ-tubulin (Sigma T6557) and HSP90 (Cell Signaling #4874). Pcx was detected using Neutravidin conjugated HRP (ThermoFisher).

3.3.7 Chromatin immunoprecipitation (ChIP)

Chromatin was cross-linked for 20 minutes in 1% formaldehyde and sonicated to 200-1000bp using a Branson digital sonifier, with microtip as described in (213). Immunoprecipitation was carried out using 10μL of polyclonal TGIF1 antiserum (81), or pre-immune serum. Bound and input fractions were analyzed by qPCR on a BioRad MyIQ cycler using Sensimix Plus SYBRgreen plus FITC mix (Bioline).

3.3.8 Statistical methods

Experiments in cell lines were at least two independent times with n=3 technical replicates, and data is shown as mean +/- sd of one experiment, unless otherwise specified. T-tests were used to compare groups for RT-qPCR and ChIP-qPCR experiments, and p-values are denoted in figure legends. Data was analyzed using Microsoft Excel and RStudio.

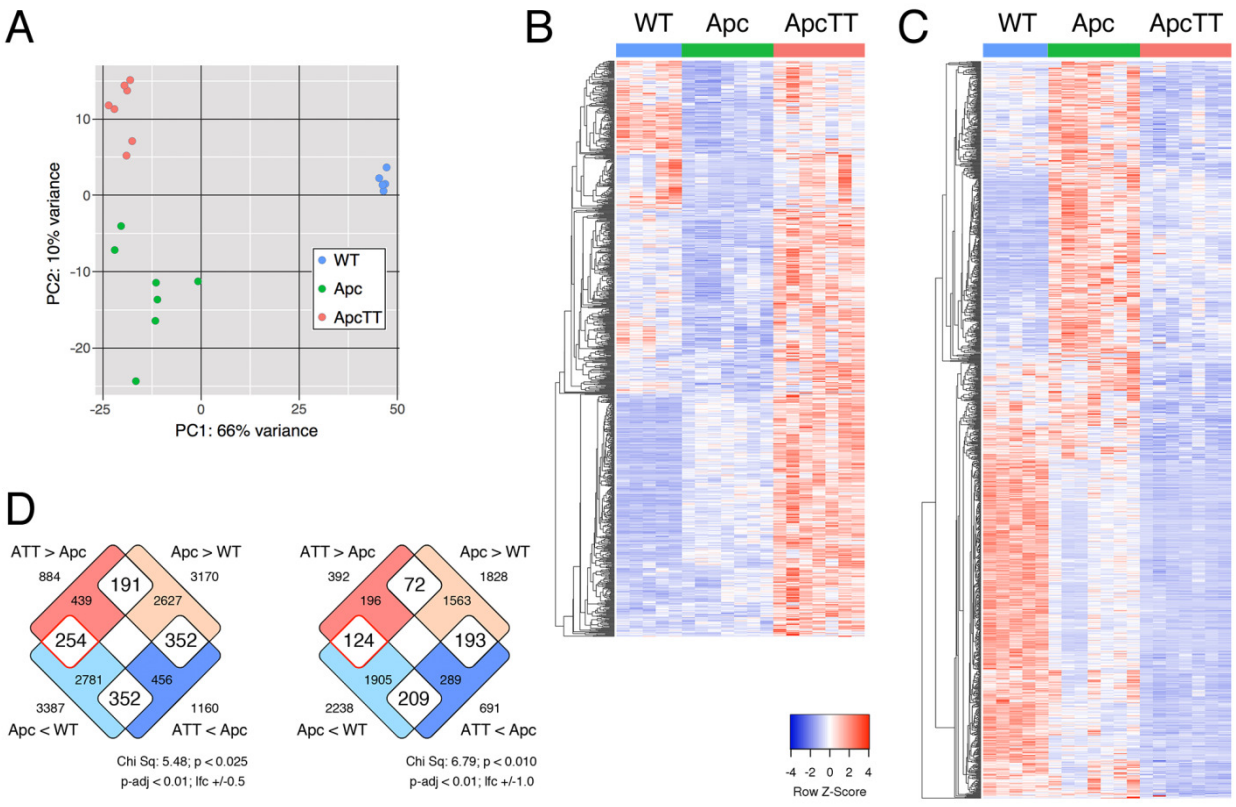
3.3 Results

3.3.1 Transcriptional changes in Tgif mutant tumors

To address how increased Tgif levels contribute to intestinal tumor growth, we performed transcriptome profiling, comparing normal wild type colon to colon tumors from *Apc* and *Apc;Tgif1;Tgif2* mice. RNA was isolated from five normal colon samples and seven tumors from mice of each of the two genotypes from both males and females. The samples from each of the three genotypes clustered separately, although there was considerable spread among the tumors, and the two tumor genotypes clustered closer to each other than to the wild types (Figure 3.1A). Some of this spread in the tumor samples may have been due to other cell types present in the tumor, such as immune cells and stromal cells, but IF analysis has shown the majority of cells present in these colon tumors were epithelial cells. To identify genes that were differently expressed, we performed pairwise comparisons using a 0.5 log2-fold change and an adjusted p-value cut-off of <0.01. This identified close to 2000 genes that were differentially expressed between the two tumor genotypes, with 884 being higher in the *Apc;Tgif1;Tgif2* than in the *Apc* tumors and 1160 with lower expression. Hierarchical clustering of each of these two gene lists suggested that, among the genes with increased expression in the *Apc;Tgif1;Tgif2* compared to the *Apc* tumors, a small fraction was also more highly expressed in wild type colon (Figure 3.1B). This is consistent with these genes being Tgif targets that are repressed in *Apc* tumors by increased Tgif expression. Among the genes with lower expression in *Apc;Tgif1;Tgif2* tumors, many are increased in the *Apc* tumors compared to wild type, consistent with loss of Tgifs reversing at least part of the *Apc* mutant gene expression program (Figure 3.1C). Despite the presence of some genes that

Figure 3.1 – Gene expression changes in Tgif mutant colon tumors.

A) Principle component analysis of RNA-seq data from normal wild type (WT) mouse colon or from colon tumors isolated from *Apc* heterozygous mice (Apc) or *Apc* heterozygous mice with homozygous deletion of both *Tgif1* and *Tgif2* (ApcTT). Heat maps are shown for all genes with significantly (log2-fold change > 0.5, p-adjusted < 0.01) higher (B) or lower (C) expression in ApcTT than in Apc. D) Venn diagrams indicating the overlap between genes that are significantly differently expressed between Apc versus WT and Apc versus ApcTT.


A
PC2: 10% variance
PC1: 66% variance
WT
Apc
ApcTT
B
WT
Apc
ApcTT
C
WT
Apc
ApcTT
Row Z-Score
D
ATT > Apc
Apc > WT
Apc < WT
ATT < Apc
884
191
3170
439
2627
254
352
2781
456
3387
352
1160
Chi Sq: 5.48; p < 0.025
p-adj < 0.01; lfc +/-0.5
392
72
1828
196
1563
124
193
1905
289
2238
209
691
Chi Sq: 6.79; p < 0.010
p-adj < 0.01; lfc +/-1.0

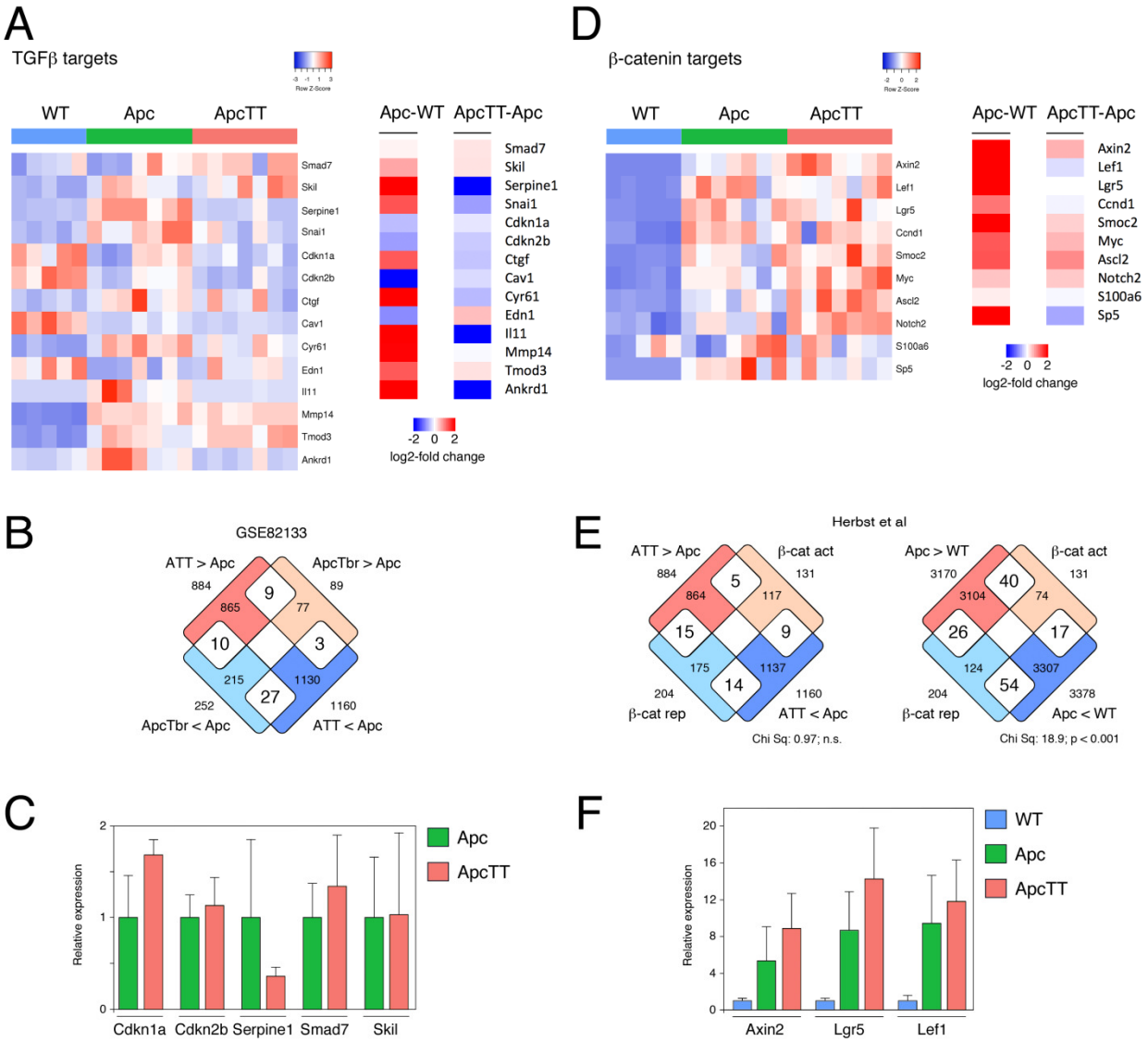
decrease in the *Apc* tumor compared to wild type and increase in *Apc;Tgif1;Tgif2* tumors, there was minimal enrichment for this class (Figure 3.1D) among genes that are significantly differently expressed in both the *Apc* to wild type (left Venn diagram) and *Apc;Tgif1;Tgif2* to *Apc* comparisons (right Venn diagram). However, more genes with higher expression in *Apc;Tgif1;Tgif2* tumors had reduced expression in *Apc* tumors compared to wild type (124/392; 31.6%) than had higher expression (72/392; 18.4%). Thus, it appears that there is a subset of differentially expressed genes that fit with being Tgif targets. However, there also appears to be significant tumor to tumor variability and a larger number of genes that do not fit a simple direct Tgif target model.

Tgifs are well characterized as repressors of TGFβ responsive transcription (81,100) and have been suggested to promote Wnt responsive gene expression (128,129). We, therefore, examined expression of genes that are known targets of these pathways. For a panel of well characterized TGFβ targets (*Smad7, Skil, Serpine1, Cdkn1a,* and *Cdkn2b*), and some additional genes that respond to TGFβ in LS1034 CRC cells (214), there was no consistent pattern in expression differences between normal colon and *Apc* tumors, and deletion of Tgifs had minimal effect (Figure 3.2A). Overlapping gene expression changes in *Apc;Tgif1;Tgif2* compared to *Apc* tumors with expression array data from mouse *Apc* colon tumors or *Apc* tumors lacking the TGFβ type 2 receptor (GSE82133; (215)) revealed minimal overlap (Figure 3.2B). qRT-PCR analysis of canonical TGFβ target genes in a set of *Apc;Tgif1;Tgif2* and *Apc* colon tumors did not show significant increases in expression in the *Tgif* mutants (Figure 3.2C).

To examine Wnt signaling we looked at expression of canonical targets of the pathway. These genes were clearly activated in *Apc* tumors compared to wild type colon

Figure 3.2 – Wnt and TGFβ target gene activity in colon tumors.

A) A heat map (z-score per gene) is shown for RNA-seq data for a panel of well characterized TGFβ target genes in addition to those shown to be regulated by TGFβ in LS1034 CRC cells. The right hand panel shows the log-2 fold change for each gene, comparing Apc tumors to wild type, and Apc;Tgif1;Tgif2 (ApcTT) tumors to Apc only. B) A Venn diagram indicating the overlap between genes with differential expression in Apc versus ApcTT tumors and those with significantly different (log2 fold change > +/- 0.5, $p < 0.05$) expression comparing Apc mouse colon tumors to those with deletion of the TGFβ type 2 receptor (Tbr in figure) as well as Apc. Data is from GSE82133. C) qRT-PCR analysis of a panel of known TGFβ target genes comparing ApcTT tumors to Apc only (n=4 per genotype). D) Heat maps are shown for expression of known Wnt/β-catenin target genes in RNA-seq data. Data is shown as in panel A. E) Overlap of genes differentially expressed in Apc versus Apc;Tgif1;Tgif2 tumors (left) or Apc versus WT tissue (right) with validated β-catenin target genes that are either activated (act) or repressed (rep) by Wnt/β-catenin signaling. Note the enrichment for genes that are β-catenin activated and increased in the Apc compared to wild type, and for β-catenin repressed and decreased in the Apc compared to wild type. In contrast, loss of Tgifs has little effect on this gene set. F) qRT-PCR analysis of a panel of known Wnt/β-catenin target genes comparing Apc;Tgif1;Tgif2 and Apc tumors to wild type (n=4 per genotype).


A
TGFβ targets
WT
Apc
ApcTT
Smad7
Skil
Serpine1
Snai1
Cdkn1a
Cdkn2b
Ctgf
Cav1
Cyr61
Edn1
Il11
Mmp14
Tmod3
Ankrd1
Apc-WT
ApcTT-Apc
-2 0 2
log2-fold change
D
β-catenin targets
Axin2
Lef1
Lgr5
Ccnd1
Smoc2
Myc
Ascl2
Notch2
S100a6
Sp5
B
GSE82133
ATT > Apc
ApcTbr > Apc
884
865
9
89
77
10
3
215
1130
27
252
1160
ApcTbr < Apc
ATT < Apc
E
Herbst et al
β-cat act
β-cat rep
884
5
131
864
117
15
9
175
1137
14
204
1160
Chi Sq: 0.97; n.s.
Apc > WT
Apc < WT
3170
40
3104
74
26
17
124
3307
54
3378
Chi Sq: 18.9; p < 0.001
C
Relative expression
Apc
ApcTT
Cdkn1a
Cdkn2b
Serpine1
Smad7
Skil
F
WT
Apc
ApcTT
Axin2
Lgr5
Lef1

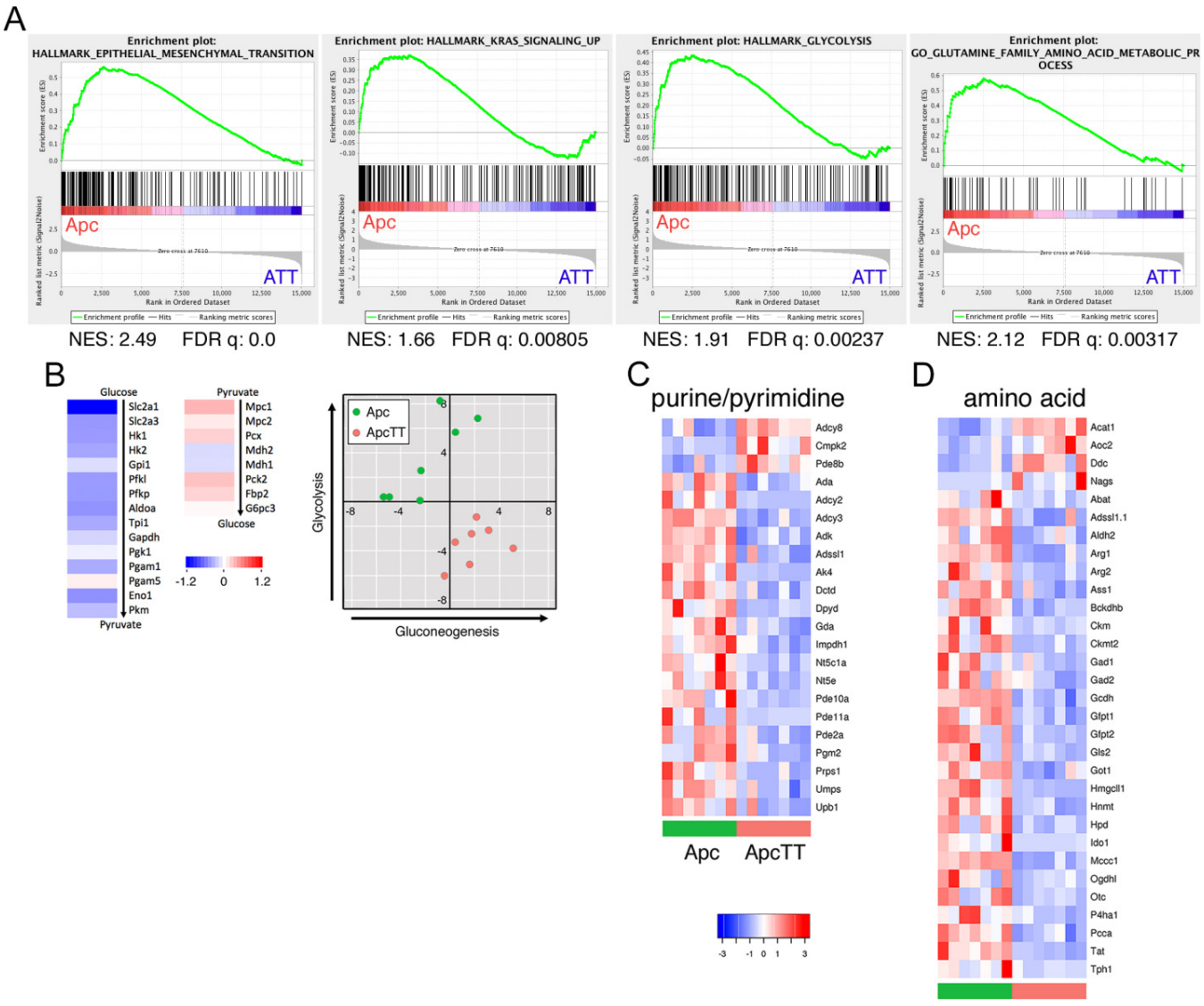
but were not further activated by deletion of *Tgifs* (Figure 3.2D). Comparing a set of β-catenin activated or repressed target genes (216) with our data showed limited overlap with expression differences between *Apc;Tgif1;Tgif2* and *Apc* tumors, whereas there was clear enrichment for these target genes in the comparison between *Apc* and WT tissue (Figure 3.2E). This was supported by qRT-PCR analysis showing increased expression of *Axin2*, *Lgr5*, and *Lef1* in *Apc* mutant tumors but no decrease in *Apc;Tgif1;Tgif2* tumors, as would be expected if Tgif1 promotes β-catenin activated gene expression (Figure 3.2F). Thus, it appears, in the context of colon tumors in mice, Tgifs do not play a major role in regulating either TGFβ or Wnt/β-catenin signaling.

3.3.2 Altered metabolic gene expression in tumors lacking Tgifs

To identify functional groups among the gene expression changes, we performed GSEA. Gene sets indicative of epithelial to mesenchymal transition (EMT) and KRAS signaling were among the most significantly enriched in the *Apc* compared to *Apc;Tgif1;Tgif2* tumors (Figure 3.3A). Surprisingly, glycolysis was also one of the most significantly enriched gene sets in the *Apc* tumors, and other metabolic signatures were enriched in *Apc* compared to *Apc;Tgif1;Tgif2* tumors (Figure 3.3A). Comparing *Apc;Tgif1;Tgif2* to *Apc* tumors, one of the most down-regulated glycolytic genes was *Slc2a1*, encoding Glut1, the major glucose transporter in the intestine. For most glycolytic enzymes, there was a more modest reduction in expression in *Apc;Tgif1;Tgif2* tumors (Figure 3.3B). Examining expression of genes encoding proteins that function to generate glucose from pyruvate revealed that these genes were generally slightly more highly expressed in the *Apc;Tgif1;Tgif2* tumors. Summing the relative expression for each tumor for a panel of glycolysis or gluconeogenesis-specific genes revealed a clear

Figure 3.3 – Altered metabolic gene expression in Tgif mutant colon tumors.

A) GSEA analysis indicates enrichment of EMT, KRAS signaling, glycolysis and glutamine metabolism in Apc tumors compared to ApcTT. The nominal enrichment score (NES) and FDR q-value are shown. B) Heat maps are shown indicating fold-change (comparing ApcTT to Apc tumors) for the glycolytic pathway and for genes involved specifically in the conversion of pyruvate to glucose. The plot to the right shows summed z-scores for a panel of genes involved only in glycolysis or in gluconeogenesis, plotted as gluconeogenesis versus glycolysis for each tumor. Heat maps are shown for all genes in the purine and pyrimidine metabolic pathways (C) or amino acid metabolic pathways (D) that are significantly differently expressed comparing Apc to ApcTT tumors.

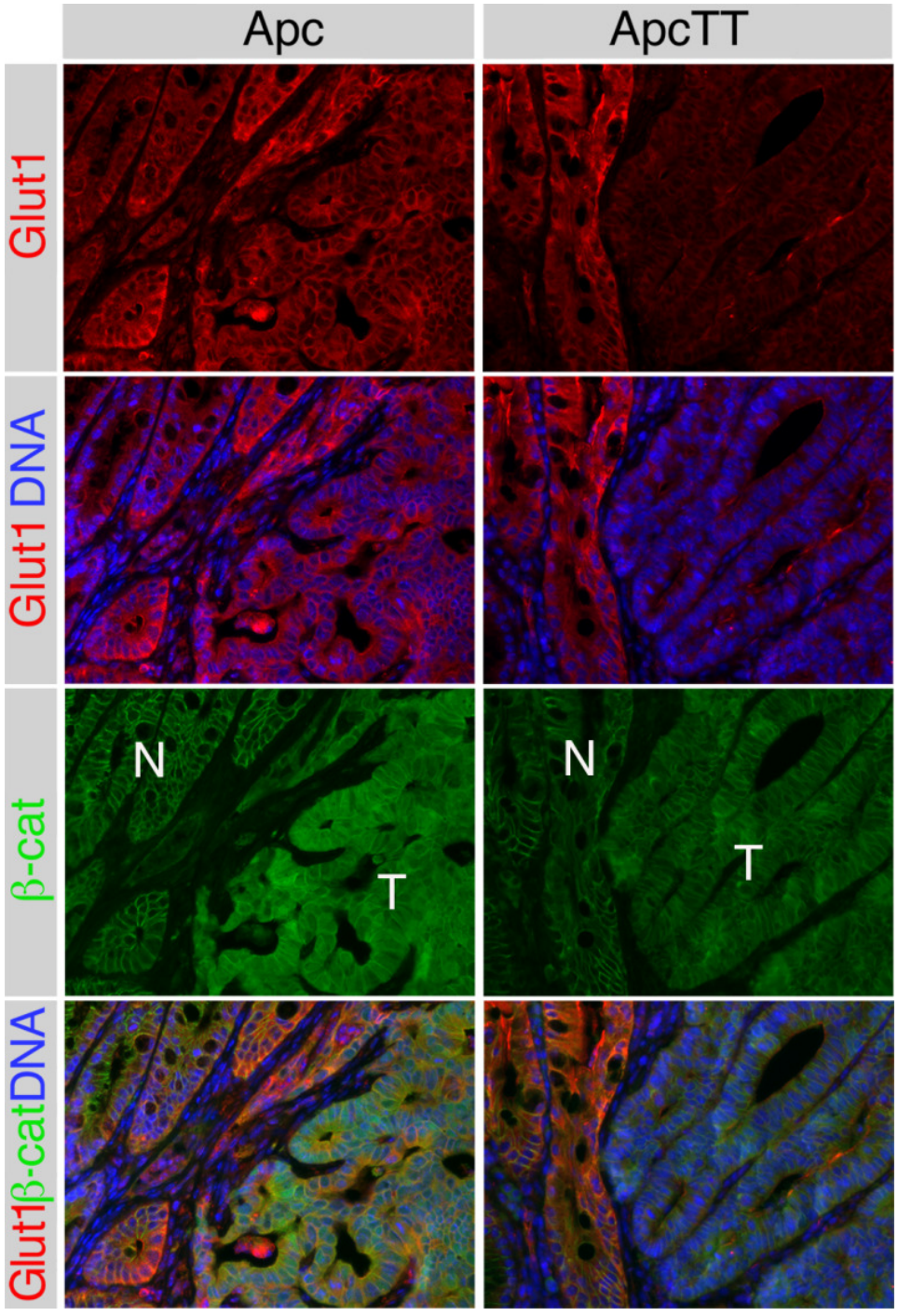

A
Enrichment plot: HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION
Enrichment plot: HALLMARK_KRAS_SIGNALING_UP
Enrichment plot: HALLMARK_GLYCOLYSIS
Enrichment plot: GO_GLUTAMINE_FAMILY_AMINO_ACID_METABOLIC_PROCESS
Apc
ATT
Rank in Ordered Dataset
Enrichment profile
Hits
Ranking metric scores
NES: 2.49 FDR q: 0.0
NES: 1.66 FDR q: 0.00805
NES: 1.91 FDR q: 0.00237
NES: 2.12 FDR q: 0.00317
B
Glucose
Slc2a1
Slc2a3
Hk1
Hk2
Gpi1
Pfkl
Pfkp
Aldoa
Tpi1
Gapdh
Pgk1
Pgam1
Pgam5
Eno1
Pkm
Pyruvate
Pyruvate
Mpc1
Mpc2
Pcx
Mdh2
Mdh1
Pck2
Fbp2
G6pc3
Glucose
-1.2
0
1.2
Apc
ApcTT
Glycolysis
Gluconeogenesis
C
purine/pyrimidine
Adcy8
Cmpk2
Pde8b
Ada
Adcy2
Adcy3
Adk
Adssl1
Ak4
Dctd
Dpyd
Gda
Impdh1
Nt5c1a
Nt5e
Pde10a
Pde11a
Pde2a
Pgm2
Prps1
Umps
Upb1
Apc
ApcTT
-3
-1
0
1
3
D
amino acid
Acat1
Aoc2
Ddc
Nags
Abat
Adssl1.1
Aldh2
Arg1
Arg2
Ass1
Bckdhb
Ckm
Ckmt2
Gad1
Gad2
Gcdh
Gfpt1
Gfpt2
Gls2
Got1
Hmgcll1
Hnmt
Hpd
Ido1
Mccc1
Ogdhl
Otc
P4ha1
Pcca
Tat
Tph1

separation of *Apc* and *Apc;Tgif1;Tgif2* tumors, consistent with the GSEA result, despite the expression of most components of these pathways (Figure 3.3B). We also examined the RNA-seq data for changes in other metabolic pathways by comparing all genes that were significantly differently expressed between *Apc* and *Apc;Tgif1;Tgif2* tumors to metabolic gene lists from KEGG. This analysis revealed reduced expression of multiple genes with links to purine and pyrimidine synthesis and amino acid metabolic pathways (Figure 3.3C-D).

Since *Slc2a1* was the most down-regulated glycolytic gene in *Apc;Tgif1;Tgif2* tumors, we examined expression of the Glut1 protein in colon tumors of each genotype by IF. Glut1 was expressed throughout normal colon and *Apc* mutant tumor tissue, with relatively little difference in expression between the two (Figure 3.4). In contrast, there was clearly lower expression of Glut1 in the *Apc;Tgif1;Tgif2* tumor tissue compared to adjacent normal tissue and to *Apc* mutant tumors (Figure 3.4). Together, these analyses suggest that loss of Tgifs from *Apc* tumors results in widespread changes in metabolic gene expression.

Figure 3.4 – Glut1 expression in normal and tumor tissue.

IF analysis shown for Glut1 and β-catenin in colon tumors [T] with adjacent normal tissue [N].


Apc
ApcTT
Glut1
Glut1 DNA
β-cat
Glut1β-catDNA
N
T
N
T

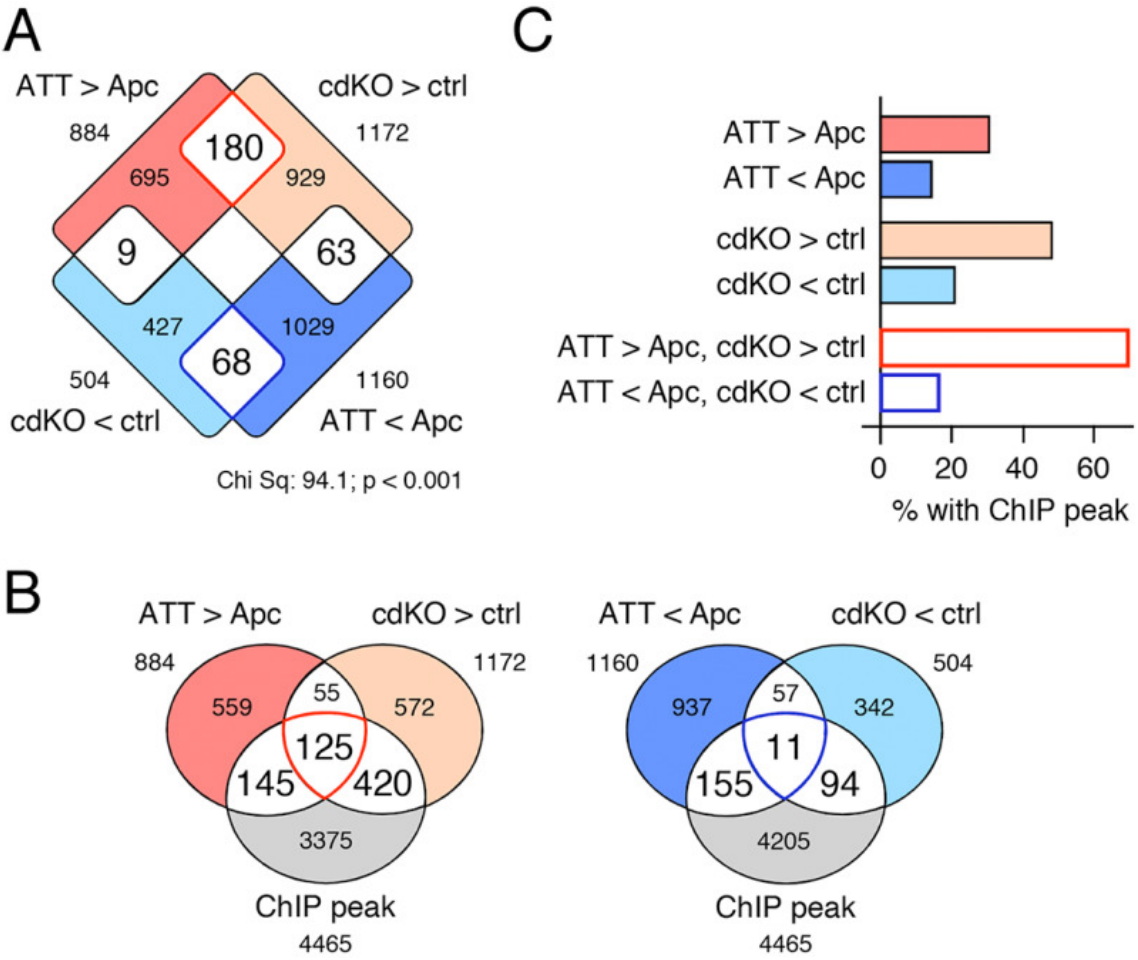
3.3.3 Tgifs repress expression of genes involved in Acetyl-CoA metabolism

The majority of metabolic gene expression changes examined so far are decreases in expression in the absence of Tgifs, suggesting these changes are unlikely to be direct Tgif targets. To identify Tgif target genes we overlapped gene expression changes found here with transcriptome profiling from wild type and conditional *Tgif1;Tgif2* null mouse embryos (111). There was relatively little overlap between these two data sets, but, among the genes that changed in both, there was a significant enrichment for genes that increased with deletion of Tgifs from embryos and tumors (Figure 3.5A). ChIP-seq analysis from mouse ES cells identified more than 16,000 potential Tgif1-bound regions across the genome (109). To enrich for higher confidence targets, we considered only the top 40% of putative Tgif1-bound regions from this analysis and overlapped this list with genes that were differently expressed in *Tgif1;Tgif2* null embryos and tumors lacking Tgifs (Figure 3.5B). This revealed a greater overlap with genes that were activated by loss of Tgifs than with genes that had lower expression in the mutants (Figure 3.5B-C). Among the genes with increased expression in both *Tgif1;Tgif2* null embryos and tumors almost 70% had high confidence ChIP peaks (Figure 5C).

Analysis of the 125 genes with ChIP-seq peaks and higher expression in both RNA-seq datasets revealed a significant enrichment for a MEIS1 consensus site (which is identical to a TGIF site) associated with these genes, consistent with the idea that they are direct Tgif targets (Figure 3.5D). Propanoate metabolism and acetyl-CoA biosynthetic process were the most significantly enriched pathways, and, among the 125 gene list,

Figure 3.5 – Identification of putative Tgif target genes.

A) Differentially expressed genes from RNA-seq data from control or *Tgif1;Tgif2* null (cdKO) day 9 mouse embryos (GSE78728) overlapped with genes that are significantly differently expressed in ApcTT versus Apc tumors. B) Genes with significantly higher (left) or lower (right) expression in either data-set were overlapped with Tgif1 ChIP-seq data from mouse ES cells (GSE55404). C) The percentage of genes from each of the indicated overlaps between expression data from embryos and tumors with ChIP-seq peaks is shown. D) EnrichR analysis of the 125 genes with increased expression in embryos and tumors that also have ChIP-seq peaks is shown.

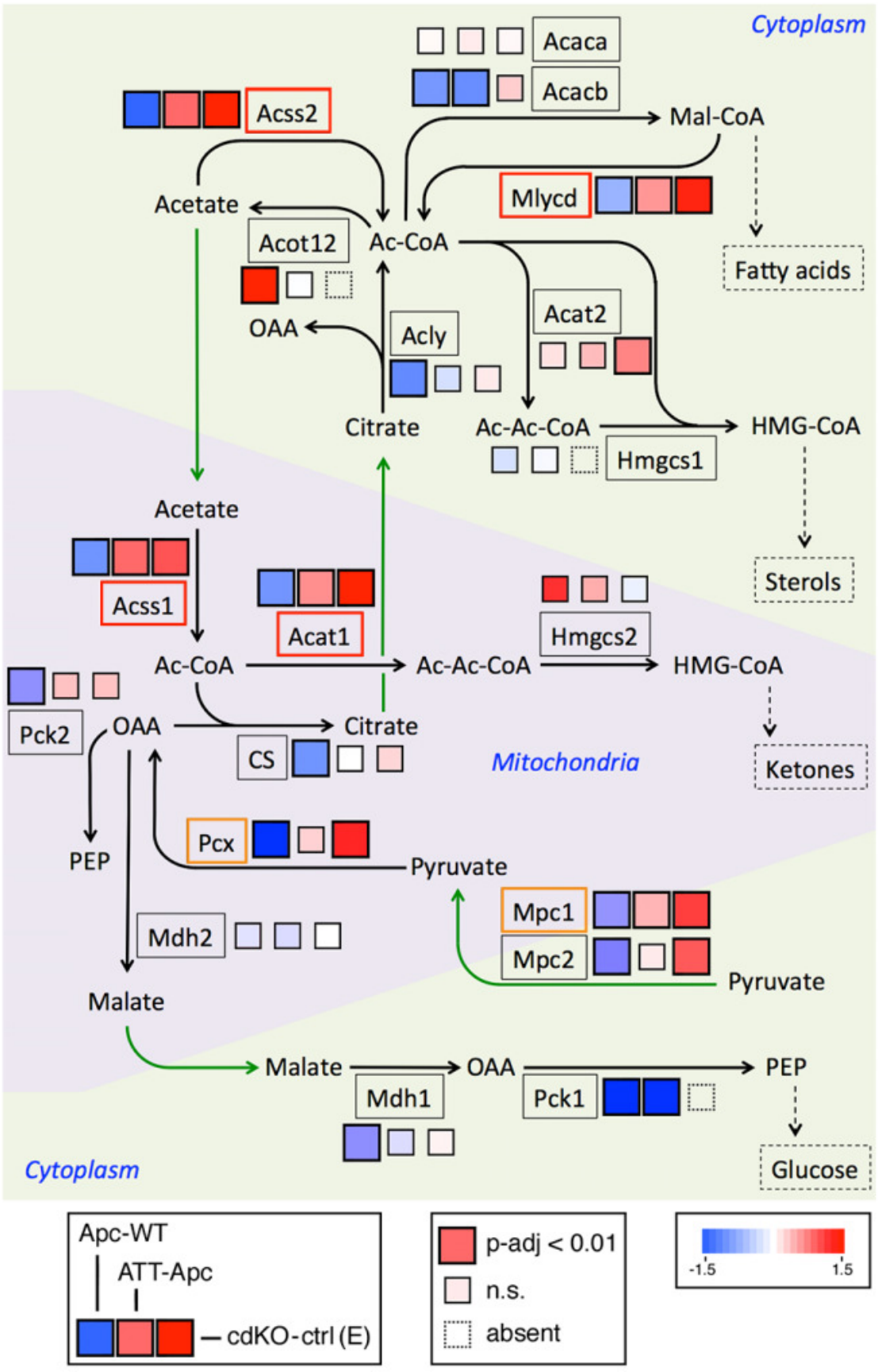

A
ATT > Apc
cdKO > ctrl
884
180
1172
695
929
9
63
427
1029
68
504
1160
cdKO < ctrl
ATT < Apc
Chi Sq: 94.1; p < 0.001
C
ATT > Apc
ATT < Apc
cdKO > ctrl
cdKO < ctrl
ATT > Apc, cdKO > ctrl
ATT < Apc, cdKO < ctrl
0
20
40
60
% with ChIP peak
B
ATT > Apc
cdKO > ctrl
884
1172
559
55
572
125
145
420
3375
ChIP peak
4465
ATT < Apc
cdKO < ctrl
1160
504
937
57
342
11
155
94
4205
ChIP peak
4465
D
Genome Browser PWMs
TGACAGNY_V$MEIS1_01
p-adj: 0.0096
V$STAT1_01
V$TGIF_01
KEGG 2016
Propanoate metabolism_Homo sapiens_hsa00640
p-adj: 0.0052
Lysosome_Homo sapiens_hsa04142
Pyruvate metabolism_Homo sapiens_hsa00620
GO Biological Process
acetyl-CoA biosynthetic process (GO:0006085)
p-adj: 0.0094
acetyl-CoA biosynthetic process from pantothenate (GO:1990181)
acetyl-CoA biosynthetic process from pyruvate (GO:0006086)

there were three genes encoding enzymes that synthesize acetyl-CoA: Acss1, Acss2, and Mlycd. To place these changes in context, we visualized expression changes for genes encoding a number of enzymes involved in acetyl-CoA metabolism as part of a metabolic pathway map. *Acss2* was significantly increased in both cdKO embryos and in tumors lacking Tgifs and decreased in *Apc* tumors compared to wild type colon (Figure 3.6).

Similarly, the mitochondrial *Acss1* was increased in Tgif mutant embryos and tumors and decreased in the *Apc* tumors. Other genes that showed this pattern included *Mlycd*, which encodes a cytosolic enzyme that converts malonyl-CoA to acetyl-CoA, and *Acat1* which generates acetoacetyl-CoA from acetyl-CoA in the mitochondria as the first step of ketone synthesis (Figure 3.6). Since there was some increase in expression of genes associated with the early stages of pyruvate metabolism (Figure 3.3B) and *Mpc1* and *Pcx* expression was increased in *Tgif1;Tgif2* null embryos, we also examined some changes in this pathway. *Mpc1* expression showed a similar pattern to the acetyl-CoA synthetic genes, as did Pcx, although the increase in Pcx expression in *Apc;Tgif1;Tgif2* tumors was not statistically significant (Figure 3.6). This analysis is consistent with the idea that Tgifs directly repress multiple genes involved in acetyl-CoA metabolism and suggests they may also play a similar function for pyruvate metabolic genes.

Figure 3.6 – Acetyl-CoA and pyruvate metabolism pathway map.

A pathway map for selected genes involved in acetyl-CoA and pyruvate metabolism is shown, with mitochondrial and cytoplasmic compartments shown separately. Black arrows indicate metabolic reactions, green arrows translocations, and the dashed arrows links to additional metabolic pathways. For each gene shown (boxed), the three colored squares represent fold changes in Apc to WT comparison (left), ApcTT to Apc tumor comparison (center) and cdKO to control embryo comparison (right). Larger boxes indicate significant change (p-adjusted < 0.01). Smaller boxes are not significant at this cut-off.


Cytoplasm
Acaca
Acacb
Mal-CoA
Acss2
Acetate
Mlycd
Acot12
Ac-CoA
Fatty acids
OAA
Acly
Acat2
Citrate
Ac-Ac-CoA
HMG-CoA
Hmgcs1
Acetate
Sterols
Acss1
Acat1
Hmgcs2
Ac-CoA
Ac-Ac-CoA
HMG-CoA
Pck2
OAA
Citrate
CS
Mitochondria
Ketones
Pcx
PEP
Pyruvate
Mpc1
Mpc2
Mdh2
Pyruvate
Malate
Malate
OAA
PEP
Mdh1
Pck1
Cytoplasm
Glucose
Apc-WT
ATT-Apc
cdKO-ctrl (E)
p-adj < 0.01
n.s.
absent
-1.5
1.5

IF analysis of colon tissue from *Apc* and *Apc;Tgif1;Tgif2* mice indicated that Acss2 expression was reduced in *Apc* mutant tumors compared to adjacent normal colon, and expression was higher in both normal and tumor tissue in the *Apc;Tgif1;Tgif2* mice (Figure 3.7A). In both small intestine and colon, we observed higher Acss2 expression, with more evident nuclear localization in the *Tgif1;Tgif2* mice compared to wild type (Figure 3.7C-D). In support of this, western blot of colon tumors indicated higher Acss2 expression in *Apc;Tgif1;Tgif2* than in *Apc* tumors, and Pcx expression was also higher in the *Tgif1;Tgif2* mutants (Figure 3.7B). To address the possibility that genes involved in acetyl-CoA and pyruvate metabolism are direct Tgif targets in multiple cell types, we tested expression of a panel of these genes by qRT-PCR in both normal small intestine and primary MEFs. All three acetyl-CoA synthetic genes and *Acat1* were significantly more highly expressed in *Tgif1;Tgif2* null small intestine than in wild type tissue (Figure 3.8A). Similarly, expression of *Pcx* and *Mpc1* was also higher in the mutant. We observed a similar pattern for five of the six genes in primary MEFs (Figure 3.8B). *Acss1* expression did not increase in MEFs, but its expression is very low in cultured cells, including primary MEFs.

Figure 3.7 – Increased Acss2 expression in Tgif mutant tumors and crypts.

A) IF analysis is shown for Acss2 and β-catenin in colon tumors with adjacent normal tissue from *Apc* mutants and *Apc;Tgif1;Tgif2* (ApcTT) mice. B) Western blot analysis of colon tumors from Apc and ApcTT mice showing expression of Acss2 and Pcx, together with Hsp90 and γ-tubulin loading controls. Molecular weight markers are shown. C-D) IF analysis is shown for Acss2 and β-catenin in normal colon (C) or small intestine (SI) (D) from wild type (WT) or Tgif1;Tgif2 conditional double knockout (T1;T2).

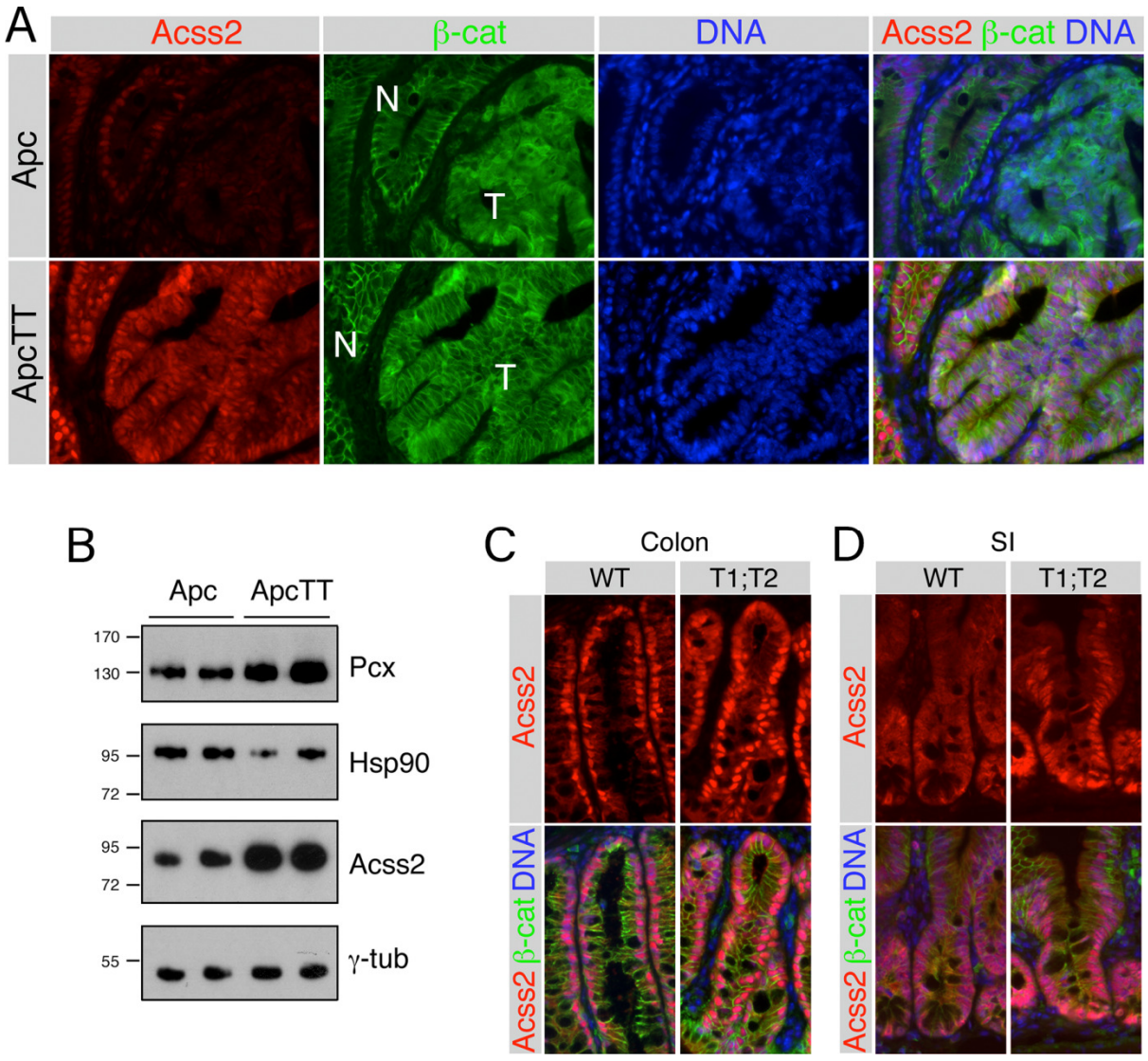
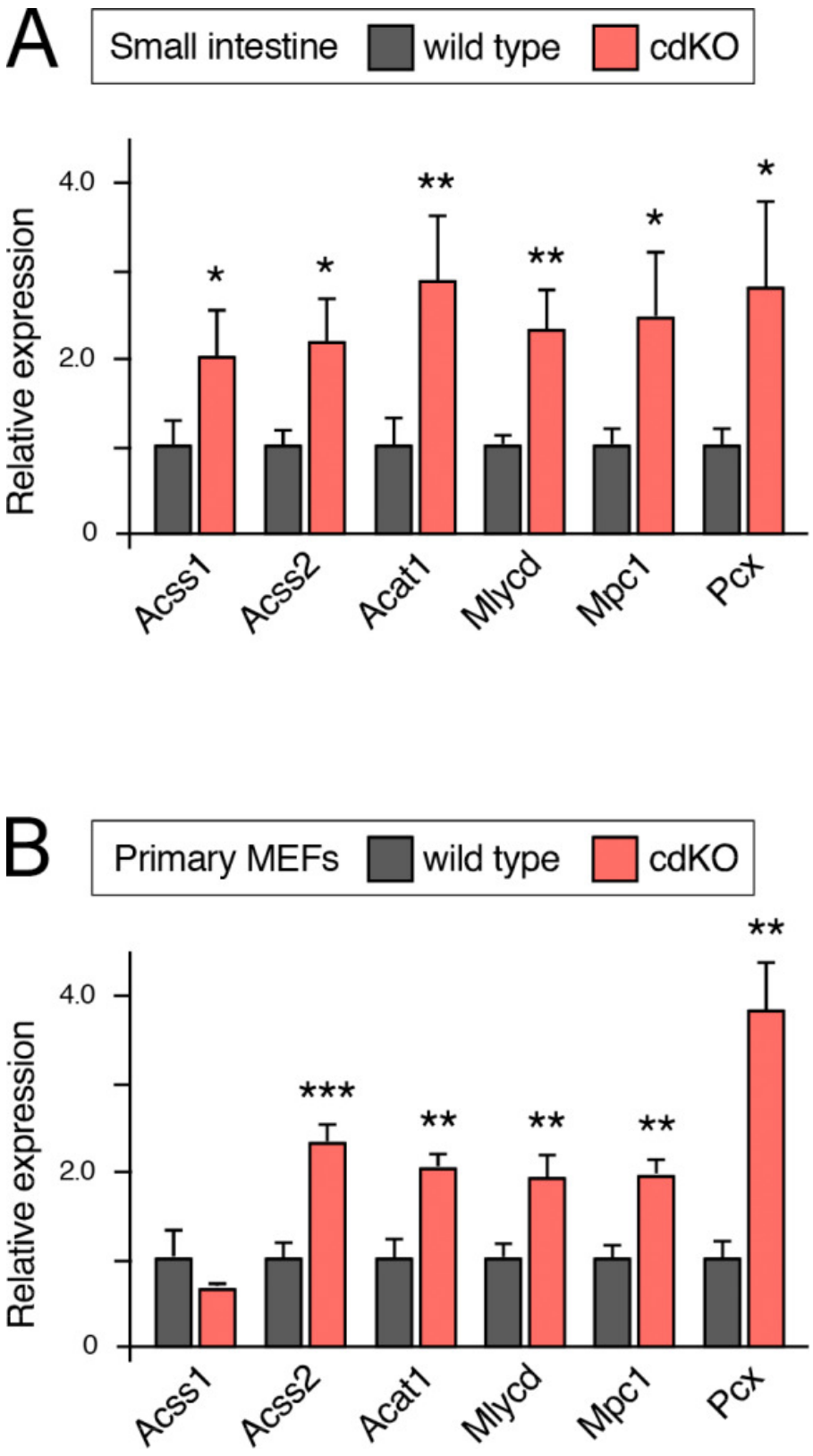

A
Acss2
β-cat
DNA
Acss2 β-cat DNA
Apc
ApcTT
N
T
N
T
B
Apc
ApcTT
170
130
Pcx
95
72
Hsp90
95
72
Acss2
55
γ-tub
C
Colon
WT
T1;T2
Acss2
Acss2 β-cat DNA
D
SI
WT
T1;T2
Acss2
Acss2 β-cat DNA

Figure 3.8 – Increased expression of acetyl-CoA and pyruvate metabolism genes in Tgif null small intestine and primary MEFs.

A) Expression of the indicated genes was analyzed by qRT-PCR from normal small intestine from wild type or cdKO (Tgif1;Tgif2 conditional double knockout) mice. B) Expression of the same genes was analyzed in wild type and cdKO primary MEFs. Expression is plotted relative to the wild type (mean +sd) of 4 and 3 replicates for intestine and MEFs. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ for comparison to wild type.


A
Small intestine
wild type
cdKO
Relative expression
4.0
2.0
0
*
*
**
**
*
*
Acss1
Acss2
Acat1
Mlycd
Mpc1
Pcx
B
Primary MEFs
wild type
cdKO
Relative expression
4.0
2.0
0
***
**
**
**
**
Acss1
Acss2
Acat1
Mlycd
Mpc1
Pcx

We next examined the sequences of the ChIP-seq peaks associated with each of these genes. In each case the potential Tgif1 bound region overlapped the transcriptional start site, and for all but *Acss1* at least two TGIF consensus sites were present (cTGTCA or TGTCAa; Figure 3.9A-B). To test Tgif1 recruitment, we performed ChIP-qPCR for the five genes that had ChIP-seq peaks with consensus TGIF sites. For *Mpc1*, we amplified two regions as the predicted peak was quite broad and had consensus sites close to each end. In chromatin from wild type small intestine, we observed significant enrichment of the putative Tgif1 binding regions from all five genes compared to a negative control region, and similar results were obtained from primary MEFs (Figure 3.9C-D). Together, these data suggest that Tgifs are direct repressors of a set of genes involved in acetyl-CoA and pyruvate metabolism.

3.3.4 TGIF repression of genes involved of Acetyl-CoA in cancer

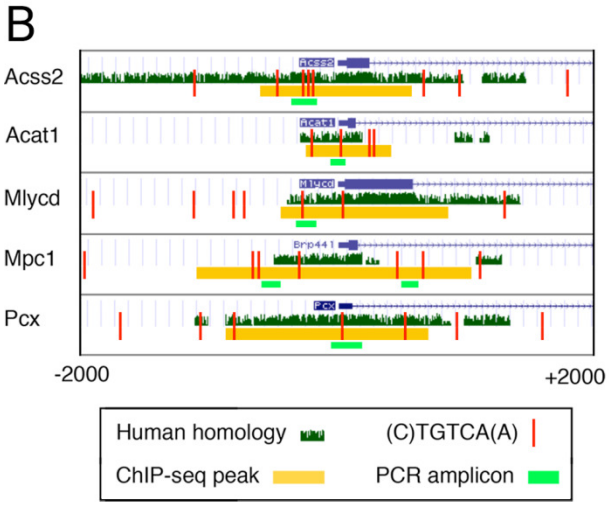
With strong evidence to suggest Tgifs directly repress genes involved in acetyl-CoA and pyruvate metabolism, we next decided to look at the expression patterns of these genes with the context of the human disease. First, we analyzed the expression profile of these genes in the human CRC cell line, HCT116, in order to confirm the expression changes of a panel of genes involved in acetyl-CoA and pyruvate metabolism seen in the small intestine and MEFs (Figure 3.8A-B). We tested expression of this panel of acetyl-CoA and pyruvate metabolism genes in HCT116 cells with and without siRNA-mediated knockdown of TGIF1 and TGIF2 by qRT-PCR. TGIF1 and TGIF2 knockdown these HCT116 cells was >70% (Figure 3.10A). Similar to both the mouse small intestine and MEFs, the three acetyl-CoA synthetic genes and *ACAT1* were significantly more highly expressed in the double knockdown cells, and this result was also true for *PC*

Figure 3.9 - Tgif1 regulation of acetyl CoA metabolic gene expression.

A) The relative positions of ChIP-seq peaks are shown, together with the percentile ranking in this data set (ranked by relative enrichment), and the number of 6/7 base matches (either cTGTCA or TGTCAa) to the TGIF consensus site and the expected number. B) The positions of the ChIP-seq peaks, qPCR amplicons, and TGIF consensus sites for each to the five genes tested are shown using UCSC genome browser views. A 4kb region centered on the transcriptional start is shown for each mouse gene, with similarity to human below. C) Tgif1 binding to each peak region was analyzed by ChIP-qPCR from normal wild type small intestine. D) Tgif1 binding in primary MEFs was analyzed by ChIP-qPCR. Chromatin was precipitated with a TGIF1 antiserum or pre-immune serum (pre-I). Data is mean + sd of triplicates and is plotted in arbitrary units with the TGIF1 IP for the negative control region (fbpk3) set equal to 1. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, for comparison to fbpk3.

A

Gene	Peak (bp)	%ile	rel to TSS	(C)TGTCA(A)	Exp
Acat1	670	17	-230/+440	4	0.6
Acss1	867	20	-319/+548		0.7
Acss2	1184	12	-597/+587	4	1.0
Mlycd	1308	2	-446/+862	2	1.1
Mpc1	2142	29	-1103/+1039	5	1.8
Pcx	1585	1	-883/+702	3	1.4



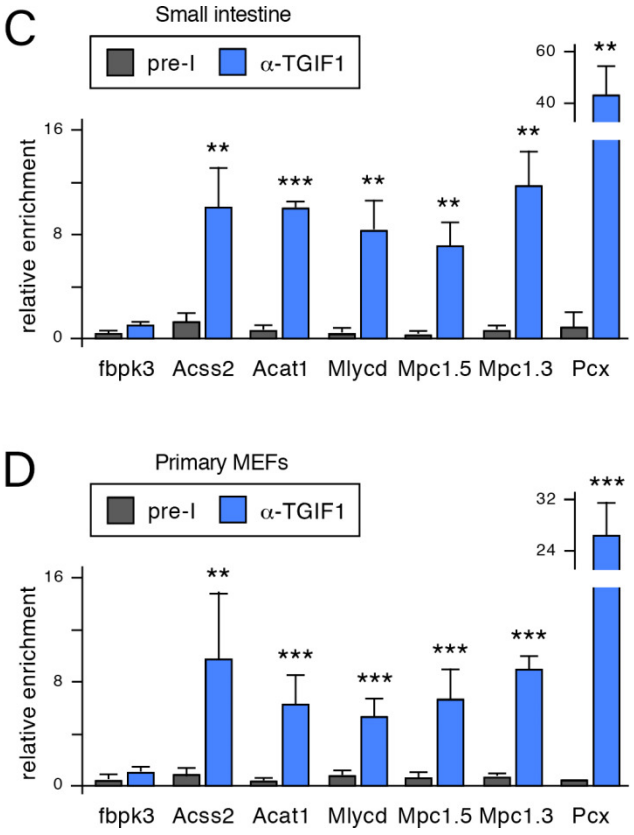
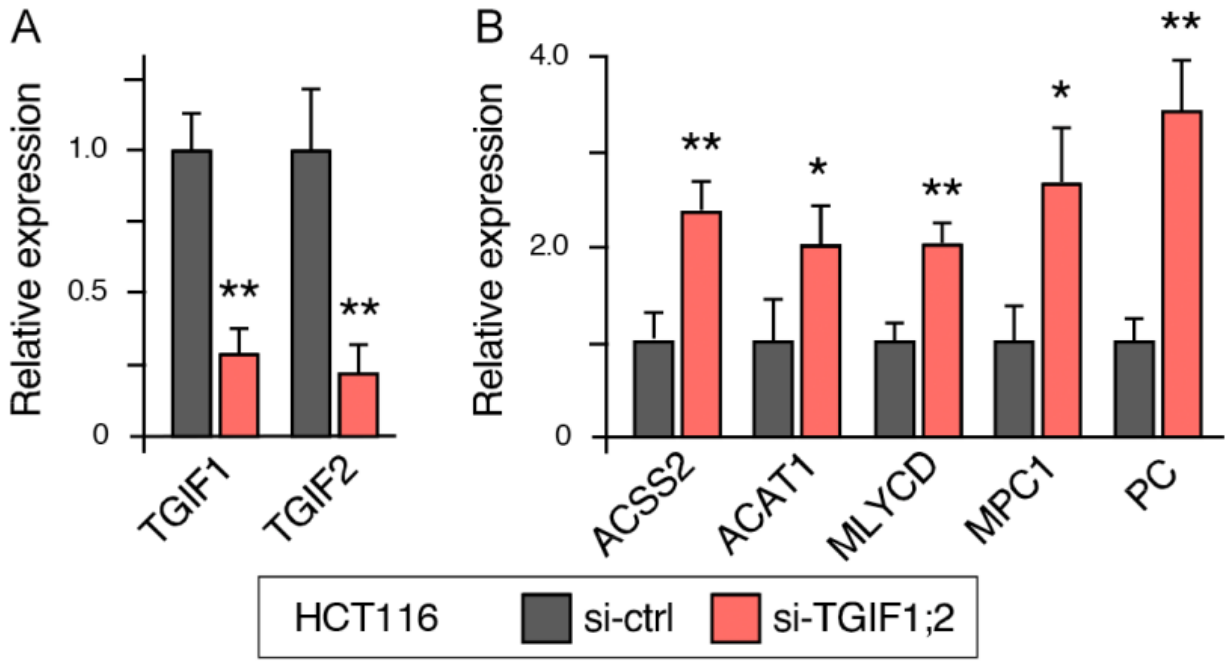


Figure 3.10 - Increased expression of acetyl-CoA and pyruvate metabolism genes in HCT116 cells with TGIF knockdown.

A) Expression of TGIF1 and TGIF2 in control (si-ctrl) and knockout (siTGIF1;2) HCT116 cells was analyzed by qRT-PCR. B) Expression of the indicated genes was analyzed by qRT-PCR in HCT116 cells with and without TGIF knockdown. Expression is plotted relative to the wild type (mean +sd) of 4 and 3 replicates for si-ctrl and si-TGIF1;2 samples. * $p < 0.05$ and ** $p < 0.01$ for comparison to si-ctrl.


A
Relative expression
1.0
0.5
0
**
**
TGIF1
TGIF2
B
Relative expression
4.0
2.0
0
**
*
**
*
**
ACSS2
ACAT1
MLYCD
MPC1
PC
HCT116
si-ctrl
si-TGIF1;2

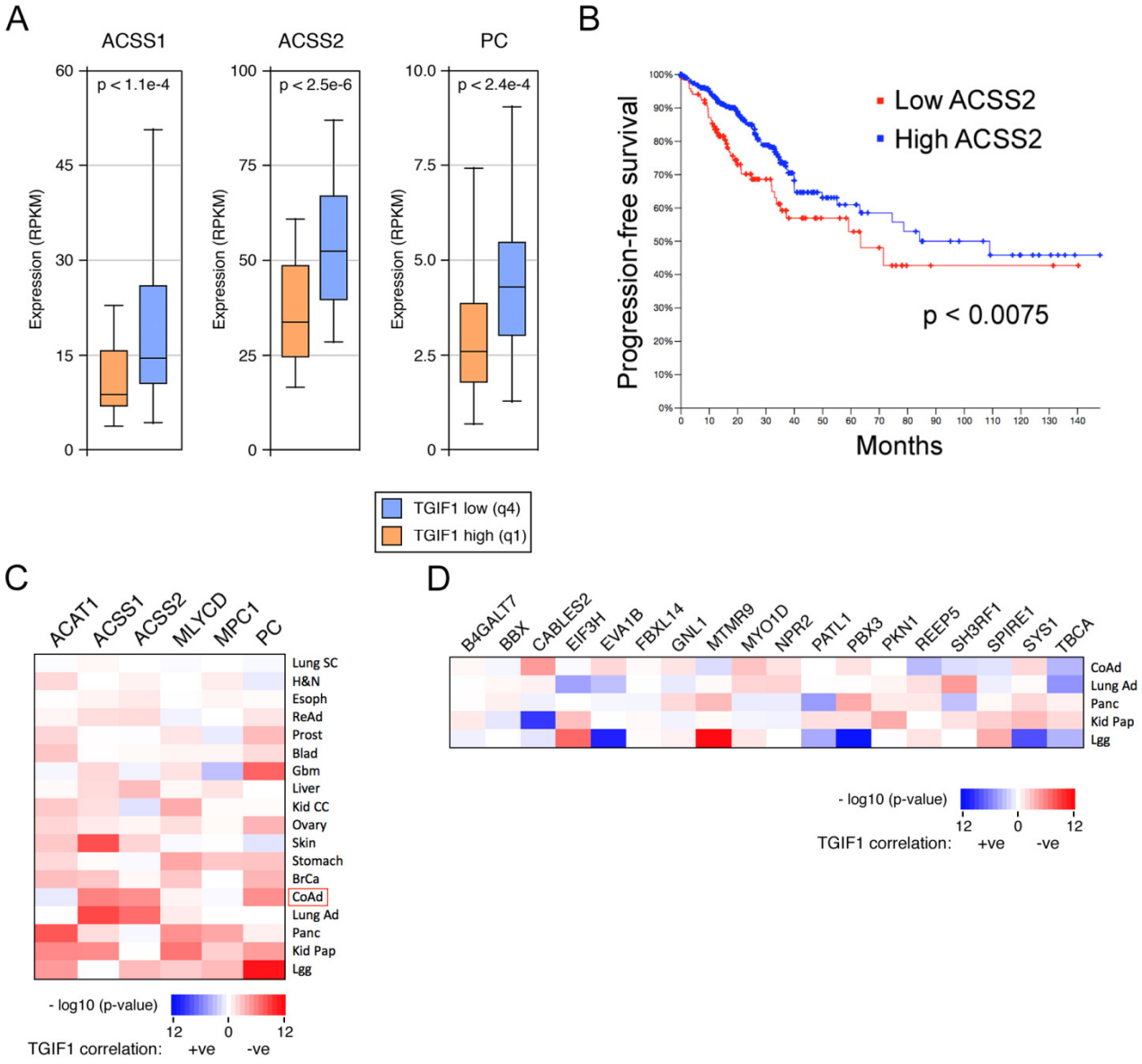
(human homolog of *Pcx*) and *MPC1* (Figure 3.10B). This result replicates the results from the small intestine and primary MEFs (Figure 3.8), suggesting the TGIF-mediated repression of acetyl-CoA and pyruvate metabolism is conserved between mice and humans within the context of CRC.

We next analyzed TCGA CRC data to see if TGIF1 levels affected expression of *ACSS1*, *ACSS2*, and *PC*, key genes involved in acetyl-CoA or pyruvate metabolism. We stratified the data into quartiles based on TGIF1 expression within the tumors. Then, we compared the expression of these three genes between the top and bottom quartiles. All three genes were significantly more highly expressed in the tumors in the bottom quartile of TGIF1 expression compared to the top quartile of tumors with TGIF1 expression (Figure 3.11A), once again agreeing with the idea that these genes are direct TGIF targets. Additionally, progression-free survival analysis from TCGA CRC data indicates patients with tumors with higher *ACSS2* expression survived longer than with lower expression as defined by z-score (Figure 3.11B), further suggesting TGIF1 and ACSS2 have opposing effects on tumor progression, likely due to TGIF1 repression of ACSS2 (Figure 3.9). Together, this data also agrees with both the previously seen gene expression and ChIP-qPCR data from mouse small intestines and primary MEFs and gene expression data from HCT116 cells.

To identify if TGIF repression of acetyl-CoA and pyruvate metabolism is a conserved function of TGIFs, we next did an analysis of pan cancer data. The correlation of expression with TGIF1 of the panel of six genes (Figure 3.8), three involved in acetyl-CoA metabolism, *ACAT1*, and the pyruvate metabolism genes, *PC* and *MPC1*, was analyzed in a eighteen different solid cancer types (Figure 3.11C). Overall, the expression

Figure 3.11 – TGIF1 expression negatively correlates with genes involved in acetyl-CoA and pyruvate metabolism in different cancer types.

A) Expression of a panel of genes is shown in the top (high) and bottom (low) quartiles of human CRCs expressing TGIF1. Data is from the published TCGA colon cancer dataset and has been stratified into quartiles by TGIF1 expression. p-values are denoted. B) Progression-free survival analysis of provisional TCGA colon cancer data is plotted for tumors with high and low expession of ACSS2. High and low expression was determined by a z-score above and below 0, respectively. C) Heat map is shown comparing the correlation of expression of TGIF1 with a panel of genes involved in acetyl-CoA and pyruvate metabolism in eighteen different solid cancer types using published TCGA data. D) Heat map is shown comparing the correlation of expression of TGIF1 with a panel of randomly generated genes in the five different solid cancer types with the highest negative correlation with acetyl-CoA and pyruvate metabolism genes from (C).


A
ACSS1
ACSS2
PC
p < 1.1e-4
p < 2.5e-6
p < 2.4e-4
Expression (RPKM)
TGIF1 low (q4)
TGIF1 high (q1)
B
Low ACSS2
High ACSS2
p < 0.0075
Progression-free survival
Months
C
ACAT1
ACSS1
ACSS2
MLYCD
MPC1
PC
Lung SC
H&N
Esoph
ReAd
Prost
Blad
Gbm
Liver
Kid CC
Ovary
Skin
Stomach
BrCa
CoAd
Lung Ad
Panc
Kid Pap
Lgg
- log10 (p-value)
TGIF1 correlation: +ve -ve
D
B4GALT7
BBX
CABLES2
EIF3H
EVA1B
FBXL14
GNL1
MTMR9
MYO1D
NPR2
PATL1
PBX3
PKN1
REEP5
SH3RF1
SPIRE1
SYS1
TBCA
CoAd
Lung Ad
Panc
Kid Pap
Lgg
- log10 (p-value)
TGIF1 correlation: +ve -ve

of these six genes negatively correlated with TGIF1 expression, especially in the top 50% of cancers with an overall negative correlation (bottom nine rows of heatmap).

Additionally, many epithelial cancer types are found within this set of nine cancers, including colon adenomas. In order to make sure these correlations between TGIF1 and the panel of genes was not occurring due to chance, we randomly selected eighteen genes and compared their correlation with TGIF1 expression in five different cancers, including CRCs (Figure 3.11D). There did not appear to be any pattern to the correlation between expression of these random genes within the given cancer types. Overall, this analysis of solid cancer types indicates TGIF regulation of acetyl-CoA and pyruvate metabolism may not only be conserved through many cancer types, but may especially be conserved within epithelial cancers.

3.4 Discussion

Using a colon cancer model as a starting point to analyze TGIF function, we provide evidence that TGIF transcription factors directly regulate genes involved in acetyl-CoA and pyruvate metabolism. We also provide evidence this regulation by TGIFs is seen in multiple different cancer types, especially in epithelial cancers. This function of TGIFs does not appear to be limited to tumor or normal tissue and may represent a key unexpected function of these transcription factors, independent of the other pathways they are known to regulate.

Our initial prediction was that Tgifs promote intestinal tumorigenesis by inhibiting TGFβ signaling. Mutating the *Tgfbr2* gene in the intestine has minimal effect, but, in concert with an *Apc* mutation, loss of *Tgfbr2* drives the transition from adenoma to invasive adenocarcinoma (217). Decreasing Tgif levels might be expected to increase the TGFβ response and limit tumor growth. However, transcriptome profiling of colon tumors revealed almost no overlap with changes in TGFβ-responsive gene expression, suggesting that at least, in this model, Tgifs are not major regulators of TGFβ signaling. In this context, it seems somewhat surprising that deleting Tgif1 and Tgif2 had such limited effect on the TGFβ response in colon tumors. However, a TGFβ-independent role for Tgifs is consistent with analysis suggesting that the majority of Tgif function may be mediated by direct binding to DNA (109). In agreement with this, structural studies show that, unlike many other homeodomain proteins, TGIF1 binds with high specificity and relatively high affinity to its cognate site (112).

In addition to direct DNA binding and SMAD-interaction, TGIF1 was proposed to activate Wnt signaling by sequestering Axins, allowing activation of Wnt/β-catenin

target genes (128). In our transcriptome data, canonical Wnt target genes show increased expression in *Apc* mutant tumors, but there is no consistent decrease in the Tgif mutant tumors, as would be expected if Tgif1 promoted β-catenin nuclear function. Like effects on TGFβ signaling, this suggests the Wnt pathway is not a major TGIF target in this colon tumor model, arguing against the two most likely models to explain a pro-tumorigenic function of Tgifs. A further link to Wnt signaling is the demonstration that TGIF1 is directly activated by Wnt/β-catenin signaling (128). Our data are consistent with this, in that Tgif1 and Tgif2 expression is increased in *Apc* mutant tumors compared to normal tissue, although we do not know if Tgif genes are β-catenin targets in the intestine.

Genome wide analysis of Tgif1 binding to chromatin in mouse ES cells revealed a very large number of binding sites (109). Comparing the genes predicted by this binding did not reveal any enrichment for genes with increased expression in Tgif1 knockdown or knockout cells. When we ranked ChIP peak enrichment scores and considered the top 40%, there was enrichment for genes activated by Tgif deletion, consistent with this being a higher confidence target gene set. Comparing the gene expression changes with deletion of Tgifs from colon tumors to our previous analysis of early mouse embryos lacking Tgifs revealed a relatively small overlap, consistent with tissue specific effects. However, there was enrichment in the overlap for genes that showed increased expression in both data sets with *Tgif1;Tgif2* deletion. Integrating this analysis with chromatin binding data identified a high confidence target gene set. In support of this, the promoters of these genes were enriched for a TGIF consensus site. Surprisingly, pathway analysis on this high confidence gene set identified acetyl-CoA metabolism as the most

significantly enriched biological process, with no apparent links to TGFβ or Wnt signaling.

Along with large metabolic shifts, the two biggest changes between *Apc* and *Apc;Tgif1;Tgif2* tumors by GSEA were in EMT and KRAS signaling. The enrichment for the EMT signature in the *Apc* tumors was initially somewhat surprising as this tumor model of CRC does not metastasize, but recent work has suggested EMT transcription factors participate in metabolic rewiring seen in tumorigenesis by promoting glycolysis (218,219). Additionally, there is evidence to suggest dysregulation of metabolic pathways, especially glycolysis, can drive EMT (137,220,221). As glycolysis was one of the other large changes we noticed by GSEA, it may not be surprising to see EMT and glycolysis enriched in the *Apc* tumors together. The change in KRAS signaling is not quite as surprising as *Apc* tumors were typically larger than *Apc;Tgif1;Tgif2* tumors, suggesting difference in proliferation between the two tumor genotypes. This difference in KRAS signature may in part account for the differences in tumor size and volume previously observed.

In addition, analysis of all gene expression changes between *Apc* tumors and *Apc* tumors lacking both Tgifs revealed changes in multiple metabolic pathways, further supporting a role for Tgifs as regulators of metabolic gene expression. Among a panel of six genes with links to acetyl-CoA and pyruvate metabolism, five had multiple consensus TGIF sites within the region identified by ChIP-seq, and we validated them as direct Tgif1 targets in both normal small intestine and primary MEFs further supporting the notion of this being a conserved core Tgif function. Our data suggest that Tgifs play a role in regulating metabolic gene expression in both normal and tumor tissues and may

mediate part of the metabolic reprogramming that occurs in colon adenomas. Analysis of a panel of diverse human cancer data sets suggests that TGIF1 may regulate metabolic genes in multiple cancers, supporting the wider relevance of this conserved function of TGIFs.

In summary, our data suggest a model in which Tgifs function in multiple cell types to limit expression of a core set of acetyl-CoA metabolic genes. In cancers where Tgif levels increase, this normal Tgif function may be co-opted by the tumor as part of the metabolic reprogramming.

Chapter 4 – General Discussion

The work done for this thesis sought to understand the role of TGIFs in CRC. We first analyzed multiple CRC datasets, including TCGA data, to confirm the upregulation of *TGIF1* and *TGIF2*. Then, using CRISPR/Cas9 mediated knockout, we generated TGIF1 KO HCT116 cells and showed they had decreased proliferation; this result was also observed in intestinal crypts in mice with differing levels of Tgif1 expression – cdKO mice had decreased proliferation while the opposite was true in *Villin*-TGIF1 transgenic mice. Using a murine model of CRC with an intestine-specific Cre-mediated deletion of *Apc*, we analyzed the role of Tgif1 and Tgif2. We observed Tgifs are upregulated in CRC, recapitulating the expression patterns seen in humans, and knockout of Tgifs or overexpression of TGIF1 had opposing impacts on tumor burden and tumor size.

With this result, we performed transcriptome profiling on the colon tumors from the mice. The results were overlapped with transcriptome profiling data from wild type and cdKO embryos (111), and this analysis strongly suggested TGIFs directly regulate genes regulating acetyl-CoA and pyruvate metabolism. To follow up on these results, we provided evidence to suggest glycolysis is downregulated in Apc;cdKO tumors through decreased expression of Glut1, and we also show increased Acss2 expression in Apc;cdKO tumors compared to Apc tumors by IF. In addition, we showed Tgif knockout or knockdown in murine MEFs, SI, and human HCT116 cells results in increased expression by RT-qPCR of six genes involved in acetyl-CoA and pyruvate metabolism, and two of the genes involved in these processes were shown to be upregulated at the protein level by western blot. Finally, we show Tgif1 directly regulates genes involved in

acetyl-CoA and pyruvate metabolism by performing ChIP-qPCR. Analysis from the transcriptome profiling, IF, western blot, RT-qPCR, and ChIP-qPCR taken together provide strong evidence Tgifs regulate acetyl-CoA and pyruvate metabolism.

Evidence for this regulatory role of TGIFs was further demonstrated by an analysis of a large panel of solid tumors which suggested TGIF expression strongly negatively correlates with a panel of genes involved in acetyl-CoA and pyruvate metabolism. Additionally, this role of TGIFs does not appear to be limited to tumor tissue, suggesting it may be a core function of TGIF transcription factors. Taken together, this work provides evidence for a novel role for TGIFs and their function in CRCs.

4.1 TGIF connection to proliferation

Previous studies on the role of TGIFs in cancer have focused on the role of TGIF1. In multiple cancer types, including breast, colon and lung cancers, TGIF1 expression has been shown to be pro-tumorigenic and pro-proliferative (124,128,129). No study has properly addressed the role of both TGIFs simultaneously within any given cancer model. As there is strong evidence to suggest TGIF1 and TGIF2 have overlapping and potentially redundant functions (100,108,111), it remains a possibility that knockout of a single TGIF could be compensated for by presence of its paralog. Thus, our double knockout of TGIFs in a CRC model more accurately has allowed us to ascertain some of their functions in CRC, and this is where our study has excelled and provided important insights.

Double knockout of both Tgifs in mouse small intestinal epithelium resulted in fewer cells and fewer proliferating cells in the intestinal crypts. In contrast, mice

overexpressing *Villin*-TGIF1 had more proliferating cells in the intestinal crypts compared to wild type animals. This result would suggest TGIFs promote proliferation, independent of a tumor state and mirrors the result obtained *in vitro* with single knockout of TGIF1 in HCT116 cells. In two TGIF1 null colonies generated by CRISPR/Cas9 deletion, both grew significantly slower than wild type cells, further suggesting TGIFs promote proliferation.

Unfortunately, we were unable to generate TGIF1 & TGIF2 double knockout in HCT116 cells. Generating these cells would have allowed us to eliminate the possibility of overlapping functions of TGIFs. The inability to generate the double KO of TGIFs might suggest TGIFs are necessary for HCT116 cell survival. However, given we were able to generate viable cdKO mice with no Tgif expression in the intestines, this possibility seems unlikely. The inability to generate double TGIF KO HCT116 cells could also indicate double KO of TGIFs in HCT116 cells may cause these cells to senesce, indicating we could generate the double KO but would not be able to grow or use them. Additionally, two other possibilities remain – the double knockout of TGIF1 and TGIF2 is possible in HCT116 cells, but they grow so slowly that they are difficult to identify and screen, and, secondly, not enough colonies were screened. If generation of TGIF1 & TGIF2 double knockout HCT116 cells is difficult to achieve, screening 29 colonies may not have been enough to determine it was not possible to generate these cells. Additionally, there is evidence that a complete Tgif1 or Tgif2 knockout mouse is viable, though a double knockout mouse is not (111,119,222). While evidence for a full body double knockout of TGIFs shows it is not possible, double knockout is possible in the intestines. This would suggest TGIFs are not essential for intestinal homeostasis.

Taken together, it would seem that TGIF function in intestines is not essential but could promote proliferation, something tumors could potentially exploit.

Once we shifted to the CRC tumor model utilizing *Villin*-Cre with Tgif knockout and TGIF1 overexpression, we observed clear differences in tumor burden. Mice with TGIF1 overexpression had both more tumors and more overall larger tumors compared to *Apc* mutant mice, while mice with *Tgif1* or *Tgif1;Tgif2* knockout within an *Apc* background had both fewer tumors and fewer larger tumors. We also observed a stepwise effect on tumor burden and tumor size as we went from single to double deletion of Tgifs. This data, again, agrees with data previously published which suggested TGIF1 is pro-tumorigenic and promotes tumor proliferation (129). Our data also goes beyond that conclusion by suggesting deletion or overexpression of Tgifs may have a stepwise type effect on CRC tumors, with respect both to tumor size and burden, providing evidence of a potential compensatory function of Tgifs.

In addition, one of the biggest changes between *Apc* and *Apc;Tgif1;Tgif2* tumors through transcriptome profiling and subsequent GSEA was an enrichment of KRAS signaling in *Apc* tumors. KRAS is a well-known oncogene whose mutation results in aberrant and dysregulated cellular proliferation, and the KRAS/MAPK pathway has been found to be mutated in 66% of human CRCs. While there is a link between Tgif expression and proliferation, no direct link between Tgifs and Kras signaling is currently known. It is somewhat surprising to see and enrichment for Kras signaling in *Apc* tumors compared to *Apc;Tgif1;Tgif2* tumors, but this differential expression of Kras signaling within the context of Tgif KO in tumors may in part explain the tumor size and volume differences we observed.

The unanswered question here is how latent levels of Tgif expression are able to affect tumorigenesis and/or tumor growth. This is unknown, and the answer would be crucial in understanding CRC tumor biology. One of the easiest ways to address this would be to analyze Tgif expression in normal crypts and identify if there is an overlap with intestinal stem cells (ISCs). This co-expression would be very important for two reasons. Firstly, it has previously been shown that stem cells upregulate TGIF (109), and if Tgifs were upregulated in intestinal stem cells, it would recapitulate that previous finding. Secondly, it is widely believed ISCs are the cells of origin for CRCs (15,23). Thus, evidence of Tgif upregulation in ISCs in the absence of a tumor would strongly suggest the level of Tgif expression in these cells of origin for CRC would have a priming effect for tumor growth and progression.

This potential priming effect for tumor growth and progression would be simple to test in HCT116 double TGIF1 and TGIF2 knockout cells, if we could generate them. It would be worth trying to screen many more double KO colonies in order to do some very simple experiments with them. An alternate strategy to generate HCT116 cells without TGIF1 or TGIF2 expression, a shRNA mediated knockdown of TGIF2 in TGIF1 KO cells, could be used. A combination of experiments including a simple growth assay with serial replating, colony forming assay in soft agar and on plastic, and xenografts in mice with double knockout and wild type cells would provide the results to assess the role TGIFs have on proliferation and tumorigenicity both *in vitro* and *in vivo*, and these experiments could be competed rather rapidly once the double knockout cells could be generated.

4.2 Tgif disconnect with TGFβ and Wnt signaling

4.2.1 Tgifs do not appear to regulate TGFβ signaling in our CRC model

TGIFs have been well characterized as repressors, beginning with their discovery as RXR repressors (81,82,97,107,108,113). Much of the work to understand the function of TGIFs has been done within the context of TGFβ signaling (81,82,110,112,120,204), so much so that TGIFs perhaps have been misnamed TGFβ-induced factor (129,222,223). TGIFs have been shown to interact with SMADs independent of DNA binding to its consensus site (81,112), and TGIFs compete with SMAD coactivators. Repression of TGIF-bound SMADs is further enhanced by recruitment of other corepressors by TGIFs (107,108). However, unlike other TGFβ inhibitors such as SMAD7 or SKIL (224,225), there is little evidence to suggest TGFβ signaling directly regulates TGIF expression. Taken together, this would suggest that while TGIFs are able to limit TGFβ signaling, TGFβ signaling does not induce TGIFs, and a feedback mechanism between the two has yet to be shown to exist.

Our initial hypothesis was that Tgifs would promote intestinal tumorigenesis in our mouse model by inhibiting TGFβ signaling. *Tgfbr2* mutations, within the context of an *Apc* CRC tumor model, are able to facilitate the transition from adenoma to invasive carcinoma (217). Thus, it would be reasonable to think that decreased expression of Tgifs would result in increased TGFβ response, limiting tumor size. However, very surprisingly, analysis of our RNA-seq data of well characterized TGFβ target genes saw no consistent changes in expression between normal tissue and *Apc* tumors in which Tgifs are upregulated. Even more surprising was the result that Tgif knockout in tumors had little to no effect on expression of TGFβ targets. Thus, it does not appear Tgifs are

major regulators of TGFβ signaling within this model of CRC. This is in contrast to the effect of Arkadia (*Rnf111*), a ubiquitin E3 that promotes TGFβ signaling by driving degradation of the Ski and Skil (SnoN) SMAD corepressors. *Rnf111* deletion increased tumor numbers in a chemical carcinogen mouse colon cancer model, increased Skil expression and reduced the TGFβ response (226). Thus, altered SMAD corepressor levels can affect CRC tumor progression, although it remains possible that other Arkadia substrates contribute.

Within this context, it is certainly surprising that deletion of Tgif1 and Tgif2 had such a minimal effect on TGFβ signaling in colon tumors. However, this result is consistent with published studies which suggested that the majority of TGIF function may be through direct DNA binding (109–111), and this data is corroborated by another structural study which showed TGIF1 has both high affinity and specificity for its consensus site (112,227). Therefore, while it was initially surprising our data does not show changes in TGFβ-responsive genes with changes in Tgif levels, this result suggests Tgifs may have a TGFβ independent function within the context of CRC (more on this later).

4.2.2 Tgifs do not appear to regulate WNT signaling in our CRC model

A novel role of TGIF1, independent of direct DNA binding and TGFβ inhibition, was proposed by one study which provided evidence to suggest TGIF1 activates Wnt signaling by sequestering Axins in the nucleus (128). This blocks the formation of the β-catenin destruction complex, allowing β-catenin to translocate to the nucleus and drive Wnt target genes. This study was done primarily in a breast cancer model. A second group also published a study with evidence to suggest TGIF1 activates Wnt signaling in a

CRC model, but this study did not find evidence of Axin sequestration (129). We investigated the link between Wnt and Tgifs in our model of CRC, and transcriptome profiling showed increased expression of Wnt target genes in the *Apc* tumors compared to normal tissue. However, there was no decrease in the expression of Wnt target genes in the tumors lacking both Tgifs, as would be expected if Tgifs promoted β-catenin mediated transcriptional regulation. Thus, Tgifs do not appear to regulate Wnt signaling in our CRC model, similar to its lack of effect on TGFβ signaling

It remains possible that any effect on Wnt signaling is a cell type specific function of TGIF1, a notion that is consistent with the significant differences in gene expression changes seen in different cell types and tissues with reduced TGIF function. It is also possible TGIF1 promotes β-catenin function, but its effect is masked by the overriding pathway activation caused by *Apc* mutation. However, this still argues against a Tgif effect on tumor promotion via β-catenin in this mouse model. These results, taken together, strongly suggest that the two models which most likely explain the pro-tumorigenic role of Tgifs in CRC are incorrect, indicating the pro-tumorigenic role of Tgifs in CRC may be due to the genes they directly regulate, independent of Wnt and TGFβ signaling.

Another link between Wnt signaling and TGIF1 was demonstrated by showing TGIF1 is directly activated by β-catenin (128). Our RNA-seq data is consistent with this as Tgif1 and Tgif2 expression is increased in *Apc* tumors compared to normal tissue, but we do not have evidence to suggest β-catenin directly activates Tgifs in the intestines. One of the next steps for this project would be to perform ChIP for the β-catenin/Tcf co-activating complex within the Tgif promoter in *Apc* tumors and normal intestine. This

would provide direct evidence that Wnt signaling regulates Tgif expression, corroborating a previously published study (128). Additionally, if we could ChIP the β-catenin/TCF complex to the Tgif promoter in normal tissue, it would provide strong evidence to suggest Wnt signaling is a regulator of Tgifs outside of the tumor context. This would be both a novel and important finding as little is currently known about how Tgifs are transcriptionally regulated.

4.3 Tgif regulation of metabolism

After finding little evidence to suggest Tgifs were regulating Wnt or TGFβ signaling within our CRC model, we compared gene expression enrichment between Tgif1 wildtype and null tumors using published ChIP-seq data from Lee et al. 2015 (109). Within this data set, there were over 16,000 ChIP-seq peaks for Tgif1. In order to narrow our focus and consider genes likely to be Tgif1 direct targets, we considered the top 40% of genes with ChIP peak enrichment scores with Tgif1 deletion. This resulted in a set of genes which we considered to be high confidence Tgif1 targets. Additionally, there were few genes enriched in both *Tgif1;Tgif2* null CRC tumors and a previous analysis conducted by the Wotton lab in early mouse embryos lacking Tgifs (111), strongly suggesting Tgifs may have tissue specific effects. However, the genes that did overlap between these two data sets, when combined with the Tgif1 ChIP-seq data, yielded strong evidence of a high confidence Tgif1 target gene set. Furthermore, these high confidence genes had TGIF1 consensus sites in their promoters, providing strong evidence for TGIF1 regulation of these genes.

To our surprise, the genes highly upregulated with high confidence ChIP peaks were genes involved in acetyl-CoA and pyruvate metabolism, with no connection to TGFβ or Wnt signaling (as previously discussed). Additionally, transcriptome analysis of *Apc* and *Apc;Tgif1;Tgif2* tumors suggested large scale metabolic shifts, providing additional evidence Tgifs regulate expression of genes involved in cancer metabolism. When we analyzed a panel of six genes involved in acetyl-CoA and pyruvate metabolism, five had TGIF consensus sites within their promoter regions. We were then able to validate these genes as Tgif1 targets in multiple tissue types, normal small intestine, and primary MEFs. This suggested that, unlike the majority of changes seen with Tgif deletion in CRC tumors, the increased expression of acetyl-CoA and pyruvate metabolic genes is not cell type specific, with evidence it occurs in mouse embryos, primary MEFs, normal small intestine, and colon tumors. This strongly suggests that regulation of acetyl-CoA and pyruvate metabolism may be a fundamental, yet unexpected role of Tgifs. However, even if this is an unexpected role of Tgifs, we provide evidence to suggest it is a conserved core function of Tgifs.

To investigate this further, we analyzed the expression of ACSS1, ACSS2, and PC in human TCGA CRC data. After splitting the tumors into quartiles by TGIF1 expression, we noted that the expression of these three genes was increased in the lowest quartile compared to the uppermost quartile. This was a somewhat surprising result as high ACSS2 levels previously have been seen in malignant tumors (188), suggesting tumors may become dependent on acetate to generate ACSS2-mediated acetyl-CoA for the generation of fatty acids and overall growth and survival. Additionally, in brain tumors and triple-negative breast cancers, ACSS2 expression was shown to correlate with

tumor grade and survival, with higher expression of ACSS2 leading to a worse prognosis (176,188).

Reconciling these studies with our data suggested the link between ACSS2 and tumor severity phenotype may be cancer or tissue-specific. We provide evidence for this when we performed a progression free survival analysis on TCGA CRC data for ACSS2. The results showed tumors with higher ACSS2 expression had better progression free survival, and fit into our larger overall model in which Tgif expression leads to a more aggressive tumor phenotype and represses Acss2 expression. This result from CRCs is somewhat confusing as it goes against conventional wisdom, but it may demonstrate a novel finding about ACSS2 within the context of CRC, i.e. ACSS2 expression in human CRC tumors may be more associated with less aggressive tumors and normal tissue compared to other cancers. However, tumors developed in our model of CRC did not develop past the adenoma stage, and it remains possible ACSS2 expression could increase a later tumor stage, though the progression free survival analysis would argue against that.

One reason high ACSS2 expression may be beneficial for progression free survival is that the colonic lumen is filled with microbes which can produce high levels of short chain fatty acids, such as butyrate and acetate (228,229). Short-chain fatty acids are a major energy source for colonocytes. Thus, the microbiome of the colon may have a unique impact on colonic energy homeostasis compared to other tissue types, perhaps explaining why increased ACSS2 expression in human CRC results in better progression free survival while the opposite may be true for other tissues. It is possible, for example, that decreased expression of ACSS2 in a tumor would limit fatty acid synthesis, favoring

glycolysis and the Warburg effect. Thus, these results suggest that Tgif regulation of Acss2 and other enzymes involved in fatty acid synthesis might be an important function of Tgifs, especially in the colon and CRC, where fatty acid metabolism is important (more on this later).

Although we observed extensive changes in expression of metabolic genes, relatively few were increased in the Tgif mutant tumors, suggesting the majority of changes may be indirect effects. Attempting to place potential direct Tgif target genes in context suggests that Tgifs repress anabolic metabolism rather than catabolism (see Figure 3.6). For example, Tgif repression of mitochondrial *Acss1* and *Acat1* would be expected to limit ketone synthesis, and generation of acetyl-CoA from acetate would be reduced via repression of both *Acss1* and *Acss2*. In the absence of Tgifs, anabolic metabolism may favor synthesis of ketones and sterols, and the utilization of pyruvate to generate other metabolic intermediates. These direct effects of Tgifs are reminiscent of the shift in cancer cells towards the generation of metabolic intermediates that can drive tumor growth.

Reprogramming of metabolic gene expression is recognized as one of the hallmarks of cancer. In addition to an increased reliance on glycolysis, termed the Warburg effect (230), there is extensive rewiring of energy metabolism in cancer cells (137,231). Comparison of gene expression between *Apc* tumors and those lacking Tgifs shows enrichment of glycolysis and glutamine metabolism in the *Apc* tumors that have high Tgif expression, and higher expression of genes involved in nucleotide and amino acid metabolism. Recent work suggests metabolic reprogramming occurs at the adenoma stage of CRC (232), and it appears this is downstream of activation of oncogenes, such as

KRAS or BRAF, and requires high MYC expression (232,233). Thus it would appear that Tgifs are required components for this metabolic shift to repress some acetyl-CoA and pyruvate metabolism genes, but they do not appear to be necessary for the large-scale metabolic shifts seen in tumors.

Additionally, there is evidence to suggest EMT may play a role in the metabolic reprogramming of tumors. EMT was the top enriched gene signature by GSEA, and this was quite surprising as the tumor model we used does not metastasize. Evidence from the literature would suggest EMT can regulate some metabolic pathways, including glycolysis (234) and oxidative phosphorylation (235). *SNAI1*, a well-known EMT transcription factor prepresses fructose-1,6-bisphosphate 1 (FBP1) which favors increased glucose uptake (236), glycolysis, and flux of metabolites through downstream glycolytic pathways, such as the pentose phosphate pathway (PPP). Additionally, *SNAI1* has been shown to repress the expression of FASN and ACC, two enzymes involved in fatty acid metabolism (237). Together, this suggests EMT can rewire tumor metabolism by increasing glycolysis and decreasing other metabolic pathways.

Complicating this is the fact that metabolic alterations can drive EMT, especially metabolic alterations within the glycolytic pathway. In fact, one study in breast cancer MCF-10A cells showed overexpression of phosphoglucose isomerase (PGI), an enzyme that converts G6P to F6P, can cause the stabilization of ZEB1 and ZEB2, two well characterized EMT drivers (238). Additionally, another study found silencing GAPDH in HCT116 cells inhibited EMT by repression of *SNAI1* (221). This, taken with evidence of EMT transcription factors influencing tumor metabolic shifts, suggests there may be a positive feedback loop between EMT and glycolysis. EMT transcription factors, such as

SNAI1 and *ZEB1*, can cause metabolic shifts in tumors in order to allow the tumor cells to survive in the tumor microenvironment while dysregulation of tumor metabolism, especially increased glycolysis and decreases in other pathways, in turn drives EMT. Metabolic stress typically causes cellular senescence (239), and EMT induction is one means by which cells could survive and proliferate in nutritionally deficient and hypoxic conditions found in tumors.

Connecting EMT to Tgifs within our tumor model is not directly obvious. We provide direct evidence to suggest Tgifs act independently from EMT as we show Tgifs ChIP to five genes involved in acetyl-CoA and pyruvate metabolism. One connection between Tgifs and EMT is the repression of fatty acid synthesis. As previously discussed, short chain fatty acids, especially butyrate, are abundant in the colon due to the effects of the microbiome. Thus, normal colonocytes may have increased expression of enzymes involved in fatty acid synthesis. EMT transcription factors have been shown to decrease expression of enzymes involved in fatty acid synthesis, and we show Tgifs directly bind and repress transcription of enzymes involved in fatty acid synthesis. Thus, it may be that the enrichment for EMT we see in *Apc* tumors may be partially due to the lack of Tgif repression of fatty acid synthesis in *Apc;Tgif1;Tgif2* tumors. In addition, it may be Tgif repression of genes involved in fatty acid synthesis is an important function of Tgifs in CRC specifically. However, it remains possible that a decrease in glycolysis seen in the *Apc;Tgif1;Tgif2* tumors may help explain the difference in EMT signature between the two tumor genotypes. As there is evidence to suggest there is a positive feedback look between glycolysis and EMT, one simple theory to explain the EMT difference in the tumors would be the enrichment for glycolysis in *Apc* tumors. Taken together, our data

may suggest Tgif expression is pro-EMT, but this is likely through an indirect mechanism.

Tgifs appear to play a role in regulating metabolic gene expression in both tumor and normal tissues, and we provide evidence to suggest this is conserved in multiple cancer types. We first provide evidence via RT-qPCR in HCT116 WT and *TGIF1;TGIF2* knockdown cells that the five of the six genes we analyzed in murine small intestine and primary MEFs were all upregulated with TGIF knockdown. We then showed analysis of 18 solid tumor types for TGIF1 expression correlation with a panel of acetyl-CoA and pyruvate metabolic genes. We identified a negative expression correlation between the two in many of the cancers, and this was especially pronounced in cancers with an epithelial cell of origin. This again provides evidence to suggest a core function of TGIF1 is to repress these genes, independent of tissue type. It also provides evidence that the regulatory role of Tgifs we see in our CRC model is not necessarily limited to CRC but likely occurs in other types of cancer as well, particularly in epithelial cancers. Further work is required to understand the metabolic regulation by Tgifs in not only CRC but in other types of cancer as well.

The model of CRC we used to develop these tumors gives us an insight into what Tgifs are doing within early states of tumorigenesis. This is a novel study, and the results are quite surprising. To reiterate, Tgifs do not appear to regulate TGFβ or Wnt signaling and instead appear to regulate metabolism, specifically fatty acid/acetyl-CoA synthesis and pyruvate metabolism. Additionally, this Tgif-mediated metabolic switch in *Apc* tumors may be pro-EMT, and this would again be a novel function of Tgifs. In sum, our

work within this mouse model not only has provided new insights into Tgif function in tumorigenesis, but opened up new avenues for Tgif research in CRCs.

Here, the next immediate steps to strengthen evidence for this novel role of Tgif-mediated metabolic reprogramming include further analysis of the panel of six genes we analyzed which regulate acetyl-CoA and pyruvate metabolism. A bulk of our presented evidence was a combination of western blots for Acss2 and Pcx, and one panel of IF for Acss2. Assessing protein levels and subcellular localization both in tumor and wild type intestinal tissue with and without *Tgif1;Tgif2* knockout would be very informative and provide direct evidence to validate Tgif regulation of these targets in addition to the bioinformatics approach. Additionally, it would validate our finding that Tgifs regulate these genes in normal and tumor tissues. Performing western blots and RT-qPCR in human samples for these genes involved in acetyl-CoA and pyruvate metabolism in addition to TGIFs would not only provide evidence for TGIF regulation of these genes in the human disease, but it would also strengthen the idea that these genes Tgifs regulate is a core, conserved function across species.

4.4 Potential Medium & Long Term Projects

4.4.1 Metabolomics experiments

One of the next steps for this project is to perform metabolomics experiments utilizing mass spectrometry on both normal intestines and tumors in *Apc* and *Apc;Tgif1;Tgif2* mice to assess the levels of different metabolites, e.g. acetate, acetyl-CoA, pyruvate, and lactate. Acss2 turns acetate into acetyl-CoA, so we would expect to see decreased levels of acetate and increased levels of acetyl-CoA in *Tgif1;Tgif2* null

tissue with perhaps lower levels of acetate and increased levels of acetyl-CoA in *Apc;Tgif1;Tgif2* tumors compared to normal small intestine. We would also expect to see opposing results with pyruvate and lactate, similar to acetate and acetyl-CoA. Tumors undergoing aerobic respiration typically turn pyruvate into lactate in order to reduce ROS (137,138), and Tgifs appear to directly negatively regulate genes involved in pyruvate metabolism. Thus, we would expect to see increased levels of lactate and decreased levels of pyruvate in tissues expressing Tgifs, with more lactate present in tumor tissue than in normal tissue.

Additionally, full scale metabolomics on murine colon tumors, HCT116 cells with and without *TGIF1;TGIF2* KO, and human tumor samples would be very useful set of experiments. The results of these experiments would provide raw levels of metabolites within these different tissue types and validate the expected results of the experiments proposed above which analyze levels of a panel of metabolites. Full scale metabolomics would also show levels of metabolites in different metabolic pathways, providing evidence for the downstream impacts of Tgif-mediated regulation of metabolic genes. In addition, overlapping the results of these full-scale metabolomics experiments would concretely show a conserved function of Tgif across species and model systems.

These experiments are important because they would provide direct evidence and a functional consequence of Tgif-mediated regulation of metabolic genes and their outputs. Additionally, overlapping the results would provide more evidence that the acetyl-CoA and pyruvate metabolic gene regulation by Tgifs is independent of tumorigenesis, and this is a core function of Tgifs. These experiments additionally could be informative by showing differences in metabolite levels between normal and tumor

tissue in the presence or absence of Tgifs, thus showing the metabolic shifts due to Tgifs during tumorigenesis.

4.4.2 Identification of additional primary effects of Tgif knockout in our mouse model of CRC

We provide evidence that Tgifs directly regulate the expression of a few genes involved in acetyl-CoA and pyruvate metabolism. However, this is by no means the only set of genes Tgifs regulate, and it is possible Tgifs regulate other genes involved in both metabolism and other cellular processes. It would be very informative to identify other genes Tgifs directly regulate to obtain a fuller picture of their role in CRCs. Performing ChIP-seq for Tgifs in normal and tumor tissue with and without Tgif expression would be the experiment to identify genes directly regulated by Tgifs. ChIP-seq results would reveal a more complete picture of Tgif regulation, and provide evidence for novel functions of Tgif. Additionally, these results would show changes in gene regulation by Tgifs between normal and tumor tissue which could provide interesting insights into changes and differences in Tgif function based on tissue type. Finally, these results could also provide evidence for secondary effects of Tgifs. Further analysis of Tgif ChIP-seq data potentially could show enrichment for a class of genes whose repression could also participate in metabolic reprogramming or in some other cellular pathway.

4.4.3 Identification of secondary effects of Tgif knockout in our mouse model of CRC

While transcriptome analysis of *Apc* and *Apc;Tgif1;Tgif2* tumors showed large changes in expression of metabolic genes, including genes involved in purine/pyrimidine

biosynthesis and amino acid metabolism, only a few genes increased in Tgif mutant tumors. In addition, one of the biggest metabolic shifts in the *Apc* tumors was the shift to glycolysis, with the upregulation of Glut1. We did not see any evidence to suggest Tgifs directly regulate expression of *Slc2a1*, the gene transcribed to create Glut1, even though immunofluorescence analysis *Apc;Tgif1;Tgif2* tumors showed decreased expression of Glut1 compared to *Apc tumors*. This suggests most of the changes observed in the Tgif mutant tumors may be due to indirect effects of Tgif knockout. It remains possible Tgifs are not directly regulating the pathways previously mentioned but are instead driving a secondary oncogenic pathway which results in the upregulation of metabolic genes we see in *Apc* mutant tumors.

One way to identify potential secondary effects of Tgif knockout in tumors would be to identify if there are any transcription factors which could function as repressors enriched in the metabolic genes upregulated in *Apc* tumors. This could be done using oPossum, a tool that can be used to identify overrepresented transcription factor binding sites in large data sets (240–242). With the results of this analysis, it would be straightforward to identify if expression of this transcription factor(s) which acts as a repressor was low in *Apc* tumors in which Tgif expression is increased, and higher in *Apc;Tgif1;Tgif2* tumors. This would provide indirect evidence to suggest Tgifs regulate these repressors. Then, analyzing the promoter region of these genes for Tgif binding sites and subsequent ChIP-seq/ChIP-QPCR data would provide evidence Tgifs regulate these repressors, thus allowing transcription of the once repressed metabolic genes in *Apc* tumors. These results would be important because they would provide evidence that Tgifs regulate tumor metabolism both directly through repression of genes involved in acetyl-

CoA and pyruvate metabolism and indirectly through potential repression of transcription factors repressing metabolic genes upregulated in *Apc* tumors.

4.4.4 Generation of a new mouse model of CRC

Problems with our mouse model

There are some critical issues with the model we used and presented in this thesis. First and foremost, the vast majority of the tumors in these animals were present in the small intestine, with few tumors developing in the colon. Secondly, the genetic background of the mice can have effects on the data generated through strain specific modifiers (12); therefore, these studies need to be done on100% inbred animals in order to mitigate variations in tumor multiplicity and size due to genetic background. The need to keep mice inbred in order to mitigate effects of strain background does not faithfully represent the human disease. The tumors that do develop in the intestines do not progress beyond the adenoma stage (12). This, again, does not accurately model the human disease. Additionally, it means we are unable to identify the role Tgifs may have in more aggressive tumors. Finally, the majority of the phenotypic effect we observed in this model occurred in the small intestine, though we did see a reduction in tumor volume in the colon between *Apc* and *Apc;Tgif1;Tgif2* mutant tumors. Given these reasons, *Apc* mutant mice expressing *Villin*-Cre may not be the best model in which to study the function of Tgifs.

A new model

The ideal mouse model of CRC would have the same hallmark features as the human disease, including similar molecular mechanisms of tumorigenesis and disease

pathology, genetic heterogeneity within a sporadic tumor that specifically develops in the colon, high penetrance, a latency period, growth from an adenoma to a metastatic adenocarcinoma with metastases to lung, liver, and/or lymph nodes (243,244). Additional aspects of this ideal mouse model would include the ability for researchers to manipulate one gene or condition at a time within the context of the many mutations which arise during the course of tumorigenesis, and the ability to monitor tumors *in vivo* (244,245). It has certainly been very difficult to generate mouse models that satisfy all of these requirements, and many different models have been generated, many of which address a few, but not all of the requirements laid out above (12,246).

A recently published study developed a mouse model that addressed many of these aforementioned requirements, and it would be a useful model in which Tgif function could be studied at multiple stages of CRC, from adenoma to invasive adenocarcinoma to metastases. This mouse model has loxP sites flanking exon 14 of *Apc*, loxP sites flanking exons 2-10 of *Tp53*, and loxP sites flanking Tet-inducible *Kras*^{G12D} allele and was established within a *Villin*-Cre^{ERT2} background (245). *Villin*-Cre normally is expressed within the epithelium of both the small intestine and colon; to make expression colon-specific, a 4-OH-tamoxifen-ethanol colonic enema was used to drive the *Villin*-Cre^{ERT2}. The Tet-inducible *Kras*^{G12D} allele was expressed with administration of doxycycline in the drinking water. After a latency period of approximately six weeks, tumors at all stages from adenomas to metastatic adenocarcinomas were detected, and metastases were observed in the liver, lung, and lymph nodes.

Interestingly, metastases were only observed when the mutant *Kras* was expressed; however, when doxycycline was removed and mutant *Kras* expression

stopped, those animals did not have metastases, and it was observed tumors which were adenocarcinomas reverted to adenomas, strongly suggesting mutant *Kras* is required for metastasis (245). Whole-exome sequencing of these tumors indicated primary tumors were heterogenous for $Kras^{G12D}$ while metastases were homogenous for the mutant *Kras* allele, further providing evidence $Kras^{G12D}$ drives tumor progression to metastasis. Additionally, TGFβ signaling was shown to mediate Kras-induced invasion, through upregulation of nuclear pSmad2, and every mouse with metastases had at least one primary carcinoma which had invaded entirely through the colon, strongly suggesting that this invasion and extravasation exhibited by carcinomas is required for metastasis (245).

This mouse model, iKAP (245), is clearly a superior model to the *Villin*-Cre;Apc^{CKO} we used, and it has the potential to allow a researcher to understand more deeply and completely the function of Tgifs, or any gene, in CRC. While this model does develop a few tumors in the distal portion of the small intestine (in approximately 10% of animals), the majority of the tumors these animals develop are found in the colon (245). Additionally, Tgif function can be assessed in all stages of tumors (through doxycycline addition or subtraction) through RNA-seq or ChIP-seq, and these results can be compared to each other. For example, one could ask if the metabolic changes driven by Tgifs in an adenoma are the same as metabolic changes in an invasive cancer and in a distal metastatic site. The results of these metabolomics experiments would help validate our findings described here, and would additionally show the evolving role of Tgif regulation of metabolism within the context of tumor progression.

It remains possible that Tgifs may have different functions at different states of tumorigenesis – this has yet to have been tested. It may be that Tgifs participate in metabolic rewiring in adenomas and may have a different role in carcinomas. Additionally, as TGFβ, known to induce invasion and metastasis in cancers (73), appears to facilitate Kras-mediated invasion, it would be informative to study if Tgifs regulate EMT through its known role as an TGFβ repressor. This work would provide evidence for another function of Tgifs in tumor progression, independent of metabolic regulation. However, as previously discussed, Tgifs may promote EMT through downregulation of fatty acid synthesis, in a manner independent of TGFβ signaling. The results of these studies could have potential clinical and therapeutic relevance. Overall, the iKAP model of CRC appears to be robust and mimics the human disease, and it may prove to be a model in which it is easy to test the functions of Tgifs within CRC.

References

1. Siegel RL, Miller KD, Jemal A. Cancer Statistics , 2018. CA Cancer J Clin. 2018;00(00).
2. Cronin KA, Lake AJ, Scott S, Sherman RL, Noone AM, Howlader N, et al. Annual Report to the Nation on the Status of Cancer, part I: National cancer statistics. Cancer. 2018;124(13):2785–800.
3. Kuipers EJ, Grady WM, Lieberman D, Seufferlein T, Sung JJ, Boelens PG, et al. COLORECTAL CANCER. PMC [Internet]. 2016 [cited 2018 Sep 10]; Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4874655/pdf/nihms769726.pdf
4. Fearon EFR, Vogelstein B. A genetic model for colorectal tumorigenesis. Cell [Internet]. 1990 Jun 1 [cited 2014 Aug 30];61(5):759–67. Available from: http://www.ncbi.nlm.nih.gov/pubmed/2188735
5. Medema JP, Vermeulen L. Microenvironmental regulation of stem cells in intestinal homeostasis and cancer. Nature [Internet]. Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved.; 2011 Jun 16 [cited 2015 Apr 17];474(7351):318–26. Available from: http://www.nature.com/nature/journal/v474/n7351/pdf/nature10212.pdf
6. Vogelstein B, Papadopoulos N, Velculescu VE, Zhou S, Diaz LA, Kinzler KW. Cancer genome landscapes. Science [Internet]. 2013 Mar 29 [cited 2014 Jul 9];339(6127):1546–58. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23539594
7. Imada S, Murata Y, Kotani T, Hatano M, Sun C, Konno T, et al. Role of Src

family kinases in regulation of intestinal epithelial homeostasis. Mol Cell Biol [Internet]. American Society for Microbiology; 2016 Aug 22 [cited 2018 Jul 25];36(22):2811–23. Available from: http://www.ncbi.nlm.nih.gov/pubmed/27550814

8. Solanas G, Batlle E. Control of cell adhesion and compartmentalization in the intestinal epithelium. Experimental Cell Research. 2011.
9. Meineke FA, Potten CS, Loeffler M. Cell migration and organization in the intestinal crypt using a lattice-free model. Cell Prolif. 2001.
10. Sato T, Van Es JH, Snippert HJ, Stange DE, Vries RG, Van Den Born M, et al. Paneth cells constitute the niche for Lgr5 stem cells in intestinal crypts. 2011 [cited 2018 Sep 18]; Available from: https://www.nature.com/articles/nature09637.pdf
11. Clevers HC, Bevins CL. Paneth Cells: Maestros of the Small Intestinal Crypts. 2013 [cited 2018 Sep 18]; Available from: http://physiol.annualreviews.org
12. Johnson RL, Fleet JC. Animal models of colorectal cancer. Cancer Metastasis Rev [Internet]. Springer US; 2013 Jun 18 [cited 2018 Jul 25];32(1–2):39–61. Available from: http://link.springer.com/10.1007/s10555-012-9404-6
13. Xue Y, Johnson R, Desmet M, Snyder PW, Fleet JC. Generation of a transgenic mouse for colorectal cancer research with intestinal cre expression limited to the large intestine. Mol Cancer Res [Internet]. 2010 [cited 2018 Jul 25];8:1095–104. Available from: http://mcr.aacrjournals.org/
14. Madison BB, Dunbar L, Qiao XT, Braunstein K, Braunstein E, Gumucio DL. cis elements of the villin gene control expression in restricted domains of the vertical (crypt) and horizontal (duodenum, cecum) axes of the intestine. J Biol Chem. 2002

15. Barker N, Ridgway RA, Van Es JH, Van De Wetering M, Begthel H, Van Den Born M, et al. Crypt stem cells as the cells-of-origin of intestinal cancer. Nature [Internet]. 2009 [cited 2018 Sep 15];457. Available from: https://www.nature.com/articles/nature07602.pdf

16. Shen MM, Wang † X, Economides † K D, Walker ¶ D, Abate-Shen C. Progenitor Cells for the Prostate Epithelium: Roles in Development, Regeneration, and Cancer [Internet]. 2008 [cited 2018 Sep 16]. Available from: http://symposium.cshlp.org/content/73/529.full.pdf

17. Holland EC, Celestino J, Dai C, Schaefer L, Sawaya RE, Fuller GN. Combined activation of Ras and Akt in neural progenitors induces glioblastoma formation in mice. Nat Genet [Internet]. 2000 May [cited 2018 Sep 16];25(1):55–7. Available from: http://www.ncbi.nlm.nih.gov/pubmed/10802656

18. Visvader JE. Cell-of-origin and cancer stem-cell concepts are distinct. Nature [Internet]. 2011 [cited 2018 Jul 25];469. Available from: https://www.nature.com/articles/nature09781.pdf

19. Narayan S, Roy D. Role of APC and DNA mismatch repair genes in the development of colorectal cancers. Mol Cancer [Internet]. 2003 Dec 12 [cited 2018 Jul 26];2(41):1–15. Available from: http://www.ncbi.nlm.nih.gov/pubmed/14672538

20. Miller JR, M O O N RT. Signal transducti through [-catenin and speclflcatlon°o cell fate during embryogenesis [Internet]. 1996 [cited 2018 Sep 18]. Available from: http://genesdev.cshlp.org/content/10/20/2527.full.pdf

21. Dale TC. Signal transduction by the Wnt family of ligands [Internet]. Biochem. J.

1998 [cited 2018 Sep 18]. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1219034/pdf/9425102.pdf

22. Clevers H. Wnt/β-Catenin Signaling in Development and Disease. Cell [Internet]. 2006 [cited 2018 Sep 18];127(3):469–80. Available from: https://www.cell.com/action/showPdf?pii=S0092-8674%2806%2901344-4

23. Barker N, Van Es JH, Kuipers J, Kujala P, Van Den Born M, Cozijnsen M, et al. Identification of stem cells in small intestine and colon by marker gene Lgr5. Nature [Internet]. 2007 [cited 2018 Oct 23];449(7165):1003–7. Available from: https://www.hubrecht.eu/app/uploads/2017/11/nature06196.pdf

24. Smits R, Kielman MF, Breukel C, Zurcher C, Neufeld K, Jagmohan-Changur S, et al. Apc1638T: a mouse model delineating critical domains of the adenomatous polyposis coli protein involved in tumorigenesis and development. Genes Dev [Internet]. Cold Spring Harbor Laboratory Press; 1999 May 15 [cited 2018 Sep 19];13(10):1309–21. Available from: http://www.ncbi.nlm.nih.gov/pubmed/10346819

25. Polakis P. Wnt signaling and cancer [Internet]. 2000 [cited 2018 Sep 18]. Available from: www.genesdev.org

26. Laken SJ, Papadopoulos N, Petersen GM, Gruber SB, Hamilton SR, Giardiello FM, et al. Analysis of masked mutations in familial adenomatous polyposis. Proc Natl Acad Sci U S A [Internet]. National Academy of Sciences; 1999 Mar 2 [cited 2018 Sep 25];96(5):2322–6. Available from: http://www.ncbi.nlm.nih.gov/pubmed/10051640

27. Gumbiner BM. Regulation of Cadherin Adhesive Activity. J Cell Biol [Internet].

2000 [cited 2018 Sep 18];148(3):399–403. Available from: http://www.jcb.org

28. Ben-Ze'ev A, Geiger B. Differential molecular interactions of beta-catenin and plakoglobin in adhesion, signaling and cancer. Curr Opin Cell Biol [Internet]. 1998 Oct [cited 2018 Sep 18];10(5):629–39. Available from: http://www.ncbi.nlm.nih.gov/pubmed/9818174

29. Moss SF, Liu TC, Petrotos A, Hsu TM, Gold LI, Holt PR. Inward growth of colonic adenomatous polyps. Gastroenterology [Internet]. 1996 Dec [cited 2018 Sep 18];111(6):1425–32. Available from: http://www.ncbi.nlm.nih.gov/pubmed/8942720

30. Fodde R, Kuipers J, Rosenberg C, Smits R, Kielman M, Gaspar C, et al. Mutations in the APC tumour suppressor gene cause chromosomal instability. Nat Cell Biol [Internet]. 2001 Apr 1 [cited 2015 Aug 17];3(4):433–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/11283620

31. Kaplan KB, Burds AA, Swedlow JR, Bekir SS, Sorger PK, Näthke IS. A role for the Adenomatous Polyposis Coli protein in chromosome segregation. Nat Cell Biol [Internet]. 2001 Apr 1 [cited 2018 Sep 18];3(4):429–32. Available from: http://www.nature.com/articles/ncb0401_429

32. Rao C V, Yang Y-M, Swamy M V, Liu T, Fang Y, Mahmood R, et al. Colonic tumorigenesis in BubR1+/-ApcMin/+ compound mutant mice is linked to premature separation of sister chromatids and enhanced genomic instability. Proc Natl Acad Sci U S A [Internet]. 2005 Mar 22 [cited 2015 Sep 12];102(12):4365–70. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=555497&tool=pmcentr

ez&rendertype=abstract

33. García MA, Carrasco E, Aguilera M, Alvarez P, Rivas C, Campos JM, et al. The chemotherapeutic drug 5-fluorouracil promotes PKR-mediated apoptosis in a p53-independent manner in colon and breast cancer cells. PLoS One [Internet]. Public Library of Science; 2011 Jan 24 [cited 2015 Sep 30];6(8):e23887. Available from: http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0023887

34. Treatment of colon cancer by stage [Internet]. [cited 2015 Sep 30]. Available from: http://www.cancer.org/cancer/colonandrectumcancer/detailedguide/colorectal-cancer-treating-by-stage-colon

35. Cheng Y, Yang H, Chen G, Zhang Z. Molecularly targeted drugs for metastatic colorectal cancer. Drug Des Devel Ther [Internet]. 2013 Jan [cited 2015 Sep 30];7:1315–22. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3817019&tool=pmcentrez&rendertype=abstract

36. Edwards MS, Chadda SD, Zhao Z, Barber BL, Sykes DP. A systematic review of treatment guidelines for metastatic colorectal cancer. Colorectal Dis [Internet]. 2012 Feb [cited 2015 Jul 27];14(2):e31-47. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3562494&tool=pmcentrez&rendertype=abstract

37. Newmark HL, Lipkin M, Maheshwari N. Colonic hyperplasia and hyperproliferation induced by a nutritional stress diet with four components of Western-style diet. J Natl Cancer Inst [Internet]. 1990 Mar 21 [cited 2018 Sep 19];82(6):491–6. Available from: http://www.ncbi.nlm.nih.gov/pubmed/2313721

38. Magalhães B, Peleteiro B, Lunet N. Dietary patterns and colorectal cancer: Systematic review and meta-analysis. Eur J Cancer Prev. 2012;21(1):15–23.

39. Newmark HL, Yang K, Kurihara N, Fan K, HAugenlicht L, Lipkin M, et al. Western-style diet-induced colonic tumors and their modulation by calcium and vitamin D in C57Bl/6 mice: a preclinical model for human sporadic colon cancer. Carcinogenesis [Internet]. 2009 [cited 2018 Aug 14];30(1):88–92. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2722141/pdf/bgn229.pdf

40. Risio M, Lipkin M, Newmark H, Yang K, Rossini FP, Steele VE, et al. Apoptosis, cell replication, and Western-style diet-induced tumorigenesis in mouse colon. Cancer Res [Internet]. 1996 Nov 1 [cited 2018 Sep 19];56(21):4910–6. Available from: http://www.ncbi.nlm.nih.gov/pubmed/8895743

41. Bissahoyo A, Pearsall RS, Hanlon K, Amann V, Hicks D, Godfrey VL, et al. Azoxymethane is a genetic background-dependent colorectal tumor initiator and promoter in mice: effects of dose, route, and diet. Toxicol Sci [Internet]. 2005 Dec 1 [cited 2018 Sep 19];88(2):340–5. Available from: http://academic.oup.com/toxsci/article/88/2/340/1691186/Azoxymethane-Is-a-Genetic-BackgroundDependent

42. Yamada Y, Yoshimi N, Hirose Y, Kawabata K, Matsunaga K, Shimizu M, et al. Frequent beta-catenin gene mutations and accumulations of the protein in the putative preneoplastic lesions lacking macroscopic aberrant crypt foci appearance, in rat colon carcinogenesis. Cancer Res [Internet]. 2000 Jul 1 [cited 2018 Sep 19];60(13):3323–7. Available from: http://www.ncbi.nlm.nih.gov/pubmed/10910031

43. Miyaki M, Iijima T, Kimura J, Yasuno M, Mori T, Hayashi Y, et al. Frequent mutation of beta-catenin and APC genes in primary colorectal tumors from patients with hereditary nonpolyposis colorectal cancer. Cancer Res [Internet]. 1999 Sep 15 [cited 2018 Sep 19];59(18):4506–9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/10493496

44. Perše M, Cerar A. Morphological and Molecular Alterations in 1,2 Dimethylhydrazine and Azoxymethane Induced Colon Carcinogenesis in Rats. J Biomed Biotechnol [Internet]. Hindawi Publishing Corporation; 2011 [cited 2018 Aug 24];2011:14. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3018655/pdf/JBB2011-473964.pdf

45. Glauert HP, Bennink MR. Metabolism of 1,2-dimethylhydrazine by cultured rat colon epithelial cells. Nutr Cancer [Internet]. 1983 Jan 4 [cited 2018 Sep 19];5(2):78–86. Available from: http://www.tandfonline.com/doi/abs/10.1080/01635588309513782

46. Oravec CT, Jones CA, Huberman E. Activation of the colon carcinogen 1,2-dimethylhydrazine in a rat colon cell-mediated mutagenesis assay. Cancer Res [Internet]. 1986 Oct [cited 2018 Sep 19];46(10):5068–71. Available from: http://www.ncbi.nlm.nih.gov/pubmed/3756864

47. Rosenberg DW, Giardina C, Tanaka T. Mousemodels for the study of colon carcinogenesis. Carcinogenesis [Internet]. 2009 [cited 2018 Sep 19];30(2):183–96. Available from: https://academic.oup.com/carcin/article-lookup/doi/10.1093/carcin/bgn267

48. Shirai T, Nakanowatari J, Kurata Y, Fukushima S, Ito N. Different dose-response relationships in the induction of different types of colonic tumors in Wistar rats by 1,2-dimethylhydrazine. Gan [Internet]. 1983 Feb [cited 2018 Sep 19];74(1):21–7. Available from: http://www.ncbi.nlm.nih.gov/pubmed/6301917

49. Večerić Ž, Cerar A. Comparison of Wistar vs . Fischer rat in the incidence of 1 , 2-dimethylhydrazine induced intestinal tumors. 2004;38(3):227–34.

50. Perše M, Cerar A. The dimethylhydrazine induced colorectal tumours in rat - experimental colorectal carcinogenesis. Radiol Oncol. 2005;39(1):61–70.

51. Su LK, Kinzler KW, Vogelstein B, Preisinger AC, Moser AR, Luongo C, et al. Multiple intestinal neoplasia caused by a mutation in the murine homolog of the APC gene. Science [Internet]. 1992 May 1 [cited 2015 Nov 16];256(5057):668–70. Available from: http://www.ncbi.nlm.nih.gov/pubmed/1350108

52. Moser AR, Pitot HC, Dove WF. A dominant mutation that predisposes to multiple intestinal neoplasia in the mouse. Science (80-). 1990.

53. Luongo C, Moser AR, Gledhill S, Dove WF. Loss of Apc+ in intestinal adenomas from Min mice. Cancer Res [Internet]. 1994 Nov 15 [cited 2018 Sep 19];54(22):5947–52. Available from: http://www.ncbi.nlm.nih.gov/pubmed/7954427

54. McCart AE, Vickaryous NK, Silver A. Apc mice: Models, modifiers and mutants. Pathol Res Pract. 2008.

55. Silverman KA, Koratkar R, Siracusa LD, Buchberg AM. Identification of the modifier of Min 2 (Mom2) locus, a new mutation that influences Apc-induced intestinal neoplasia. Genome Res. 2002.

56. Moser AR, Dove WF, Roth KA, Gordon JI. The Min (multiple intestinal neoplasia) mutation: Its effect on gut epithelial cell differentiation and interaction with a modifier system. J Cell Biol. 1992.

57. Jasperson KW, Tuohy TM, Neklason DW, Burt RW. Hereditary and Familial Colon Cancer. Gastroenterology. 2010.

58. Saam JR, Gordon JI. Inducible gene knockouts in the small intestinal and colonic epithelium. J Biol Chem. 1999.

59. Simon TC, Roth KA, Gordon JI. Use of transgenic mice to map cis-acting elements in the liver fatty acid- binding protein gene (Fabpl) that regulate its cell lineage-specific, differentiation-dependent, and spatial patterns of expression in the gut epithelium and in the liver acinus. J Biol Chem. 1993.

60. Quaroni A, Calnek D, Quaroni E, Chandler JS. Keratin expression in rat intestinal crypt and villus cells. Analysis with a panel of monoclonal antibodies. J Biol Chem. 1991.

61. Hinoi T, Akyol A, Theisen BK, Ferguson DO, Greenson JK, Williams BO, et al. Mouse model of colonic adenoma-carcinoma progression based on somatic Apc inactivation. Cancer Res [Internet]. 2007 Oct 15 [cited 2015 Sep 12];67(20):9721–30. Available from: www.aacrjournals.org

62. El Marjou F, Janssen KP, Chang BHJ, Li M, Hindie V, Chan L, et al. Tissue-specific and inducible Cre-mediated recombination in the gut epithelium. Genesis. 2004.

63. Jackson EL, Willis N, Mercer K, Bronson RT, Crowley D, Montoya R, et al. Analysis of lung tumor initiation and progression using conditional expression of

oncogenic K-ras. Genes Dev. 2001.

64. Harada N, Tamai Y, Ishikawa T-O, Sauer B, Takaku K, Oshima M, et al. Intestinal polyposis in mice with a dominant stable mutation of the β-catenin gene [Internet]. The EMBO Journal. 1999 [cited 2018 Jul 25]. Available from: http://emboj.embopress.org/content/embojnl/18/21/5931.full.pdf

65. Valentin-Vega YA, Okano H, Lozano G. The intestinal epithelium compensates for p53-mediated cell death and guarantees organismal survival. Cell Death Differ. 2008.

66. Chytil A, Magnuson MA, Wright CVE, Moses HL. Conditional inactivation of the TGF-beta type II receptor using Cre:Lox. Genesis [Internet]. 2002 Feb [cited 2018 Sep 19];32(2):73–5. Available from: http://www.ncbi.nlm.nih.gov/pubmed/11857781

67. Colnot S, Niwa-Kawakita M, Hamard G, Godard C, Le Plenier S, Houbron C, et al. Colorectal cancers in a new mouse model of familial adenomatous polyposis: influence of genetic and environmental modifiers. Lab Invest [Internet]. 2004 Dec [cited 2015 Sep 12];84(12):1619–30. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15502862

68. Shibata H, Toyama K, Shioya H, Ito M, Hirota M, Hasegawa S, et al. Rapid colorectal adenoma formation initiated by conditional targeting of the APC gene. Science (80-). 1997.

69. Kuraguchi M, Wang X-P, Bronson RT, Rothenberg R, Yaw Ohene-Baah N, Lund JJ, et al. Adenomatous Polyposis Coli (APC) Is Required for Normal Development of Skin and Thymus [Internet]. [cited 2018 Aug 28]. Available from:

www.plosgenetics.org

70. Robanus-Maandag EC, Koelink PJ, Breukel C, Salvatori DCF, Jagmohan-Changur SC, Bosch CAJ, et al. A new conditional Apc-mutant mouse model for colorectal cancer. Carcinogenesis [Internet]. 2010 [cited 2018 Oct 23]; Available from: https://watermark.silverchair.com/bgq046.pdf?token=AQECAHi208BE49Ooan9kkhW_Ercy7Dm3ZL_9Cf3qfKAc485ysgAAAjAwggIsBgkqhkiG9w0BBwagggIdMIICGQIBADCCAhIGCSqGSIb3DQEHATAeBglghkgBZQMEAS4wEQQMGhfERpTOwfCpnLrQAgEQgIIB49A3lyQl2XThO2Dy3xkATLNDhJkxnOTDnz_OJGuM1ODAznTl

71. Massagué J, Gomis RR. The logic of TGFβ signaling. FEBS Lett [Internet]. Wiley-Blackwell; 2006 May 22 [cited 2018 Jul 25];580(12):2811–20. Available from: http://www.ncbi.nlm.nih.gov/pubmed/16678165

72. Massagué J. TGFβ signalling in context. Nat Rev Mol Cell Biol [Internet]. 2012 Oct [cited 2014 Jul 11];13(10):616–30. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4027049&tool=pmcentrez&rendertype=abstract

73. Massagué J. TGFbeta in Cancer. Cell [Internet]. 2008 Jul 25 [cited 2015 Feb 5];134(2):215–30. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3512574&tool=pmcentrez&rendertype=abstract

74. Akhurst RJ, Hata A. Targeting the TGFβ signalling pathway in disease. Nat Rev Drug Discov [Internet]. 2012 Oct [cited 2014 Jul 15];11(10):790–811. Available from:

http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3520610&tool=pmcentrez&rendertype=abstract

75. Calon A, Espinet E, Palomo-Ponce S, Tauriello DVF, Iglesias M, Céspedes MV, et al. Dependency of Colorectal Cancer on a TGF-β-Driven Program in Stromal Cells for Metastasis Initiation. Cancer Cell [Internet]. Cell Press; 2012 Nov 13 [cited 2018 Jul 25];22(5):571–84. Available from: https://www.sciencedirect.com/science/article/pii/S1535610812003558?via%3Dihub

76. Shi Y, Massagué J. Mechanisms of TGF-beta signaling from cell membrane to the nucleus. Cell [Internet]. 2003 Jun 13 [cited 2015 May 18];113(6):685–700. Available from: http://www.ncbi.nlm.nih.gov/pubmed/12809600

77. Tsukazaki T, Chiang TA, Davison AF, Attisano L, Wrana JL. SARA, a FYVE domain protein that recruits Smad2 to the TGFβ receptor. Cell. 1998;

78. Massagué J, Seoane J, Wotton D. Smad transcription factors. Genes Dev [Internet]. Cold Spring Harbor Laboratory Press; 2005 Dec 1 [cited 2015 Feb 25];19(23):2783–810. Available from: http://www.ncbi.nlm.nih.gov/pubmed/16322555

79. Liu X, Sun Y, Weinberg RA, Lodish HF. Ski/Sno and TGF-beta signaling. Cytokine Growth Factor Rev [Internet]. 2001 Mar [cited 2018 Sep 19];12(1):1–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/11312113

80. Wang W, Mariani F V, Harland RM, Luo K. Ski represses bone morphogenic protein signaling in Xenopus and mammalian cells. Proc Natl Acad Sci USA [Internet]. 2000 Dec 19 [cited 2018 Sep 19];97(26):14394–9. Available from:

http://www.pnas.org/cgi/doi/10.1073/pnas.97.26.14394

81. Wotton D, Lo RS, Lee S, Massagué J, Massague J. A Smad transcriptional corepressor. Cell [Internet]. 1999 Apr 2 [cited 2015 Sep 21];97(1):29–39. Available from: https://ac.els-cdn.com/S0092867400807126/1-s2.0-S0092867400807126-main.pdf?_tid=0baac737-1ac3-4561-b0ff-fc59ce52b1bd&acdnat=1532621854_afe940969d30f682cccf0d80af385e6e

82. Wotton D, Lo RS, Swaby L-AC, Massagué J. Multiple Modes of Repression by the Smad Transcriptional Corepressor TGIF* [Internet]. 1999 [cited 2018 Jul 26]. Available from: http://www.jbc.org/

83. Wotton D, Knoepfler PS, Laherty CD, Eisenman RN, Massagué J. The Smad transcriptional corepressor TGIF recruits mSin3. Cell Growth Differ [Internet]. 2001 Sep [cited 2015 Sep 12];12(9):457–63. Available from: http://www.ncbi.nlm.nih.gov/pubmed/11571228

84. Gomis RR, Alarcón C, Nadal C, Van Poznak C, Massagué J. C/EBPbeta at the core of the TGFbeta cytostatic response and its evasion in metastatic breast cancer cells. Cancer Cell [Internet]. 2006 Sep [cited 2018 Sep 19];10(3):203–14. Available from: http://linkinghub.elsevier.com/retrieve/pii/S1535610806002479

85. Chen C-R, Kang Y, Siegel PM, Massagué J. E2F4/5 and p107 as Smad cofactors linking the TGFbeta receptor to c-myc repression. Cell [Internet]. 2002 Jul 12 [cited 2018 Sep 19];110(1):19–32. Available from: http://www.ncbi.nlm.nih.gov/pubmed/12150994

86. Scandura JM, Boccuni P, Massague J, Nimer SD. Transforming growth factor beta-induced cell cycle arrest of human hematopoietic cells requires p57KIP2 up-

regulation. Proc Natl Acad Sci U S A. 2004.

87. Ruzinova MB, Benezra R. Id proteins in development, cell cycle and cancer. Trends Cell Biol [Internet]. 2003 [cited 2018 Sep 10];13(8):410. Available from: http://ticb.trends.com0962-8924/03/$-seefrontmatterq

88. Ying QL, Nichols J, Chambers I SA. BMP Induction of Id Proteins Suppresses Differentiation and Sustains Embryonic Stem Cell Self-Renewal in Collaboration with STAT3. Cell [Internet]. 2003 [cited 2018 Nov 29]; Available from: https://ac.els-cdn.com/S009286740300847X/1-s2.0-S009286740300847X-main.pdf?_tid=221116cc-920c-4e87-b383-f0a557826b33&acdnat=1543469787_4a58e1ec756c51fcd4bd350c863e4059

89. Kang Y, Chen CR, Massagué J. A self-enabling TGFβ response coupled to stress signaling: Smad engages stress response factor ATF3 for Id1 repression in epithelial cells. Mol Cell. 2003.

90. Tang B, Yoo N, Vu M, Mamura M, Nam J-S, Ooshima A, et al. Transforming growth factor-beta can suppress tumorigenesis through effects on the putative cancer stem or early progenitor cell and committed progeny in a breast cancer xenograft model. Cancer Res [Internet]. 2007 Sep 15 [cited 2018 Sep 21];67(18):8643–52. Available from: http://cancerres.aacrjournals.org/cgi/doi/10.1158/0008-5472.CAN-07-0982

91. Mani SA, Guo W, Liao MJ, Eaton EN, Ayyanan A, Zhou AY, et al. The Epithelial-Mesenchymal Transition Generates Cells with Properties of Stem Cells. Cell. 2008.

92. Thuault S, Valcourt U, Petersen M, Manfioletti G, Heldin C-HH, Moustakas A.

Transforming growth factor-beta employs HMGA2 to elicit epithelial-mesenchymal transition. J Cell Biol [Internet]. 2006 Jul 17 [cited 2015 Sep 16];174(2):175–83. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2064178&tool=pmcentrez&rendertype=abstract

93. Derynck R, Akhurst RJ. Differentiation plasticity regulated by TGF-beta family proteins in development and disease. Nat Cell Biol. 2007.

94. Shipitsin M, Campbell LL, Argani P, Weremowicz S, Bloushtain-Qimron N, Yao J, et al. Cancer Cell Molecular Definition of Breast Tumor Heterogeneity. [cited 2018 Sep 11]; Available from: https://www.cell.com/action/showPdf?pii=S1535-6108%2807%2900029-3

95. Ozdamer B, Bose R, Barrios-Rodiles M, Wang H-R, Zhang Y, Wrana JL. Regulation of the Polarity Protein Par6 by TGFb Receptors Controls Epithelial Cell Plasticity. Science (80-) [Internet]. 2005 [cited 2018 Aug 2];307(5715):1603–9. Available from: www.sciencemag.orgsciencevol307http://science.sciencemag.org/

96. Viloria-Petit AM, David L, Jia JY, Erdemir T, Bane AL, Pinnaduwage D, et al. A role for the TGFbeta-Par6 polarity pathway in breast cancer progression. Proc Natl Acad Sci U S A [Internet]. 2009 Aug 18 [cited 2015 Sep 21];106(33):14028–33. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2729014&tool=pmcentrez&rendertype=abstract

97. Bertolino E, Reimund B, Wildt-Perinic D, Clerc RG. A novel homeobox protein

which recognizes a TGT core and functionally interferes with a retinoid-responsive motif. J Biol Chem [Internet]. 1995 Dec 29 [cited 2015 Sep 21];270(52):31178–88. Available from: http://www.ncbi.nlm.nih.gov/pubmed/8537382

98. Bürglin TR. Analysis of TALE superclass homeobox genes (MEIS, PBC, KNOX, Iroquois, TGIF) reveals a novel domain conserved between plants and animals. Nucleic Acids Res [Internet]. 1997 Nov 1 [cited 2015 Sep 21];25(21):4173–80. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=147054&tool=pmcentrez&rendertype=abstract

99. Mukherjee K, Bürglin TR. Comprehensive analysis of animal TALE homeobox genes: new conserved motifs and cases of accelerated evolution. J Mol Evol [Internet]. 2007 Aug 30 [cited 2018 Sep 22];65(2):137–53. Available from: http://link.springer.com/10.1007/s00239-006-0023-0

100. Wotton D, Taniguchi K. Functions of TGIF homeodomain proteins and their roles in normal brain development and holoprosencephaly. Am J Med Genet Part C Semin Med Genet [Internet]. Wiley Online Library; 2018;178(2):128–39. Available from: http:https://doi.org/10.1002/ajmg.c.31612

101. Gehring WJ, Affolter M, Burglin T. Homeodomain Proteins. Annu Rev Biochem [Internet]. 1994 Jun [cited 2018 Sep 22];63(1):487–526. Available from: http://www.ncbi.nlm.nih.gov/pubmed/7979246

102. Gehring WJ, Qian YQ, Billeter M, Furukubo-Tokunaga K, Schier AF, Resendez-Perez D, et al. Homeodomain-DNA recognition. Cell [Internet]. 1994 Jul 29 [cited 2018 Sep 22];78(2):211–23. Available from:

http://www.ncbi.nlm.nih.gov/pubmed/8044836

103. Bürglin TR, Affolter M. Homeodomain proteins: an update. Chromosoma [Internet]. 2016 Jun 13 [cited 2018 Sep 22];125(3):497–521. Available from: http://link.springer.com/10.1007/s00412-015-0543-8

104. Passner JM, Ryoo HD, Shen L, Mann RS, Aggarwal AK. Structure of a DNA-bound Ultrabithorax-Extradenticle homeodomain complex. Nature [Internet]. 1999 Feb 25 [cited 2018 Sep 22];397(6721):714–9. Available from: http://www.nature.com/articles/17833

105. Piper DE, Batchelor AH, Chang CP, Cleary ML, Wolberger C. Structure of a HoxB1-Pbx1 heterodimer bound to DNA: role of the hexapeptide and a fourth homeodomain helix in complex formation. Cell [Internet]. 1999 Feb 19 [cited 2018 Sep 22];96(4):587–97. Available from: http://www.ncbi.nlm.nih.gov/pubmed/10052460

106. Taniguchi K, Anderson AE, Sutherland AE, Wotton D. Loss of Tgif function causes holoprosencephaly by disrupting the SHH signaling pathway. PLoS Genet [Internet]. 2012 Jan [cited 2013 Nov 20];8(2):e1002524. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3285584&tool=pmcentrez&rendertype=abstract

107. Melhuish TA, Wotton D. The interaction of the carboxyl terminus-binding protein with the Smad corepressor TGIF is disrupted by a holoprosencephaly mutation in TGIF. J Biol Chem [Internet]. JBC Papers in Press; 2000 Dec 15 [cited 2015 Sep 12];275(50):39762–6. Available from: http://www.jbc.org/

108. Melhuish TA, Gallo CM, Wotton D. TGIF2 interacts with histone deacetylase 1

and represses transcription. J Biol Chem [Internet]. 2001 Aug 24 [cited 2015 Sep 12];276(34):32109–14. Available from: http://www.jbc.org/

109. Lee BK, Shen W, Lee J, Rhee C, Chung H, Kim KY, et al. Tgif1 Counterbalances the Activity of Core Pluripotency Factors in Mouse Embryonic Stem Cells. Cell Rep [Internet]. The Authors; 2015;13(1):52–60. Available from: http://dx.doi.org/10.1016/j.celrep.2015.08.067

110. Zerlanko BJ, Bartholin L, Melhuish TA, Wotton D. Premature senescence and increased TGFβ signaling in the absence of Tgif1. PLoS One [Internet]. 2012 Jan [cited 2015 Sep 12];7(4):e35460. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3325954&tool=pmcentrez&rendertype=abstract

111. Anderson AE, Taniguchi K, Hao Y, Melhuish TA, Shah A, Turner SD, et al. Tgif1 and Tgif2 Repress Expression of the RabGAP Evi5l. Mol Cell Biol [Internet]. 2017 [cited 2018 Sep 23];37(5). Available from: http://mcb.asm.org/

112. Guca E, Suñol D, Suñol S, Ruiz L, Konkol A, Cordero J, et al. TGIF1 homeodomain interacts with Smad MH1 domain and represses TGF-signaling. Nucleic Acids Res [Internet]. 2018 [cited 2018 Sep 14];(1). Available from: https://academic.oup.com/nar/advance-article-abstract/doi/10.1093/nar/gky680/5061025

113. Bartholin L, Powers SE, Melhuish TA, Lasse S, Weinstein M, Wotton D. TGIF inhibits retinoid signaling. Mol Cell Biol [Internet]. 2006 Feb [cited 2015 Sep 12];26(3):990–1001. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1347013&tool=pmcen

trez&rendertype=abstract

114. Melhuish TA, ¤☯ KT, Wotton D. Tgif1 and Tgif2 Regulate Axial Patterning in Mouse. 2016 [cited 2018 Sep 23]; Available from: https://journals.plos.org/plosone/article/file?id=10.1371/journal.pone.0155837&type=printable

115. Solomon BD, Gropman A, Muenke M. Holoprosencephaly Overview [Internet]. GeneReviews®. 1993 [cited 2018 Sep 23]. Available from: http://www.ncbi.nlm.nih.gov/pubmed/20301702

116. Jin J-Z, Gu S, McKinney P, Ding J. Expression and functional analysis of Tgif during mouse midline development. Dev Dyn [Internet]. 2006 Feb [cited 2015 Sep 21];235(2):547–53. Available from: http://www.ncbi.nlm.nih.gov/pubmed/16284942

117. Mar L, Hoodless PA. Embryonic fibroblasts from mice lacking Tgif were defective in cell cycling. Mol Cell Biol [Internet]. 2006 Jun 1 [cited 2018 Sep 23];26(11):4302–10. Available from: http://mcb.asm.org/cgi/doi/10.1128/MCB.02156-05

118. Shen J, Walsh CA. Targeted disruption of Tgif, the mouse ortholog of a human holoprosencephaly gene, does not result in holoprosencephaly in mice. Mol Cell Biol [Internet]. 2005 May 1 [cited 2018 Sep 23];25(9):3639–47. Available from: http://mcb.asm.org/cgi/doi/10.1128/MCB.25.9.3639-3647.2005

119. Bartholin L, Melhuish TA, Powers SE, Goddard-Léon S, Treilleux I, Sutherland AE, et al. Maternal Tgif is required for vascularization of the embryonic placenta. 2008.

120. Tateossian H, Morse S, Parker A, Mburu P, Warr N, Acevedo-Arozena A, et al. Otitis media in the Tgif knockout mouse implicates TGFβ signalling in chronic middle ear inflammatory disease. Hum Mol Genet [Internet]. 2013 Jul 1 [cited 2018 Sep 23];22(13):2553–65. Available from: https://academic.oup.com/hmg/article-lookup/doi/10.1093/hmg/ddt103

121. Powers SE, Taniguchi K, Yen W, Melhuish T a, Shen J, Walsh C a, et al. Tgif1 and Tgif2 regulate Nodal signaling and are required for gastrulation. Development [Internet]. 2010 Jan [cited 2013 Nov 20];137(2):249–59. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2799159&tool=pmcentrez&rendertype=abstract

122. Hayashi S, Lewis P, Pevny L, McMahon AP. Efficient gene modulation in mouse epiblast using a Sox2Cre transgenic mouse strain. Mech Dev [Internet]. 2002 Dec [cited 2018 Sep 23];119 Suppl 1:S97–101. Available from: http://www.ncbi.nlm.nih.gov/pubmed/14516668

123. Taniguchi K, Anderson AE, Melhuish TA, Carlton AL, Manukyan A, Sutherland AE, et al. Genetic and Molecular Analyses indicate independent effects of TGIFs on Nodal and Gli3 in neural tube patterning. Eur J Hum Genet [Internet]. 2017 [cited 2018 Sep 23];25:208–15. Available from: www.informatics.jax.org/

124. Wang Y, Wang H, Gao H, Xu B, Zhai W, Li J, et al. Elevated expression of TGIF is involved in lung carcinogenesis. Tumour Biol [Internet]. 2015 Jun 20 [cited 2015 Sep 11]; Available from: http://www.ncbi.nlm.nih.gov/pubmed/26091794

125. Nakakuki K, Imoto I, Pimkhaokham A, Fukuda Y, Shimada Y, Imamura M, et al. Novel targets for the 18p11.3 amplification frequently observed in esophageal

squamous cell carcinomas. Carcinogenesis [Internet]. 2002 Jan [cited 2018 Sep 23];23(1):19–24. Available from: http://www.ncbi.nlm.nih.gov/pubmed/11756219

126. Imoto I, Pimkhaokham A, Watanabe T, Saito-Ohara F, Soeda E, Inazawa J. Amplification and Overexpression of TGIF2, a Novel Homeobox Gene of the TALE Superclass, in Ovarian Cancer Cell Lines. Biochem Biophys Res Commun [Internet]. 2000 Sep 16 [cited 2018 Sep 23];276(1):264–70. Available from: http://www.ncbi.nlm.nih.gov/pubmed/11006116

127. Willer A, Jakobsen JS, Ohlsson E, Rapin N, Waage J, Billing M, et al. TGIF1 is a negative regulator of MLL-rearranged acute myeloid leukemia. Leukemia [Internet]. 2014 [cited 2018 Jul 31];29:1018–31. Available from: www.nature.com/leu

128. Zhang M-Z, Ferrigno O, Wang Z, Ohnishi M, Prunier C, Levy L, et al. TGIF Governs a Feed-Forward Network that Empowers Wnt Signaling to Drive Mammary Tumorigenesis. Cancer Cell [Internet]. 2015 Apr 13 [cited 2015 Jul 6];27(4):547–60. Available from: http://dx.doi.org/10.1016/j.ccell.2015.03.002

129. Wang J, Qi Z, Li Y, Zhao H, Chen Y, Fu W. TGFb induced factor homeobox 1 promotes colorectal cancer development through activating Wnt/B-catenin signaling. Oncotarget [Internet]. 2017 [cited 2018 Jul 31];8(41):70214–25. Available from: file:///C:/Users/Anant/Downloads/19603-282885-4-PB.pdf

130. Warburg O. über den Stoffwechsel der Carcinomzelle. Klin Wochenschr [Internet]. Springer-Verlag; 1925 Mar [cited 2018 Sep 23];4(12):534–6. Available from: http://link.springer.com/10.1007/BF01726151

131. Warburg O, Wind F, Negelein E. THE METABOLISM OF TUMORS IN THE

BODY. J Gen Physiol [Internet]. Rockefeller University Press; 1927 Mar 7 [cited 2018 Sep 23];8(6):519–30. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19872213

132. Almuhaideb A, Papathanasiou N, Bomanji J. 18F-FDG PET/CT imaging in oncology. Ann Saudi Med [Internet]. King Faisal Specialist Hospital and Research Centre; 2011 [cited 2018 Sep 23];31(1):3–13. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21245592

133. Thompson CB. Rethinking the regulation of cellular metabolism. Cold Spring Harb Symp Quant Biol [Internet]. Cold Spring Harbor Laboratory Press; 2011 Jan 1 [cited 2018 Sep 23];76:23–9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22429931

134. Lindsten T, Golden JA, Zong W-X, Minarcik J, Harris MH, Thompson CB. The proapoptotic activities of Bax and Bak limit the size of the neural stem cell pool. J Neurosci [Internet]. 2003 Dec 3 [cited 2018 Sep 23];23(35):11112–9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/14657169

135. Rathmell JC, Heiden MGV, Harris MH, Frauwirth KA, Thompson CB. In the Absence of Extrinsic Signals, Nutrient Utilization by Lymphocytes Is Insufficient to Maintain Either Cell Size or Viability. Mol Cell [Internet]. Cell Press; 2000 Sep 1 [cited 2018 Sep 23];6(3):683–92. Available from: https://www.sciencedirect.com/science/article/pii/S1097276500000666

136. Rathmell JC, Fox CJ, Plas DR, Hammerman PS, Cinalli RM, Thompson CB. Akt-Directed Glucose Metabolism Can Prevent Bax Conformation Change and Promote Growth Factor-Independent Survival. Mol Cell Biol [Internet]. 2003

[cited 2018 Sep 23];23(20):7315–28. Available from: http://mcb.asm.org/

137. Pavlova NN, Thompson CB. The Emerging Hallmarks of Cancer Metabolism. Cell Metab [Internet]. Elsevier Inc.; 2016 Jan 12 [cited 2018 Jul 25];23(1):27–47. Available from: http://dx.doi.org/10.1016/j.cmet.2015.12.006

138. Cairns R, Harris I, McCracken S, Mak T. Cancer cell metabolism. Cold Spring Harb Symp Quant Biol [Internet]. 2011;76:299–311. Available from: http://dx.doi.org/10.1101/sqb.2011.76.012856

139. Semenza GL. Defining the role of hypoxia-inducible factor 1 in cancer biology and therapeutics. Oncogene [Internet]. Nature Publishing Group; 2010 Feb [cited 2018 Oct 28];29(5):625–34. Available from: http://www.nature.com/articles/onc2009441

140. Bruick RK, McKnight SL. A conserved family of prolyl-4-hydroxylases that modify HIF. Science [Internet]. 2001 Nov 9 [cited 2018 Sep 23];294(5545):1337–40. Available from: http://www.sciencemag.org/cgi/doi/10.1126/science.1066373

141. Maxwell PH, Wiesener MS, Chang G-W, Clifford SC, Vaux EC, Cockman ME, et al. The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. Nature [Internet]. 1999 May 20 [cited 2018 Sep 23];399(6733):271–5. Available from: http://www.ncbi.nlm.nih.gov/pubmed/10353251

142. van Uden P, Kenneth NS, Rocha S. Regulation of hypoxia-inducible factor-1α by NF-κB. Biochem J [Internet]. 2008 Jun 15 [cited 2018 Sep 23];412(3):477–84. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18393939

143. Kilic-Eren M, Boylu T, Tabor V. Targeting PI3K/Akt represses Hypoxia inducible

factor-1α activation and sensitizes Rhabdomyosarcoma and Ewing's sarcoma cells for apoptosis. Cancer Cell Int [Internet]. 2013 [cited 2018 Sep 23];13(1):36. Available from: http://cancerci.biomedcentral.com/articles/10.1186/1475-2867-13-36

144. Richard DE, Berra E, Gothié E, Roux D, Pouysségur J. p42/p44 mitogen-activated protein kinases phosphorylate hypoxia-inducible factor 1alpha (HIF-1alpha) and enhance the transcriptional activity of HIF-1. J Biol Chem [Internet]. 1999 Nov 12 [cited 2018 Sep 23];274(46):32631–7. Available from: http://www.ncbi.nlm.nih.gov/pubmed/10551817

145. Agani F, Jiang B-H. Oxygen-independent regulation of HIF-1: novel involvement of PI3K/AKT/mTOR pathway in cancer. Curr Cancer Drug Targets [Internet]. 2013 Mar [cited 2018 Sep 23];13(3):245–51. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23297826

146. Courtnay R, Ngo DC, Malik N, Ververis K, Tortorella SM, Karagiannis TC. Cancer metabolism and the Warburg effect: the role of HIF-1 and PI3K. Mol Biol Rep [Internet]. 2015 Apr 18 [cited 2018 Sep 23];42(4):841–51. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25689954

147. Muz B, De La Puente P, Azab F, Azab AK. Hypoxia Dovepress The role of hypoxia in cancer progression, angiogenesis, metastasis, and resistance to therapy. 2015 [cited 2018 Aug 28]; Available from: http://dx.doi.org/10.2147/HP.S93413

148. Schito L, Semenza GL. Hypoxia-Inducible Factors: Master Regulators of Cancer Progression. TRENDS in CANCER [Internet]. 2016 [cited 2018 Sep 4];2:758–70. Available from: http://dx.doi.org/10.1016/j.trecan.2016.10.016

149. Carmeliet P, Jain RK. Molecular mechanisms and clinical applications of angiogenesis. Nature [Internet]. 2011 May 19 [cited 2018 Sep 23];473(7347):298–307. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21593862

150. Carmeliet P, Jain RK. Principles and mechanisms of vessel normalization for cancer and other angiogenic diseases. Nat Rev Drug Discov [Internet]. 2011 Jun 1 [cited 2018 Sep 23];10(6):417–27. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21629292

151. Carmeliet P. Angiogenesis in life, disease and medicine. Nature [Internet]. 2005 Dec 15 [cited 2018 Sep 23];438(7070):932–6. Available from: http://www.ncbi.nlm.nih.gov/pubmed/16355210

152. Semenza GL. HIF-1 mediates metabolic responses to intratumoral hypoxia and oncogenic mutations. J Clin Invest [Internet]. American Society for Clinical Investigation; 2013 Sep 3 [cited 2018 Sep 23];123(9):3664–71. Available from: https://www.jci.org/articles/view/67230

153. Luo W, Hu H, Chang R, Zhong J, Knabel M, O'Meally R, et al. Pyruvate Kinase M2 Is a PHD3-Stimulated Coactivator for Hypoxia-Inducible Factor 1. Cell [Internet]. Cell Press; 2011 May 27 [cited 2018 Sep 23];145(5):732–44. Available from: https://www.sciencedirect.com/science/article/pii/S0092867411004363

154. Ying H, Kimmelman AC, Lyssiotis CA, Hua S, Chu GC, Fletcher-Sananikone E, et al. Oncogenic Kras Maintains Pancreatic Tumors through Regulation of Anabolic Glucose Metabolism. 2012 [cited 2018 Sep 13]; Available from: https://ac.els-cdn.com/S0092867412003522/1-s2.0-S0092867412003522-main.pdf?_tid=7edbceef-1fec-45fd-9c0e-

18fa2abc54eb&acdnat=1536865681_c47bf4cbeca87f3de8637a7546cdf9e3

155. Jiang P, Du W, Wang X, Mancuso A, Gao X, Wu M, et al. p53 regulates biosynthesis through direct inactivation of glucose-6-phosphate dehydrogenase. Nat Cell Biol [Internet]. 2011 Mar 20 [cited 2018 Sep 24];13(3):310–6. Available from: http://www.nature.com/doifinder/10.1038/ncb2172

156. Spiro RG. Protein glycosylation: nature, distribution, enzymatic formation, and disease implications of glycopeptide bonds. Glycobiology [Internet]. Oxford University Press; 2002 Apr 1 [cited 2018 Sep 24];12(4):43R–56R. Available from: https://academic.oup.com/glycob/article-lookup/doi/10.1093/glycob/12.4.43R

157. Wellen KE, Lu C, Mancuso A, Lemons JMS, Ryczko M, Dennis JW, et al. The hexosamine biosynthetic pathway couples growth factor-induced glutamine uptake to glucose metabolism. Genes Dev [Internet]. Cold Spring Harbor Laboratory Press; 2010 Dec 15 [cited 2018 Sep 24];24(24):2784–99. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21106670

158. Itkonen HM, Minner S, Guldvik IJ, Sandmann MJ, Tsourlakis MC, Berge V, et al. O-GlcNAc transferase integrates metabolic pathways to regulate the stability of c-MYC in human prostate cancer cells. Cancer Res [Internet]. American Association for Cancer Research; 2013 Aug 15 [cited 2018 Sep 24];73(16):5277–87. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23720054

159. Locasale JW, Grassian AR, Melman T, Lyssiotis CA, Mattaini KR, Bass AJ, et al. Phosphoglycerate dehydrogenase diverts glycolytic flux and contributes to oncogenesis. Nat Genet Vol [Internet]. 2011 [cited 2018 Sep 13];43(9). Available from: https://www.nature.com/articles/ng.890.pdf

160. Possemato R, Marks KM, Shaul YD, Pacold ME, Kim D, Birsoy K, et al. Functional genomics reveal that the serine synthesis pathway is essential in breast cancer. Nature [Internet]. Nature Publishing Group; 2011 Aug 18 [cited 2018 Sep 24];476(7360):346–50. Available from: http://www.nature.com/articles/nature10350

161. Tibbetts AS, Appling DR. Compartmentalization of Mammalian Folate-Mediated One-Carbon Metabolism. Annu Rev Nutr [Internet]. Annual Reviews ; 2010 Jul 20 [cited 2018 Sep 24];30(1):57–81. Available from: http://www.annualreviews.org/doi/10.1146/annurev.nutr.012809.104810

162. Fan J, Ye J, Kamphorst JJ, Shlomi T, Thompson CB, Rabinowitz JD. Quantitative flux analysis reveals folate-dependent NADPH production. Nature [Internet]. Nature Publishing Group; 2014 Jun 4 [cited 2018 Sep 13];510(7504):298–302. Available from: http://www.nature.com/articles/nature13236

163. Ye J, Fan J, Venneti S, Wan Y-W, Pawel BR, Zhang J, et al. Serine catabolism regulates mitochondrial redox control during hypoxia. Cancer Discov [Internet]. American Association for Cancer Research; 2014 Dec 1 [cited 2018 Sep 13];4(12):1406–17. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25186948

164. Akram M. Citric Acid Cycle and Role of its Intermediates in Metabolism. Cell Biochem Biophys [Internet]. Springer US; 2014 Apr 26 [cited 2018 Sep 24];68(3):475–8. Available from: http://link.springer.com/10.1007/s12013-013-9750-1

165. Birsoy K, Wang T, Chen WW, Freinkman E, Abu-Remaileh M, Sabatini DM. An

Essential Role of the Mitochondrial Electron Transport Chain in Cell Proliferation Is to Enable Aspartate Synthesis. Cell [Internet]. Cell Press; 2015 Jul 30 [cited 2018 Sep 24];162(3):540–51. Available from: https://www.sciencedirect.com/science/article/pii/S0092867415008533

166. Sullivan LB, Gui DY, Hosios AM, Bush LN, Freinkman E, Vander Heiden MG. Supporting Aspartate Biosynthesis Is an Essential Function of Respiration in Proliferating Cells. Cell [Internet]. Cell Press; 2015 Jul 30 [cited 2018 Sep 24];162(3):552–63. Available from: https://www.sciencedirect.com/science/article/pii/S0092867415008545

167. Li J, Cheng J-X. Direct visualization of de novo lipogenesis in single living cells. Sci Rep [Internet]. Nature Publishing Group; 2014 May 29 [cited 2018 Sep 24];4(1):6807. Available from: http://www.nature.com/articles/srep06807

168. Menendez JA, Lupu R. Fatty acid synthase and the lipogenic phenotype in cancer pathogenesis. Nat Rev Cancer [Internet]. Nature Publishing Group; 2007 Oct 1 [cited 2018 Sep 24];7(10):763–77. Available from: http://www.nature.com/articles/nrc2222

169. Pietrocola F, Galluzzi L, Bravo-San Pedro JM, Madeo F, Kroemer G, Manuel J, et al. Acetyl Coenzyme A: A Central Metabolite and Second Messenger. Cell Metab [Internet]. 2015 [cited 2018 Aug 28]; Available from: http://dx.doi.org/10.1016/j.cmet.2015.05.014

170. Choudhary C, Weinert BT, Nishida Y, Verdin E, Mann M. The growing landscape of lysine acetylation links metabolism and cell signalling. Nat Rev Mol Cell Biol [Internet]. Nature Publishing Group; 2014 Aug 1 [cited 2018 Sep 24];15(8):536–

50. Available from: http://www.nature.com/articles/nrm3841

171. Lee J V, Carrer A, Shah S, Snyder NW, Wei S, Venneti S, et al. Article Akt-Dependent Metabolic Reprogramming Regulates Tumor Cell Histone Acetylation. Cell Metab [Internet]. 2014 [cited 2018 Sep 7];20:306–19. Available from: http://dx.doi.org/10.1016/j.cmet.2014.06.004

172. Schug ZT, Peck B, Jones DT, Zhang Q, Grosskurth S, Alam IS, et al. Acetyl-CoA Synthetase 2 Promotes Acetate Utilization and Maintains Cancer Cell Growth under Metabolic Stress. Cancer Cell [Internet]. Cell Press; 2015 Jan 12 [cited 2018 Sep 24];27(1):57–71. Available from: https://www.sciencedirect.com/science/article/pii/S153561081400511X

173. Shi L, Tu BP. Acetyl-CoA and the regulation of metabolism: mechanisms and consequences. Curr Opin Cell Biol [Internet]. 2015 [cited 2018 Aug 28];33:125–31. Available from: http://dx.doi.org/10.1016/j.ceb.2015.02.003

174. Yoshii Y, Furukawa T, Yoshii H, Mori T, Kiyono Y, Waki A, et al. Cytosolic acetyl-CoA synthetase affected tumor cell survival under hypoxia: the possible function in tumor acetyl-CoA/acetate metabolism. Cancer Sci [Internet]. Wiley/Blackwell (10.1111); 2009 May 1 [cited 2018 Sep 24];100(5):821–7. Available from: http://doi.wiley.com/10.1111/j.1349-7006.2009.01099.x

175. Yoshii Y, Waki A, Furukawa T, Kiyono Y, Mori T, Yoshii H, et al. Tumor uptake of radiolabeled acetate reflects the expression of cytosolic acetyl-CoA synthetase: implications for the mechanism of acetate PET. Nucl Med Biol [Internet]. Elsevier; 2009 Oct 1 [cited 2018 Sep 24];36(7):771–7. Available from: https://www.sciencedirect.com/science/article/pii/S0969805109001504

176. Mashimo T, Pichumani K, Vemireddy V, Hatanpaa KJ, Singh DK, Sirasanagandla S, et al. Acetate Is a Bioenergetic Substrate for Human Glioblastoma and Brain Metastases. Cell [Internet]. Cell Press; 2014 Dec 18 [cited 2018 Sep 24];159(7):1603–14. Available from: https://www.sciencedirect.com/science/article/pii/S0092867414015025

177. Björnson E, Mukhopadhyay B, Asplund A, Kunos G, Nielsen J, Mardinoglu A, et al. Stratification of Hepatocellular Carcinoma Patients Based on Acetate Utilization Resource Stratification of Hepatocellular Carcinoma Patients Based on Acetate Utilization. CELREP [Internet]. 2015 [cited 2018 Aug 7];13:2014–26. Available from: http://dx.doi.org/10.1016/j.celrep.2015.10.045http://dx.doi.org/10.1016/j.celrep.2015.10.045ThisisanopenaccessarticleundertheCCBYlicense

178. Rysman E, Brusselmans K, Scheys K, Timmermans L, Derua R, Munck S, et al. Tumor and Stem Cell Biology De novo Lipogenesis Protects Cancer Cells from Free Radicals and Chemotherapeutics by Promoting Membrane Lipid Saturation. 2010 [cited 2018 Sep 24]; Available from: http://cancerres.aacrjournals.org/

179. Ramsey MR, Sharpless NE. ROS as a tumour suppressor? Nat Cell Biol [Internet]. 2006 Nov 1 [cited 2018 Sep 24];8(11):1213–5. Available from: http://www.ncbi.nlm.nih.gov/pubmed/17077852

180. Takahashi A, Ohtani N, Yamakoshi K, Iida S, Tahara H, Nakayama K, et al. Mitogenic signalling and the p16INK4a–Rb pathway cooperate to enforce irreversible cellular senescence. Nat Cell Biol [Internet]. Nature Publishing Group; 2006 Nov 8 [cited 2018 Sep 24];8(11):1291–7. Available from:

http://www.nature.com/articles/ncb1491

181. Bauer DE, Hatzivassiliou G, Zhao F, Andreadis C, Thompson CB. ATP citrate lyase is an important component of cell growth and transformation. Oncogene [Internet]. Nature Publishing Group; 2005 Sep 13 [cited 2018 Sep 24];24(41):6314–22. Available from: http://www.nature.com/articles/1208773

182. Berwick DC, Hers I, Heesom KJ, Moule SK, Tavare JM. The identification of ATP-citrate lyase as a protein kinase B (Akt) substrate in primary adipocytes. J Biol Chem [Internet]. American Society for Biochemistry and Molecular Biology; 2002 Sep 13 [cited 2018 Sep 24];277(37):33895–900. Available from: http://www.ncbi.nlm.nih.gov/pubmed/12107176

183. Chirala SS, Wakil SJ. Structure and function of animal fatty acid synthase. Lipids [Internet]. Springer-Verlag; 2004 Nov [cited 2018 Sep 25];39(11):1045–53. Available from: http://doi.wiley.com/10.1007/s11745-004-1329-9

184. Chajès V, Cambot M, Moreau K, Lenoir GM, Joulin V. Acetyl-CoA carboxylase α is essential to breast cancer cell survival. Cancer Res [Internet]. 2006 [cited 2018 Sep 14];66(10):5287–94. Available from: www.aacrjournals.org

185. Flavin R, Peluso S, Nguyen PL, Loda M. Fatty acid synthase as a potential therapeutic target in cancer. 2010 [cited 2018 Sep 14]; Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3197858/pdf/nihms-207228.pdf

186. Migita T, Narita T, Nomura K, Miyagi E, Inazuka F, Matsuura M, et al. ATP Citrate Lyase: Activation and Therapeutic Implications in Non-Small Cell Lung Cancer. Cancer Res [Internet]. 2008 [cited 2018 Sep 14];68:8547–54. Available from: www.aacrjournals.org

187. Milgraum LZ, Witters LA, Pasternack GR, Kuhajda2 FP. Enzymes of the Fatty Acid Synthesis Pathway Are Highly Expressed in in Situ Breast Carcinoma' [Internet]. 1997 [cited 2018 Sep 14]. Available from: http://clincancerres.aacrjournals.org/content/clincanres/3/11/2115.full.pdf

188. Comerford SA, Huang Z, Du X, Wang Y, Cai L, Witkiewicz AK, et al. Acetate Dependence of Tumors. Cell [Internet]. Cell Press; 2014 Dec 18 [cited 2018 Sep 25];159(7):1591–602. Available from: https://www.sciencedirect.com/science/article/pii/S0092867414014512

189. Takahashi H, McCaffery JM, Irizarry RA, Boeke JD. Nucleocytosolic Acetyl-Coenzyme A Synthetase Is Required for Histone Acetylation and Global Transcription. Mol Cell [Internet]. Cell Press; 2006 Jul 21 [cited 2018 Sep 25];23(2):207–17. Available from: https://www.sciencedirect.com/science/article/pii/S1097276506003844

190. Cai L, Sutter BM, Li B, Tu BP. Acetyl-CoA Induces Cell Growth and Proliferation by Promoting the Acetylation of Histones at Growth Genes. Mol Cell [Internet]. Cell Press; 2011 May 20 [cited 2018 Sep 25];42(4):426–37. Available from: https://www.sciencedirect.com/science/article/pii/S1097276511003327#bib34

191. Shi L, Tu BP. Acetyl-CoA induces transcription of the key G1 cyclin CLN3 to promote entry into the cell division cycle in Saccharomyces cerevisiae. [cited 2018 Sep 25]; Available from: www.pnas.org/cgi/doi/10.1073/pnas.1302490110

192. Wellen KE, Hatzivassiliou G, Sachdeva UM, Bui T V, Cross JR, Thompson CB. ATP-citrate lyase links cellular metabolism to histone acetylation. Science [Internet]. American Association for the Advancement of Science; 2009 May 22

[cited 2018 Sep 25];324(5930):1076–80. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19461003

193. Shimazu T, Hirschey MD, Newman J, He W, Shirakawa K, Le Moan N, et al. Suppression of oxidative stress by β-hydroxybutyrate, an endogenous histone deacetylase inhibitor. Science [Internet]. American Association for the Advancement of Science; 2013 Jan 11 [cited 2018 Sep 25];339(6116):211–4. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23223453

194. Moussaieff A, Rouleau M, Kitsberg D, Cohen M, Levy G, Barasch D, et al. Glycolysis-Mediated Changes in Acetyl-CoA and Histone Acetylation Control the Early Differentiation of Embryonic Stem Cells. Cell Metab [Internet]. Cell Press; 2015 Mar 3 [cited 2018 Sep 25];21(3):392–402. Available from: https://www.sciencedirect.com/science/article/pii/S1550413115000546

195. Sutendra G, Kinnaird A, Dromparis P, Paulin R, Stenson TH, Haromy A, et al. A Nuclear Pyruvate Dehydrogenase Complex Is Important for the Generation of Acetyl-CoA and Histone Acetylation. Cell [Internet]. Cell Press; 2014 Jul 3 [cited 2018 Sep 25];158(1):84–97. Available from: https://www.sciencedirect.com/science/article/pii/S0092867414006746

196. Markowitz S, Wang J, Myeroff L, Parsons R, Sun L, Lutterbaugh J, et al. Inactivation of the type II TGF-beta receptor in colon cancer cells with microsatellite instability. Science. 1995.

197. Grady WM, Myeroff LL, Swinler SE, Rajput A, Thiagalingam S, Lutterbaugh JD, et al. Mutational inactivation of transforming growth factor beta receptor type II in microsatellite stable colon cancers. Cancer Res [Internet]. 1999 Jan 15 [cited 2015

Oct 3];59(2):320–4. Available from: http://cancerres.aacrjournals.org/content/59/2/320.abstract

198. Vogelstein B, Fearon ER, Hamilton SR, Kern SE, Preisinger AC, Leppert M, et al. Genetic Alterations during Colorectal-Tumor Development. N Engl J Med. 1988;

199. Hyman CA, Bartholin L, Newfeld SJ, Wotton D. Drosophila TGIF proteins are transcriptional activators. Mol Cell Biol [Internet]. 2003 Dec [cited 2015 Sep 12];23(24):9262–74. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=309625&tool=pmcentrez&rendertype=abstract

200. Razzaque MS, Atfi A. TGIF function in oncogenic Wnt signaling. Biochim Biophys Acta - Rev Cancer [Internet]. Elsevier; 2016 Apr 1 [cited 2018 Jul 25];1865(2):101–4. Available from: https://www.sciencedirect.com/science/article/pii/S0304419X15000736?via%3Dihub

201. Hao Y, Bjerke GA, Pietrzak K, Melhuish TA, Han Y, Turner SD, et al. TGFβ signaling limits lineage plasticity in prostate cancer. Yegnasubramanian S, editor. PLoS Genet [Internet]. 2018 May 21 [cited 2018 Oct 15];14(5):e1007409. Available from: http://dx.plos.org/10.1371/journal.pgen.1007409

202. Parini P, Melhuish TA, Wotton D, Larsson L, Ahmed O, Eriksson M, et al. Overexpression of transforming growth factor β induced factor homeobox 1 represses NPC1L1 and lowers markers of intestinal cholesterol absorption. Atherosclerosis [Internet]. 2018 Aug [cited 2018 Oct 3];275:246–55. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0021915018311791

203. Kaiser S, Park Y-K, Franklin JL, Halberg RB, Yu M, Jessen WJ, et al. Transcriptional recapitulation and subversion of embryonic colon development by mouse colon tumor models and human colon cancer. Genome Biol [Internet]. 2007 Jan [cited 2015 Sep 21];8(7):R131. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2323222&tool=pmcentrez&rendertype=abstract

204. Seo SR, Lallemand F, Ferrand N, Pessah M, L'Hoste S, Camonis J, et al. The novel E3 ubiquitin ligase Tiul1 associates with TGIF to target Smad2 for degradation. EMBO J. 2004.

205. Seo SR, Ferrand N, Faresse N, Prunier C, Abécassis L, Pessah M, et al. Nuclear Retention of the Tumor Suppressor cPML by the Homeodomain Protein TGIF Restricts TGF-β Signaling. Mol Cell. 2006.

206. Patro R, Duggal G, Love MI, Irizarry RA, Kingsford C. Salmon provides fast and bias-aware quantification of transcript expression. Nat Methods. 2017.

207. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 2014.

208. Chen EY, Tan CM, Kou Y, Duan Q, Wang Z, Meirelles G, et al. Enrichr: interactive and collaborative HTML5 gene list enrichment analysis tool. BMC Bioinformatics [Internet]. 2013 Apr 15 [cited 2018 Oct 15];14(1):128. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23586463

209. Kuleshov M V., Jones MR, Rouillard AD, Fernandez NF, Duan Q, Wang Z, et al. Enrichr: a comprehensive gene set enrichment analysis web server 2016 update. Nucleic Acids Res. 2016.

210. Babicki S, Arndt D, Marcu A, Liang Y, Grant JR, Maciejewski A, et al. Heatmapper: web-enabled heat mapping for all. Nucleic Acids Res. 2016.

211. Mootha VK, Lindgren CM, Eriksson K-F, Subramanian A, Sihag S, Lehar J, et al. PGC-1alpha-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. Nat Genet [Internet]. 2003 Jul 15 [cited 2018 Oct 15];34(3):267–73. Available from: http://www.nature.com/articles/ng1180

212. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, et al. Gene set enrichment analysis: A knowledge-based approach for interpreting genome-wide expression profiles. Proc Natl Acad Sci [Internet]. 2005 Oct 25 [cited 2018 Oct 15];102(43):15545–50. Available from: http://www.ncbi.nlm.nih.gov/pubmed/16199517

213. Bjerke GA, Hyman-Walsh C, Wotton D. Cooperative Transcriptional Activation by Klf4, Meis2, and Pbx1. Mol Cell Biol. 2011.

214. Labbé E, Lock L, Letamendia A, Gorska AE, Gryfe R, Gallinger S, et al. Transcriptional cooperation between the transforming growth factor-β and Wnt pathways in mammary and intestinal tumorigenesis. Cancer Res. 2007.

215. Miguchi M, Hinoi T, Shimomura M, Adachi T, Saito Y, Niitsu H, et al. Gasdermin C is upregulated by inactivation of transforming growth factor β receptor type II in the presence of mutated Apc, promoting colorectal cancer proliferation. PLoS One. 2016.

216. Herbst A, Jurinovic V, Krebs S, Thieme SE, Blum H, Göke B, et al. Comprehensive analysis of β-catenin target genes in colorectal carcinoma cell lines

with deregulated Wnt/β-catenin signaling. BMC Genomics. 2014.

217. Muñoz NM, Upton M, Rojas A, Washington MK, Lin L, Chytil A, et al. Transforming growth factor beta receptor type II inactivation induces the malignant transformation of intestinal neoplasms initiated by Apc mutation. Cancer Res [Internet]. 2006 Oct 15 [cited 2015 Sep 12];66(20):9837–44. Available from: http://www.ncbi.nlm.nih.gov/pubmed/17047044

218. Dong C, Yuan T, Wu Y, Wang Y, Fan TWM, Miriyala S, et al. Article Loss of FBP1 by Snail-Mediated Repression Provides Metabolic Advantages in Basal-like Breast Cancer. Cancer Cell [Internet]. Elsevier Inc.; 2013;23(3):316–31. Available from: http://dx.doi.org/10.1016/j.ccr.2013.01.022

219. Shiraishi T, Verdone JE, Huang J, Kahlert UD, Hernandez JR, Torga G, et al. Glycolysis is the primary bioenergetic pathway for cell motility and cytoskeletal remodeling in human prostate and breast cancer cells. Oncotarget. 2015;

220. Li J, Wang Y, Li QG, Xue JJ, Wang Z, Yuan X, et al. Downregulation of FBP1 promotes tumor metastasis and indicates poor prognosis in gastric cancer via regulating epithelial-mesenchymal transition. PLoS One. 2016.

221. Liu K, Tang Z, Huang A, Chen P, Liu P, Yang J, et al. Glyceraldehyde-3-phosphate dehydrogenase promotes cancer growth and metastasis through upregulation of SNAIL expression. Int J Oncol. 2017.

222. Hneino M, François A, Buard V, Tarlet G, Abderrahmani R, Blirando K, et al. The TGF-β/Smad repressor TG-interacting factor 1 (TGIF1) plays a role in radiation-induced intestinal injury independently of a Smad signaling pathway. PLoS One [Internet]. 2012 Jan [cited 2015 Sep 11];7(5):e35672. Available from:

http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3342305&tool=pmcentrez&rendertype=abstract

223. Liu X, Hubchak SC, Browne JA, Schnaper HW. Epidermal growth factor inhibits transforming growth factor-β-induced fibrogenic differentiation marker expression through ERK activation. 2014 [cited 2018 Jul 26]; Available from: http://dx.doi.org/10.1016/j.cellsig.2014.05.018

224. Nakao A, Afrakhte M, Morén A, Nakayama T, Christian JL, Heuchef R, et al. Identification of Smad7, a TGFβ-inducible antagonist of TGF-β signalling. Nature. 1997.

225. Stroschein SL, Wang W, Zhou S, Zhou Q, Luo K. Negative feedback regulation of TGF-beta signaling by the SnoN oncoprotein. Science [Internet]. 1999 Oct 22 [cited 2018 Oct 26];286(5440):771–4. Available from: http://www.ncbi.nlm.nih.gov/pubmed/10531062

226. Sharma V, Antonacopoulou AG, Tanaka S, Panoutsopoulos AA, Bravou V, Kalofonos HP, et al. Enhancement of TGF-β signaling responses by the E3 ubiquitin ligase Arkadia provides tumor suppression in colorectal cancer. Cancer Res. 2011.

227. Li S, Hu R, Yao H, Long D, Luo F, Zhou X, et al. Characterization of the interaction interface and conformational dynamics of human TGIF1 homeodomain upon the binding of consensus DNA. Biochim Biophys Acta - Proteins Proteomics [Internet]. Elsevier; 2018 Oct [cited 2018 Sep 14];1866(10):1021–8. Available from: https://linkinghub.elsevier.com/retrieve/pii/S1570963918301171

228. Pryde SE, Duncan SH, Hold GL, Stewart CS, Flint HJ. The microbiology of

butyrate formation in the human colon. FEMS Microbiol Lett [Internet]. 2002 Dec 17 [cited 2018 Nov 18];217(2):133–9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/12480096

229. Donohoe DR, Garge N, Zhang X, Sun W, O'connell TM, Bunger MK, et al. The microbiome and butyrate regulate energy metabolism and autophagy in the mammalian colon. Cell Metab [Internet]. 2011 [cited 2018 Nov 18];13:517–26. Available from: https://ac.els-cdn.com/S1550413111001434/1-s2.0-S1550413111001434-main.pdf?_tid=9c2a76c1-f769-4c44-b8c0-0a434033bad0&acdnat=1542599510_acb64002c7acb8dc32fc1525bd61292d

230. Warburg O. On Origin of Cancer Cells. Science (80-). 1956.

231. Hanahan D, Weinberg RA. Leading Edge Review Hallmarks of Cancer: The Next Generation. 2011 [cited 2018 Aug 5]; Available from: https://ac.els-cdn.com/S0092867411001279/1-s2.0-S0092867411001279-main.pdf?_tid=e7683333-6f21-449a-bb01-946e7b466a0e&acdnat=1533524537_250b2a02c4c36600bc1dd70d4631f225

232. Satoh K, Yachida S, Sugimoto M, Oshima M, Nakagawa T, Akamoto S, et al. Global metabolic reprogramming of colorectal cancer occurs at adenoma stage and is induced by MYC. Proc Natl Acad Sci. 2017.

233. Hutton JE, Wang X, Zimmerman LJ, Slebos RJC, Trenary IA, Young JD, et al. Oncogenic KRAS and BRAF Drive Metabolic Reprogramming in Colorectal Cancer. Mol Cell Proteomics. 2016.

234. Masin M, Vazquez J, Rossi S, Groeneveld S, Samson N, Schwalie PC, et al. GLUT3 is induced during epithelial-mesenchymal transition and promotes tumor

cell proliferation in non-small cell lung cancer. Cancer Metab. 2014.

235. Sun Y, Daemen A, Hatzivassiliou G, Arnott D, Wilson C, Zhuang G, et al. Metabolic and transcriptional profiling reveals pyruvate dehydrogenase kinase 4 as a mediator of epithelial-mesenchymal transition and drug resistance in tumor cells. Cancer Metab. 2014.

236. Sciacovelli M, Frezza C. Metabolic reprogramming and epithelial-to-mesenchymal transition in cancer. 2017;284:3132–44.

237. Jiang L, Xiao L, Sugiura H, Huang X, Ali A, Kuro-O M, et al. Metabolic reprogramming during TGFβ1-induced epithelial-to-mesenchymal transition. Oncogene. 2015.

238. Ahmad A, Aboukameel A, Kong D, Wang Z, Sethi S, Chen W, et al. Phosphoglucose isomerase/autocrine motility factor mediates epithelial-mesenchymal transition regulated by miR-200 in breast cancer cells. Cancer Res. 2011.

239. Zheng L, Cardaci S, Jerby L, Mackenzie ED, Sciacovelli M, Johnson TI, et al. Fumarate induces redox-dependent senescence by modifying glutathione metabolism. Nat Commun. 2015.

240. Kwon AT, Arenillas DJ, Hunt RW, Wasserman WW. oPOSSUM-3: Advanced Analysis of Regulatory Motif Over-Representation Across Genes or ChIP-Seq Datasets. G3: Genes|Genomes|Genetics [Internet]. 2012 Sep [cited 2018 Oct 24];2(9):987–1002. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22973536

241. Ho Sui SJ, Fulton DL, Arenillas DJ, Kwon AT, Wasserman WW. oPOSSUM:

integrated tools for analysis of regulatory motif over-representation. Nucleic Acids Res [Internet]. 2007 Jul [cited 2018 Oct 24];35(suppl_2):W245–52. Available from: http://www.ncbi.nlm.nih.gov/pubmed/17576675

242. Ho Sui SJ, Mortimer JR, Arenillas DJ, Brumm J, Walsh CJ, Kennedy BP, et al. oPOSSUM: identification of over-represented transcription factor binding sites in co-expressed genes. Nucleic Acids Res [Internet]. 2005 Jun 2 [cited 2018 Oct 24];33(10):3154–64. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15933209

243. Taketo MM, Edelmann W. Mouse Models of Colon Cancer. Gastroenterology. 2009.

244. Jonkers J, Berns A. CONDITIONAL MOUSE MODELS OF SPORADIC CANCER. Nat Rev Cancer. 2002.

245. Boutin AT, Liao W-T, Wang M, Hwang SS, Karpinets T V, Cheung H, et al. Oncogenic Kras drives invasion and maintains metastases in colorectal cancer. Genes Dev [Internet]. 2017 [cited 2018 Oct 23]; Available from: http://www.genesdev.org/cgi/doi/10.1101/gad.293449.116.

246. Romano G, Chagani S, Kwong LN. The path to metastatic mouse models of colorectal cancer. Oncogene [Internet]. 2018 [cited 2018 Oct 23];37:2481–9. Available from: https://doi.org/10.1038/s41388-018-0155-x