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Requirement and Function of Hippo Pathway Signaling in the Mammalian Gastrointestinal Tract: A Dissertation

Jennifer L. Cotton
University of Massachusetts Medical School

REQUIREMENT AND FUNCTION OF HIPPO PATHWAY SIGNALING IN THE MAMMALIAN GASTROINTESTINAL TRACT

A Dissertation Presented

By

Jennifer Lynne Cotton

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This work was undertaken in the Graduate School of Biomedical Sciences
Program in Cancer Biology

The signature of the Thesis Advisor signifies validation of Dissertation content

_____
Junhao Mao, Ph.D., Thesis Advisor

The signatures of the Dissertation Defense Committee signify completion and approval as to style and content of the Dissertation

_____
JeanMarie Houghton, M.D., Ph.D., Member of Committee

_____
Andrew Leiter, M.D., Ph.D., Member of Committee

_____
Dannel McCollum, Ph.D., Member of Committee

_____
Ramesh Shivdasani, M.D., Ph.D., External Member of Committee

The signature of the Chair of the Committee signifies that the written dissertation meets the requirements of the Dissertation Committee

_____
Leslie Shaw, Ph.D., Chair of Committee

The signature of the Dean of the Graduate School of Biomedical Sciences signifies that the student has met all graduation requirements of the School.

_____
Anthony Carruthers, Ph.D.,
Dean of the Graduate School of Biomedical Sciences

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DEDICATION

This work is dedicated to all the patients who are still waiting for a cure

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ABSTRACT

In cancer, aberrant activation of developmental signaling pathways such as the Hippo Pathway has been shown to drive proliferation and invasion of cancer cells. Therefore, understanding the normal function of the Hippo Pathway during embryonic development can provide critical insight into how aberrant activity contributes to tumorigenesis. This dissertation explores the role of the Hippo Pathway members YAP and TAZ in gastrointestinal (GI) development and tumorigenesis. I use mouse genetics to systematically dissect the roles of YAP/TAZ in the endoderm-derived gastrointestinal epithelia and mesoderm-derived gastrointestinal mesenchyme during mammalian development. In the GI epithelium, I demonstrate that YAP/TAZ are dispensable for development and homeostasis. However, YAP/TAZ are required for Wnt pathway-driven tumorigenesis. I find that YAP/TAZ are direct transcriptional targets of Wnt/TCF4 signaling. In the GI mesenchyme, I describe a previously unknown requirement for YAP/TAZ activity during mammalian GI development. YAP/TAZ are involved in normal GI mesenchymal differentiation and function as transcriptional co-repressors in a progenitor cell population. In this way, YAP/TAZ act as molecular gatekeepers prior to Hedgehog-mediated differentiation into smooth muscle cells. This work unveils a previously unknown requirement for Hippo pathway signaling in the mammalian GI tract and a novel mechanism wherein YAP/TAZ function as transcriptional co-repressors to maintain a mesenchymal progenitor cell population.

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LIST OF ABBREVIATIONS

4-OH	4-hydroxy
Acta2	α-Smooth Muscle Actin
Actg2	γ-Smooth Muscle Actin
AIP	Anterior Intestinal Portal
Akt	RAC-alpha serine/threonine-protein kinase
Amot	Angiomotin
AMOTL2	Angiomotin like 2
AMPK	5' AMP-activated protein kinase
ANKRD1	Ankyrin repeat domain 1
ANXA10	Annexin A10
A-P	Anterior-posterior
AP1	Activating protein-1
APC	Adenomatous polyposis coli
Apc^{Min}	Adenomatous Polyposis Coli, multiple intestinal neoplasia
Axl	AXR1-like protein gene
Barx1	BARX homeobox 1
BCC	Basal cell carcinoma
BMP	Bone morphogenetic
BMPR1A	Bone morphogenetic protein receptor, type 1A gene
Braf	B-Raf Proto-Oncogene
BSA	Bovine serum albumin
CAFs	Cancer associated fibroblasts
CD	Crohn's disease
CD31	Cluster of differentiation 31/Platelet endothelial cell adhesion molecule
CD44	Cell Surface Glycoprotein CD44
CDX1	Caudal type homeobox 1
CDX2	Caudal type homeobox 2
CGNPs	Cerebellar granuale neuron precursor cells
Chd4	Chromodomain Helicase DNA Binding Protein 4
ChIP	Chromatin Immunoprecipitation
ChIP-qPCR	ChIP quantitative polymerase chain reaction
ChIPseq	ChIP sequencing
CIP	Caudal intestine portal
CK1	Casein kinase 1
CMV	Cytomegalovirus

CRC	Colorectal cancer
Cre	Causes recombination
CreER	Cre recombinase - estrogen receptor T2
CS	Cowden's Syndrome
CTGF	Connective tissue growth factor
CYR61	Cysteine rich angiogenic inducer 61
DAB	3,3'-diaminobenzidine
DAPI	4',6-diamidino-2-phenylindole
DA-β-catenin	Dominantly active β-catenin
Ddit4	DNA damage inducible transcript 4 gene
DEN	Diethylnitrosoamine
DMSO	Dimethyl sulfoxide
DN-TCF4	Dominant-negative TCF4
dpc	Days post coitum
Dpp	Decapentaplegic
DSS	Dextran sodium sulfate
DVL	Dishevelled
EB	Enteroblast
EC	Enterocyte
ECM	Extracellular matrix
EE	Enteroendocrine
EGFR	Epidermal growth factor receptor
EMT	Epithelial to mesenchymal transition
Ep	Epithelia
eRMS	Embryonal rhabdomyosarcoma
ESCs	Embryonic stem cells
EtOH	Ethanol
FAP	Familial Adenomatous Polyposis
FGF	Fibroblast growth factor
Fkh6	Forkhead homologue 6
GEMMs	Genetically engineered mouse models
GI	Gastrointestinal
Gli	Glioma-Associated Oncogene Homolog
GPCRs	G-coupled protein receptors
GSK3β	Glycogen Synthase Kinase 3 Beta
GWAS	Genome-wide associated studies
H&E	Hematoxylin and eosin
H/K-ATPase	Hydrogen potassium ATPase

H2O2	Hydrogen peroxide
H3K27ac	Acetylation at the 27th lysine residue of histone protein 3
H3K4me1	Histone H3 (mono methyl K4)
H3K4me3	Histone H3 tri methyl K4
HCC	Hepatocellular carcinoma
HEK293A	Human Embryonic Kidney 293 cells
HHEX	Hematopoietically expressed homeobox
Hhip	Hedgehog Interacting Protein
Hpo	Hippo
Hppy	Happyhour
HSCs	Hepatic stem cells
HXRXXS	Histidine-x-Arginine-x-x-Serine
IBD	Inflammatory bowel disease
ICM	Inner cell mass
IF	Immunofluorescence
IHC	Immunohistochemistry
Ihh	Indian Hedgehog
Int	Intestine
IRES	Internal ribosome entry site
ISCs	Intestinal stem cells
Jak	Janus Kinase
JPS	Juvenile Polyposis Syndrome
Ki67	Antigen identified by monoclonal antibody Ki-67
Kras	Kirsten Rat Sarcoma Viral Oncogene Homolog
LATS 1	Large tumor suppressor 1
LATS2	Large tumor suppressor 2
Lgr5	Leucine-Rich Repeat Containing G Protein-Coupled Receptor 5
Liv	Liver
LKB1	Liver kinase B1
LoxP	Locus of crossing over, P1
LPA	Lysophosphatidic acid
LRP5/6	Low-density lipoprotein-related receptors 5 and 6
MAP4K	Mitogen-activated protein kinase kinase kinase kinase
Mats	Mob As a Tumor Suppressor
MB	Medulloblastoma
Me	Mesenchyme
MEFs	Mouse embryonic fibroblasts
MeOH	Methanol

MLH1	MutL Homolog 1 gene
MLH2	PMS1 homolog 1 gene
mRNA	Messenger RNA
MSH6	MutS homolog 6 gene
Msn	Misshapen
MST1	Mammalian sterile-20-like kinase 1
MST2	Mammalian sterile-20-like kinase 2
mTmG	Membrane-targeted tdTomato/membrane-targeted EGFP
mTOR	Mechanistic target of rapamycin
Myh11	Smooth muscle myosin heavy chain
Myocd	Myocardin
NBF	Neutral buffered formalin
NG2	Neural/glial antigen 2
Nkx2.5	NK2 homeobox 5 gene
Nkx3.2	NK3 homeobox 2 gene
NLS	N-terminal nuclear localization signal
NSCLC	Non-Small Cell Lung Cancer
NURD	Nucleosome Remodeling Deacetylase
OCT	Optimal cutting temperature compound
P30	Postnatal day 30
p53	Tumor Protein P53 gene
p62	Nucleoporin p62
pAkt-S473	Phospho-V-Akt Murine Thymoma Viral Oncogene Serine473
PDAC	Pancreatic ductal adenocarcinoma
PDGFA	Platelet Derived Growth Factor Subunit A
PDGFB	Platelet Derived Growth Factor Subunit B
PDGFRα	Platelet Derived Growth Factor Receptor Alpha
PDGFRβ	Platelet Derived Growth Factor Receptor Beta
Pdx1	Pancreatic and duodenal homeobox 1
PI3K	Phosphatidylinositol 3-kinase
PJS	Peutz Jeghers Syndrome
PPxY	Proline-Proline-x–Tyrosine
pS6	Phospho-Ribosomal Protein S6
PSM2	PMS1 homolog 2 gene
Ptch	Patched
PTEN	Phosphatase and tensin homolog
RA	Retinoic Acid
RASSF2	Ras Association Domain Family Member 2

Rb	Retinoblastoma
RNAseq	RNA sequencing
R-SMAD	receptor-regulated SMAD
S1P	Sphingosine 1-phosphophate
SAG	Smoothened agonist
Sav1	Salvador
Sd	Scalloped
sFRPs	Secreted Frizzled-related proteins
Shh	Sonic hedgehog
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
SM22α	Smooth Muscle Protein 22-Alpha
SMAD	Mothers Against DPP Homolog
SMC	Smooth muscle cells
SMMHC	Smooth muscle myosin heavy chain
SMO	Smoothened
SNPs	Single-nucleotide polymorphisms
SOX2	SRY (sex determining region Y)-box 2
Sox9	SRY-Box 9
SRF	Serum response factor
SSCs	Skeletal stem cells
St	Stomach
STAT	Signal transducer and activator of transcription
STK11	Serine/threonine kinase 11
Sufu	Suppressor Of Fused Homolog
TAOK	Thousand-and-one amino acids protein kinase
TAZ	Transcriptional co-activator with PDZ-binding motif
TCF/LEF	T-cell factor/lymphoid enhancer factor
TCF4	Transcription Factor 4
TEAD	TEA domain family member
Trail	TNF-related apoptosis-inducing ligand gene
TSS	Transcription start site
Ubc	Ubiquitin C
UC	Ulcerative colitis
VGLL4	Vestigial-like protein 4
VSIG1	V-Set And Immunoglobulin Domain Containing 1
Wnt	Wingless-Type MMTV Integration Site Family
Wts	Warts

WW	Tryptophan-Tryptophan
WW45	Salvador family WW domain containing protein 1
WWTR1	Transcriptional co-activator with PDZ-binding motif
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
YAP	Yes-associated protein
Yki	Yorkie
α-SMA	Alpha- Smooth Muscle Actin
β-TrCP	Beta-Transducin Repeat Containing E3 Ubiquitin Protein Ligase

PREFACE

The majority of the work presented in this dissertation, including all mouse model generation and dissection, represents my own work at the University of Massachusetts in the lab of Dr. Junhao Mao, with the following exceptions listed below.

The data presented in Chapters II and III represent a single publication which is under revision. The following authors contributed to this manuscript:
Jennifer L. Cotton, Qi Li, Lifang Ma, Joo-Seop Park, Jiayi Wang, Jianhong Ou, Lihua J. Zhu, Y. Tony Ip, Randy L. Johnson, and Junhao Mao

CHAPTER II:

Fig. 2.3.A-F, Fig. 2.4.A-D: I generated the $Villin^{Cre}Yap^{flox/flox}Taz^{flox/flox}$ animals. Dr. Qi Li and I dissected the mice together and performed IHC. Dr. Qi Li imaged the slides. Dr. Qi Li isolated epithelia from animals, purified RNA, and performed qPCR analysis.
Fig. 2.5.A,B: Dr. Jianhong Ou performed bioinformatics analysis to compare ChIPseq data sets and contributed these panels.
Fig. 2.5.C: Dr. Jiayi Wang performed ChIP and contributed this panel.
Fig. 2.6.A, Fig. 2.7A: Lifang Ma performed DN-TCF4 transfection and qPCR. Lifang Ma also performed shRNA knockdown and soft agar colony assay.

Fig. 2.6.B-D: I generated the $Ubc^{CreER}Yap^{flox/flox}Taz^{flox/flox}$ animals. Dr. Qi Li dissected the animals, and then isolated and cultured intestinal organoids. Dr. Qi Li performed the Wnt3a, 4OH-TM, and DA-β-catenin experiments and contributed these panels.

Fig. 2.7.B-C: I generated the $Ah^{Cre}Apc^{flox/+}$ and $Ah^{Cre}Apc^{flox/+}Yap^{flox/flox}Taz^{flox/flox}$ animals and induced Cre expression with β-napthoflavone injection. Dr. Qi Li and I dissected the animals together. Dr Qi Li performed the qPCR analysis. Dr. Qi Li and I both performed IHC and imaged the slides.

CHAPTER III:

Fig. 3.1.B-C: I generated the mice in this experiment. Dr. Qi Li and I dissected the animals together. Dr. Qi Li performed western blotting analysis.

Fig. 3.22: I generated and dissected mutant embryos, and performed crosslinking. Dr. Joo-Seop Park performed immunoprecipitation. I performed qPCR and analyzed the data.

Fig. 3.23.A: Dr. Jianhong Ou performed sequence alignment and contributed this panel.

APPENDIX A:

Fig. A.7.A: Dr. He Huang isolated RNA from $Villin^{Cre}R26^{Smad7/+}$ polyps. Dr. Jianhong Ou performed data set comparison and analysis.

APPENDIX B:

No contributing authors for these figures.

CHAPTER I

Introduction

Overview

In embryogenesis, a finely tuned molecular symphony directs a single totipotent stem cell to develop into a complex multicellular organism. Stem cells differentiate into dedicated cell lineages with specialized functions. Proliferation and growth expands cell populations into new tissues. Cell polarity determines the patterning which gives rise to the caudal-rostral axis, dorsal-ventral, and left-right axis. Cells migrate in a tightly regulated movement during gastrulation to establish the germ layers. Cells undergo epithelial to mesenchymal transition (EMT) to eventually form individual organs. Blood vessel networks are generated through angiogenesis to disseminate nutrients throughout the organism. This tightly orchestrated sequence of cellular events in embryogenesis is controlled in part by developmental signaling pathways.

Developmental signaling pathways are transduction cascades that transmit cellular messages both intrinsically and extrinsically. Activation of developmental signaling pathways at specific time-points in embryogenesis tightly coordinates growth and differentiation. Conversely, termination of these signals is also critical for appropriate organismal development. For example, the Hippo Pathway is directly responsible for sensing overall liver size and halts growth once the appropriate organ size has been reached (Lee et al., 2010; Lu et al., 2010; Song et al., 2010). Other developmental signaling pathways, such as the Wnt and Hedgehog pathway, are frequently aberrantly activated in cancer. In some cases, mutations in a developmental signaling pathway may be the initial oncogenic mutation in the genetic path to tumorigenesis. For example,

mutations in the Wnt pathway member APC are common in colorectal cancer and mutations in the Hedgehog pathway member GLI are common to both basal cell carcinomas and medulloblastomas (Fearon and Vogelstein, 1990; Goodrich and Scott, 1998).

In cancer, aberrant activation of developmental signaling pathways drives proliferation and invasion of cancer cells. The hallmarks of cancer overlap with developmental processes, including angiogenesis, cell migration, EMT, and dedifferentiation (Hanahan and Weinberg, 2000). This observation has led to the hypothesis that cancer can be explained simply as development gone awry. Therefore, a detailed understanding of developmental signaling pathways during normal embryogenesis should reveal the microevolutionary advantages gained when these pathways are aberrantly activated in cancer cells. Ultimately, expanding our understanding of development will help us understand the biology of cancer as well as identify novel targets for drug therapies in patients.

In this chapter, I explore what is currently known about the etiology of colorectal cancer, including both sporadic and inherited oncogenic mutations in developmental signaling pathways, as well as genetic tools available for investigating *in vivo* tumorigenesis. Following that is a detailed discussion of the known functions of the Hippo Pathway in cancer as well as development, with a specific focus on the gastrointestinal tract. I conclude by delving into the importance of the mesenchyme in both gastrointestinal development and tumorigenesis.

Colorectal Cancer

Colorectal cancer (CRC) is currently the third leading cause of cancer-related death in the United States for both men and women. It is estimated that in 2016 alone, there will be 134,490 new colorectal cancer diagnoses and 49,190 deaths (Siegel et al., 2016). Statistically, 1 in 21 men and 1 in 23 women will develop invasive colorectal cancer in their lifetime.

Common symptoms of colorectal cancer specific to the colon include abdominal pain, bleeding, or a change in bowel habits, but can also include more vague systemic symptoms such as weight loss and fatigue (Hamilton et al., 2005). Routine screening by colonoscopy every 10 years for average patients beginning at age 50 is integral for early detection screening and better overall survival, yet colonoscopy screening is dependent on patient compliance with the screening process (Edwards et al., 2010). Just 19% of patients aged 50-75 had undergone a routine colonoscopy screening in 2000, but by 2013, this number had increased to 53% of all patients (Siegel et al., 2016). As can be predicted, as the numbers of patients participating in early-detection colonoscopy screening increased, the number of colorectal cancer cases and related deaths has decreased from 2003 to 2012.

During a routine colonoscopy, if early polyp lesions are detected, they are immediately removed and biopsied. Patients who are diagnosed with CRC in the localized disease phase have a 90% overall survival rate after 5 years (Siegel et al., 2016). This high overall survival percentage is due to the slow progression of early polyp lesion

to distant metastasis in the average patient; removal of early polyps is usually sufficient to prevent that particular lesion from progressing to metastatic disease. However, 56% of patients will be diagnosed with more advanced stage disease and face a far poorer prognosis for overall survival, with a dismal 13% overall 5 year survival rate for patients diagnosed with metastatic colorectal cancer (Siegel et al., 2016). Patients with late stage metastasis are also at higher risk for developing resistance to the current chemotherapy treatment regimes, further jeopardizing their overall survival (Edwards et al., 2010).

As such, the future of colorectal cancer research and treatment requires a two-part approach. First and foremost, early detection screening by colonoscopy is necessary for CRC prevention. However, many CRC patients with metastasis have developed resistance to chemotherapies. These patients desperately need a greater understanding of acquired resistance in the CRC cell as well as the pro-survival niche created by the tumor cell microenvironment. These breakthroughs will pave the way for more effective therapeutics that will ultimately improve patient prognosis and overall survival.

Etiology of Colorectal Cancer

Environmental Risk Factors

Colorectal cancer arises in the epithelial cells lining the gastrointestinal tract in the colon and rectum. GI epithelial cells are highly proliferative to continually replace

cells that are sloughed off as food passes through the GI tract. Due to the process of digestion, GI epithelial cells are constantly exposed to the external environment.

This constant environmental exposure explains why certain lifestyle factors significantly increase the risk for developing colorectal cancer over time. High fat diets, especially animal fats and red meat, have been shown to increase the risk of developing colorectal cancer (Haggar and Boushey, 2009). High consumption of meats cooked at high temperature, such as on a grill, increases the risk for colorectal cancer due to the generation of polycyclic hydrocarbons and heterocyclic amines during the cooking process (Santarelli et al., 2008; Sinha, 2002). Being overweight or obese also increases the risk of developing colorectal cancer, whereas regular exercise and weight loss can reduce this risk (de Jong et al., 2005). Additionally, diets lacking in fresh fruits and vegetables have been shown to increase the risk for CRC, likely due to a lack of necessary dietary fiber (Haggar and Boushey, 2009).

In addition to consumption of certain foods, cigarette smoking and alcohol use also dramatically increases the chance for developing colorectal cancer (Haggar and Boushey, 2009). The carcinogens in cigarettes can increase the risk for tumor initiation due to mutations and can also increase the rate of growth of existing cancer. Is is therefore unsurprising that approximately 12% of all deaths due to colorectal cancer are linked to cigarette smoking (Zisman et al., 2006). Alcohol consumption is also strongly correlated with elevated colorectal cancer risk, partially due to the generation of carcinogenic metabolites, such as acetaldehyde (Haggar and Boushey, 2009; Zisman et al., 2006).

As well as the consumption of carcinogens, certain pathological conditions can generate a pro-tumorigenic environment. Chronic inflammation has been positively correlated with increased cancer risk in many solid tumors; the liver inflammation in cirrhosis has been linked to hepatocellular carcinoma (HCC) and pancreatitis leads to pancreatic ductal adenocarcinoma (PDAC). Similarly, inflammatory bowel disease (IBD), including ulcerative colitis (UC) and Crohn's Disease (CD), is a strong risk factor for developing CRC (Rubin et al., 2012; Terzic et al., 2010). This chronic inflammation in the gastrointestinal tract results in approximately 20% of patients with (IBD) developing CRC over their lifetime, and more than 50% will die due to metastatic disease (Terzic et al., 2010).

Overall, due to the constant exposure to the external environment, the GI epithelia is constantly coming into contact with potential carcinogens. However, with lifestyle and diet adjustment, patients can reduce their exposure to environmental CRC risk factors.

Inherited Colon Cancer Syndromes

Some patients also have an elevated risk for developing colorectal cancer due to inheriting certain genetic mutations. Patients born with familial hereditary colon cancer syndromes comprise approximately 5% of all colorectal cancer cases, but individuals born with a familial colon cancer syndrome have 50-100% risk of developing CRC in their lifetime (Jasperson et al., 2010). Genetic testing is now available for the most

common inherited mutations and allows patients the opportunity to begin preventative screening measures once their genetic mutation is confirmed.

Familial adenomatous polyposis syndrome (FAP) is inherited in an autosomal dominant manner and is characterized by 100-1000 adenomatous polyps in the colon. FAP is caused by an inherited mutation in the *APC* gene that causes FAP patients to have elevated Wnt pathway signaling in all somatic cells (Lynch and de la Chapelle, 2003). Aberrant Wnt pathway activation in the gastrointestinal tract is sufficient to drive tumorigenesis, and FAP patients have a 100% chance of developing CRC in their lifetime due to the constitutive Wnt pathway activation. These patients therefore need to begin routine colonoscopy screening every 2 years when they reach early adolescence (Jasperson et al., 2010). Early detection screening is critical for preventing disease progression.

Lynch syndrome is another inherited familial cancer syndrome and has been linked to mutations in DNA mismatch repair enzymes such as *MLH1, MLH2, MSH6,* and *PSM2* (Hampel et al., 2008; Jasperson et al., 2010). Polyps arising in Lynch syndrome patients are characteristic of microsatellite instability due to defects in DNA repair. Patients with Lynch syndrome have a 50-80% chance of developing CRC in their lifetime, but they also have elevated risk of developing other solid tumor cancers as well, such as endometrial, stomach, ovarian, liver, and pancreatic cancer (Jasperson et al., 2010).

Patients with gastrointestinal hamartomatous polyposis syndromes such as Peutz-Jeghers Syndrome (PJS), Juvenile Polyposis Syndrome (JPS), and PTEN Hamartoma

Syndromes (such as Cowden's Syndrome (CS)) also have a 40-80% chance to develop CRC. Clinically, these patients develop hamartomatous polyps as juveniles and require regular colonoscopies to prevent obstruction and remove precancerous lesions (Chen and Fang, 2009; Jasperson et al., 2010; Jelsig et al., 2014; Shaco-Levy et al., 2016). GI hamartoma syndromes are caused by inherited mutations in tumor suppressor genes, such as *LKB1, SMAD4, BMPR1A,* and *PTEN*; patients are statistically more likely to develop other solid tumor as well (Brosens et al., 2007; Gammon et al., 2009).

For patients with inherited genetic mutations, the risk of developing CRC is so high that regular screening is an absolute necessity to extend lifespan. However, the overall percentage of CRC patients that develop CRC due to an inherited disorder is relatively low compared to the number of CRC cases caused by sporadic mutations.

Aberrant Wnt Pathway Signaling in CRC

The majority of colorectal cancers arise due to sporadic mutations in the somatic cells rather than inherited genetic mutations. Many of these sporadic mutations can be caused by exposure to carcinogens; therefore it is important to minimize exposure to environmental risk factors. As in all cancers, mutations to either inactivate a tumor suppressor or to constitutively activate an oncogene are tumorigenic—in the case of CRC, this generally happens in the gastrointestinal epithelia. The most commonly mutated gene in colorectal cancer is an inactivating mutation in the tumor suppressor

APC. Approximately 80% of all colorectal tumors have an inactivating mutation in *APC* (Kwong and Dove, 2009).

APC (adenomatous polyposis coli) protein is a main component of the Wnt pathway (Aoki and Taketo, 2007; Clevers and Nusse, 2012; Fodde, 2002; Komiya and Habas, 2008; Kwong and Dove, 2009; Novellasdemunt et al., 2015; Rowan et al., 2000). The Wnt developmental signaling pathway was first identified by a mutation in the *wingless* gene in *Drosophila melanogaster*, and is involved in regulating cell growth, migration, polarity, and differentiation during development. When Wnt pathway signaling is inactive, β-catenin is associated with the Apc-Axin-GSK3β destruction complex in the cytosol. When bound to the Apc-Axin-GSK3β destruction complex, β-catenin is phosphorylated by both CK1 and GSK3-β, and then degraded via β-TrCP and the proteasome.

Wnt pathway signaling is activated when Wnt ligand binds to the Frizzled receptor and the LRP5/6 co-receptor. Once Wnt signaling is activated, the Apc-Axin-GSK3β destruction complex is disrupted by Axin translocating to the plasma membrane to associate with Dishevelled, Frizzled, and the LRP5/6 receptors. With the disruption of the Apc-Axin-GSK3β destruction complex, β-catenin degradation is halted; this allows β-catenin protein to translocate to the nucleus. Once in the nucleus, β-catenin binds to TCF/Lef proteins to drive Wnt target gene transcription (Clevers and Nusse, 2012; Komiya and Habas, 2008; Novellasdemunt et al., 2015; Stamos and Weis, 2013).

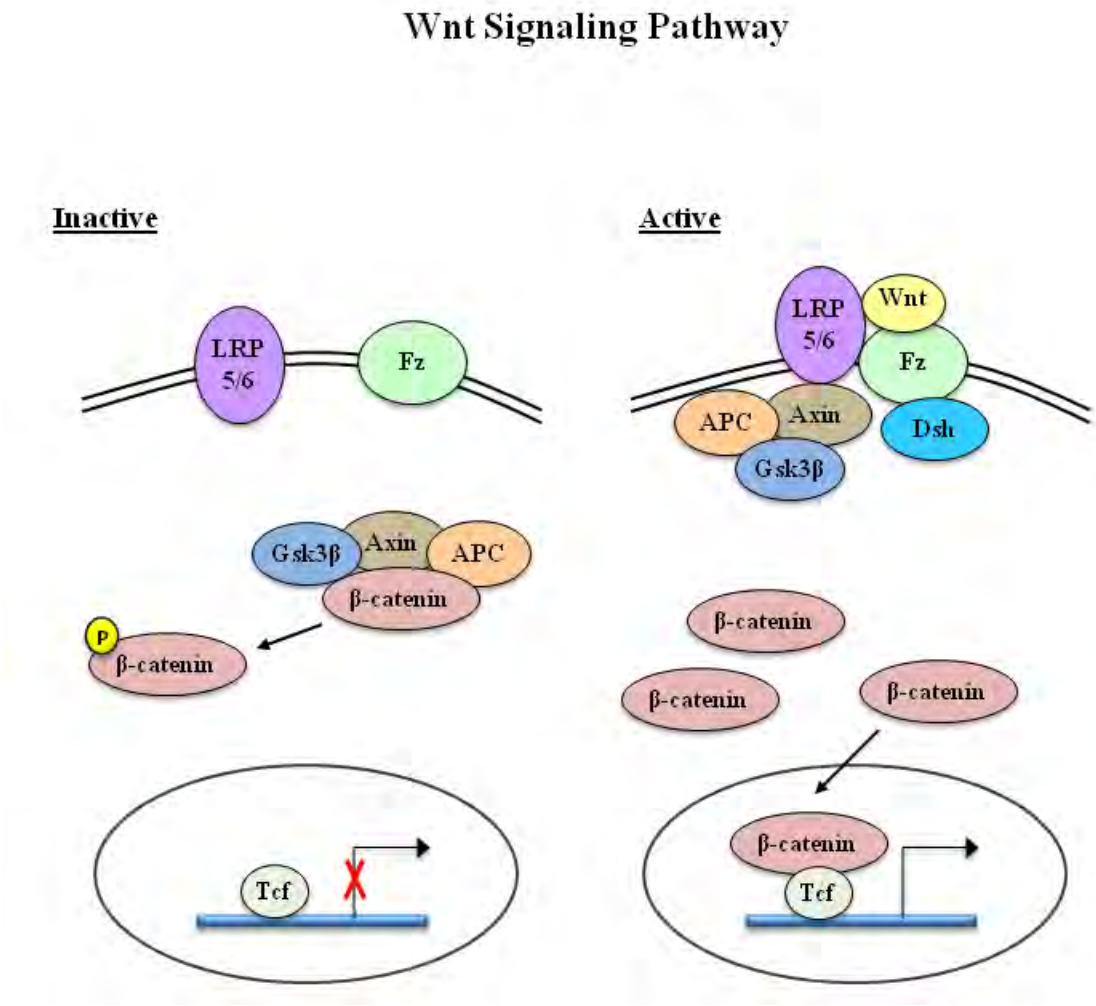


Figure 1.1. The Mammalian Wnt Pathway.
Canonical Wnt Pathway is dependent on secreted Wnt ligand for activation. When Wnt Pathway is inactive, β-catenin associates with the Destruction Complex: GSK3β, Axin, and APC in the cytoplasm. β-catenin phosphorylation is mediated by β-TrCP, and phosphorylated β-catenin is degraded via the proteasome. Wnt pathway is activated when Wnt ligand binds to Frizzled and LRP5/6. The Destruction Complex is disrupted and Axin is recruited to the plasma membrane, where it associates with LRP5/6. GSK3β is inhibited by Dishevelled (Dsh), and then β-catenin accumulates. Accumulated β-catenin translocates to the nucleus, where it associates with Tcf to drive Wnt target gene transcription.

Activated Wnt signaling is critical for intestinal stem cell maintenance. The stem cells in the intestinal crypt display elevated levels of nuclear β-catenin and Wnt target gene expression, whereas differentiated epithelial cells exhibit cytoplasmic β-catenin localization and lower expression of Wnt target genes (Radtke and Clevers, 2005). With certain mutations in the *APC* gene, the resulting mutant APC protein is unable to associate with the Apc-Axin-GSK3β destruction complex to inhibit and degrade β-catenin. The accumulation of nuclear β-catenin results in constitutive expression of Wnt pathway target genes (Radtke and Clevers, 2005). Constitutively active Wnt signaling drives proliferation of undifferentiated cells, eventually giving rise to tumors. Elevated Wnt target gene expression and accumulation of nuclear β-catenin is a hallmark of the majority of colorectal cancers (Fodde, 2002; Novellasdemunt et al., 2015; Rowan et al., 2000).

APC mutations are also the initiating step in the Vogelstein model of carcinogenesis (Fearon and Vogelstein, 1990; Powell et al., 1992; Su et al., 1992). Fearon and Vogelstein first proposed that colorectal cancer occurs through the sequential accumulations of mutations, with mutations in *APC* acting as initiating event in the sequence. In traditional adenomas, the Vogelstein model hypothesis generally holds true; loss of *APC* generates constitutively activated Wnt signaling which leads to dysplastic aberrant crypt foci (Siu et al., 1997; Yuan et al., 2001). Over time, additional mutations in other genes such as *KRAS, BRAF, SMAD4,* and *TP53* accumulate as the histology of the lesions progress from dysplasia to adenoma and eventually to adenocarcinoma (Fearon and Vogelstein, 1990; Yuan et al., 2001). Constitutively activated Wnt signaling through

sporadic *Apc* mutation contributes to tumorigenesis by generating a pro-growth environment that is more susceptible to tumorigenesis if subsequent sporadic mutations occur.

Apc-Mutant Mouse Models of Colorectal Cancer

In order to understand the contribution by different tissue compartments *in vivo* during both normal homeostasis and tumorigenesis, genetically engineered mouse models (GEMMs) represent a powerful tool available for researchers (Oshima and Oshima, 2012; Roper and Hung, 2012; Taketo and Edelmann, 2009). Mouse models allow for spatial and temporal control of gene expression in an *in vivo* system, ultimately providing a robust system to understand both normal and tumorigenic environments at the organismal level. GEMMs also allow for the investigation of the ramifications of specific genetic events *in vivo* on a time scale and cost scale that is far more amenable than human clinical trials (Roper and Hung, 2012).

The first mouse model for colorectal cancer, which is now known as the Apc^{Min} mouse, was discovered in 1990 by William Dove's group following a mutagen screen with the carcinogen ethylnitrosourea (Moser et al., 1990) and later determined to be caused by a nonsense mutation in the *Apc* gene (Su et al., 1992). The Apc^{Min} allele is transmitted via the germline in an autosomal dominant manner and yields a phenotype strikingly similar to the human FAP syndrome in both dominant inheritance pattern as well as the histological morphology of gastrointestinal polyps (Su et al., 1992). Apc^{Min}

mice allowed for in depth characterization of CRC progression in a mammalian model for the first time (Moser et al., 1990)

Because Apc^{Min} heterozygous animals die due to anemia within a few months of birth, breeding the animals is easiest when the male is carrying the mutation. Interestingly, somatic homozygous loss of *Apc* in $Apc^{Min/Min}$ animals was found to be embryonic lethal; homozygous embryos have a severe defect in the primitive ectoderm, thereby indicating the critical requirement for functional APC/Wnt signaling during early development (Moser et al., 1995).

Since the identification of the Apc^{Min} mutant, other transgenic mouse lines carrying somatic mutations in the *Apc* gene have been generated and characterized: $Apc^{1638N/+}$ (Fodde et al., 1994; Smits et al., 1997) , $Apc^{\Delta 716/+}$ (Oshima et al., 1995), $Apc^{\Delta 14/+}$ (Colnot et al., 2004), $Apc^{\Delta 15/+}$ (Robanus-Maandag et al., 2010), $Apc^{\Delta 1309/+}$ (Quesada et al., 1998), $Apc^{\Delta 508/+}$ (Kuraguchi et al., 2006), $Apc^{+/Min\text{-}FCCC}$ (Cooper et al., 2005), and $Apc^{+/\Delta e1\text{-}15}$ (Cheung et al., 2010). Additionally, an *Apc* floxed allele was generated and recapitulates the Apc^{Min} phenotype when Cre-recombination is induced in the intestinal epithelia (Shibata et al., 1997). All *Apc* mutant transgenic mice develop gastrointestinal polyps histologically similar to those in human FAP patients and patients with *APC* mutations.

Introducing a secondary mutation into an *Apc* mutant background is sufficient to accelerate tumor onset and severity in *Apc*-mouse models. Mouse models carrying mutations in either *Kras* or *Smad4* accelerate tumorigenicity when crossed into a mutant *Apc* background, although interestingly loss of the tumor suppressor *p53* does not

(Calcagno et al., 2008; Fazeli et al., 1997; Feng et al., 2011; Luo et al., 2009; Takuku et al., 1998; Trobridge et al., 2009). Overall, these experiments provide evidence that *Apc*-mutant mouse models recapitulate the canonical Vogelstein model of colorectal carcinogenesis. Therefore, these mouse models are a robust genetic tool for investigating tumor initiation and progression *in vivo*.

Hippo Signaling Pathway

While constitutively activated Wnt pathway signaling is the most common developmental pathway associated with colorectal cancer, other developmental signaling pathways have been implicated in tumorigenesis. One such developmental signaling pathway that plays a critical role in embryogenesis and is also commonly inactivated in a multitude of cancers is the Hippo Signaling Pathway. The Hippo Signaling Pathway was first characterized in *Drosophila melanogaster*, after members of the pathway were identified in a series of genetic screens for tissue growth regulation (Reddy and Irvine, 2008). Now known to be a critical regulator of organ size and growth control (Halder and Camargo, 2013; Halder and Johnson, 2011; Pan, 2010; Varelas, 2014; Zhao et al., 2010a), the Hippo Pathway takes its name from the fly *hpo* mutant; animals with mutant *hpo* in their imaginal eye discs develop oversized eyes and wrinkled head phenotype, reminiscent of the wrinkled skin of a hippopotamus (Udan et al., 2003). Mutations in Warts (Wts), another protein kinase in the Hippo Pathway, result in a similar phenotype to Hippo (Hpo) mutants. Genetic loss of either *hpo* or *wts* in developing imaginal discs

results in flies with enlarged organs as adults due to increased cellular proliferation and decreased apoptosis (Harvey et al., 2003; Jia et al., 2003; Justice et al., 1995; Pantalacci et al., 2003; Udan et al., 2003; Wu et al., 2003; Xu et al., 1995). The Hippo Pathway in *Drosophila* has since been shown to be a core protein kinase cascade pathway that restricts cellular proliferation and promotes apoptosis, thereby regulating organ and tissue size during development (Jia et al., 2003; Pantalacci et al., 2003; Wu et al., 2003).

Hpo and Wts are the core components of the Hippo Pathway along with Sav (Salvador) and Mats (Mob As a Tumor Suppressor) (Kango-Singh et al., 2002; Tapon et al., 2002). These proteins converge on the transcriptional co-activator Yorkie (Yki) to regulate gene expression of growth and proliferation targets. Yorkie does not bind to DNA directly, but rather associates with Scalloped, a TEAD family transcription factor, to drive downstream gene expression (Goulev et al., 2008; Huang et al., 2005; Wu et al., 2008).

Mechanistically, when the Hippo Pathway is active, Hpo complexes with Sav and then phosphorylates to activate Wts. Phosphorylated Wts associates with Mats and then phosphorylates Yki on three canonical Wts phosphorylation sites- serine 111, (S111), serine 168 (S168), and serine 250 (S250) (Dong et al., 2007; Huang et al., 2005). Canonical Hippo Pathway regulation of Yki is inhibitory; phosphorylation of Yki by Wts kinase inhibits Yki activity. Phosphorylated Yki is excluded from the cell nucleus and then can be sequestered by 14-3-3 proteins or sent to the proteasome for degradation (Oh and Irvine, 2008, 2009). The eye overgrowth phenotype observed in *hpo* mutant flies

hinted at the critical role that the Hippo Pathway plays in mammalian organ size and regulation.

Mammalian Hippo Signaling Pathway

The Hippo Pathway is not restricted to *Drosophila melanogaster*; in 2007 it was shown that the Hippo Pathway is also present in mammals, suggesting that it is an evolutionarily conserved pathway across species (Dong et al., 2007). The mammalian Hippo Pathway is also a kinase cascade pathway with a core set of pathway components homologous to the *Drosophila melanogaster* Hippo Pathway proteins. In the human pathway, the Hpo homologs are MST1/MST2 (Mammalian sterile-20-like kinase 1 and 2). MST1/MST2 associate with SAV1 to phosphorylate and activate the Wts homologs, LATS1/LATS2 (Large tumor suppressor 1 and 2) (Creasy and Chernoff, 1995a, b; Tao et al., 1999; Tapon et al., 2002; Yabuta et al., 2000).

Once phosphorylated, activated LATS1/LATS2 then phosphorylate the Yki homologs, YAP (Yes-associated protein) and its paralog TAZ (transcriptional co-activator with PDZ-binding motif). As in *Drosophila melanogaster*, phosphorylation of YAP/TAZ by LATS1/LATS2 drives YAP/TAZ out of the nucleus into the cytoplasm (Chan et al., 2005; Hao et al., 2008; Lei et al., 2008; Oka et al., 2008; Zhang et al., 2008; Zhao et al., 2010b; Zhao et al., 2007). Like Yorkie, YAP/TAZ cannot bind DNA directly but must instead bind to transcriptional co-activator proteins, predominantly TEAD1-4, to

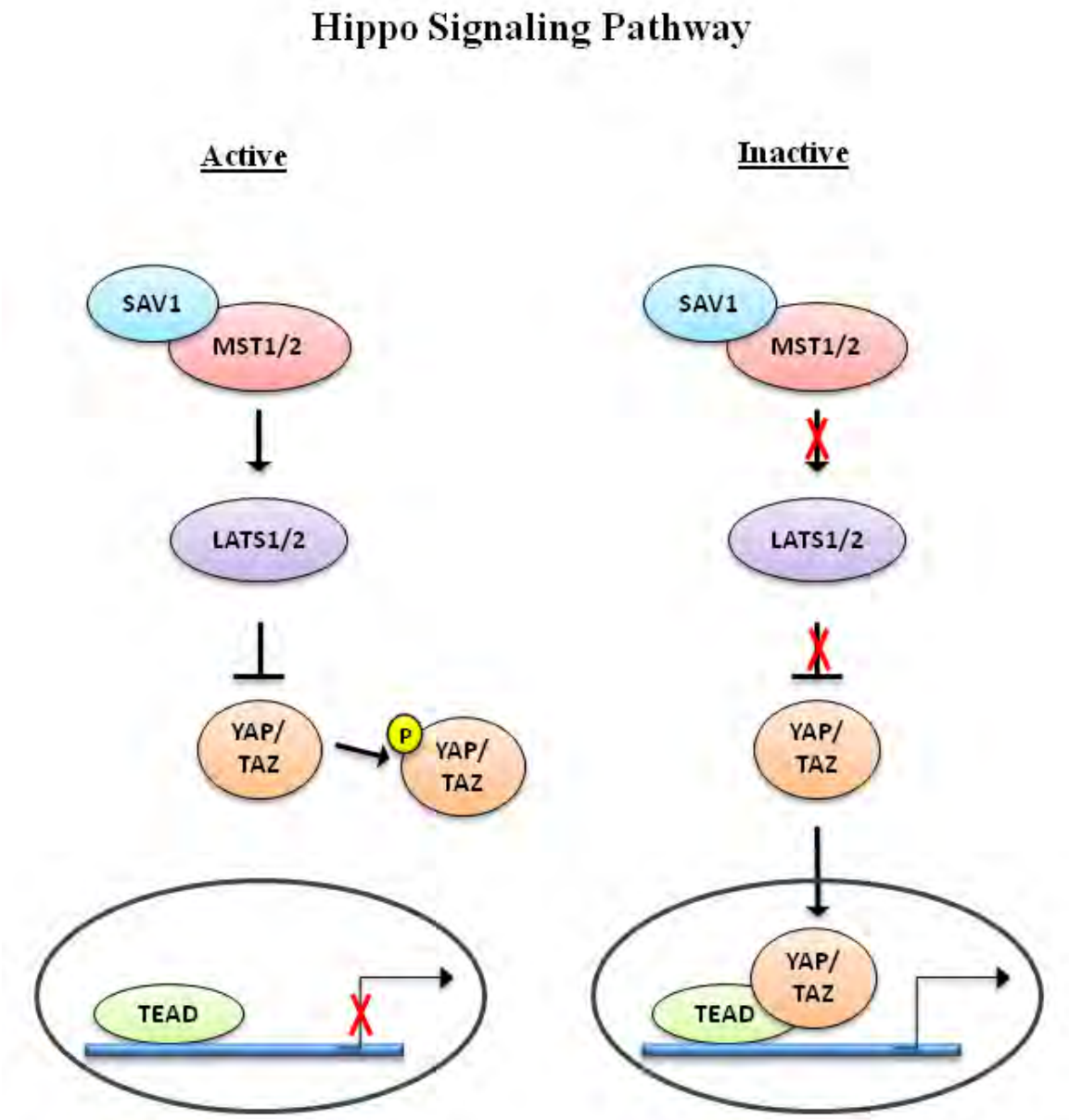


Figure 1.2. The Mammalian Hippo Signaling Pathway.
When Hippo Pathway is activated, MST1/2 phosphorylates and activates LATS1/2, which phosphorylate and inhibit YAP/TAZ. Phosphorylated YAP/TAZ are sequestered in the cytoplasm by 14-3-3 proteins and can also be degraded via the proteasome. When upstream Hippo Signaling is inactivated, LATS1/2-mediated inhibitory phosphorylation is released and YAP/TAZ translocate to the nucleus, where they associate with TEAD proteins to drive transcription of downstream target genes.

regulate transcription of downstream gene targets (Chan et al., 2009; Vassilev et al., 2001; Zhang et al., 2009). However, our lab and others have implicated AP1 as an additional YAP transcriptional co-activator in certain contexts (Liu et al., 2016; Zanconato et al., 2015).

YAP and its paralog TAZ share 45% amino acid sequence homology and are both WW-domain containing proteins; YAP contains two WW domains and TAZ contains one WW domain. WW domains recognize the proline-rich PPxY motif sequence on many transcription factors and are believed to be important for transcriptional activation. YAP/TAZ both contain N-terminal TEAD transcription factor binding domains and associate with TEAD1-4 at this binding site (Varelas, 2014). Additionally, both YAP and TAZ contain binding sites for 14-3-3 proteins. When their 14-3-3 binding sites are phosphorylated, YAP/TAZ bind to 14-3-3 proteins and are sequestered in the cytoplasm (Kanai et al., 2000; Yagi et al., 1999).

YAP is phosphorylated by LATS1/LATS2 kinase on 5 critical serine sites: S61, S109, S127, S164, and S381, all located within conserved HXRXXS recognition sequences. A sixth serine, S94, has been identified as a LATS1/LATS2 phosphorylation site, but the S94 site is more involved with YAP binding to TEAD proteins, rather than cytoplasmic sequestration (Zhao et al., 2008). A mutant YAP5SA construct, with the 5 canonical LATS1/LATS2 phosphorylation sites mutated from serine to alanine, has been shown to render YAP impervious to LATS-mediated inhibitory phosphorylation. This results in the YAP5SA protein accumulating in the cell nucleus and driving target gene transcription, resulting in upregulation of growth and invasion in cell lines (Zhao et al.,

2008). TAZ also has four canonical LATS1/LATS2 kinase recognition sequence motifs and is phosphorylated at S66, S89, S117, and S311. Mutating these four serines to alanine in TAZ4SA renders TAZ resistant to LATS-mediated inhibitory phosphorylation. Like the YAP5SA mutants, TAZ4SA results in a similar accumulation of nuclear TAZ and increase in growth (Lei et al., 2008)

When Hippo Pathway members MST1/MST2 are genetically ablated from the liver *in vivo* using Cre-Lox alleles, a liver overgrowth phenotype is observed (Lee et al., 2010; Lu et al., 2010; Song et al., 2010). Additionally, two groups showed in 2007 that doxycycline-induced liver specific overexpression of either YAP or YAP-S127A quickly induced hepatomegaly due to increase in cellular proliferation (Camargo et al., 2007; Dong et al., 2007). Importantly, this overgrowth was completely reversible; once mice were taken off doxycyline to stop expression of the YAP transgene, the mutant livers quickly returned to the same size as wildtype liver (Camargo et al., 2007; Dong et al., 2007).

These significant findings revealed that in mammals, organs sense the size they are supposed to attain during growth and development, and conclusively identified the Hippo Pathway as a master regulator of this important developmental size control mechanism.

Upstream Regulation of Hippo Pathway

The core components of the Hippo Pathway include the kinase cascade from MST1/MST2 to LATS1/LATS2 to YAP/TAZ. Beyond the core kinase cascade, a number of cellular mechanisms have been shown to interact with the core Hippo Pathway components under a myriad of conditions to ultimately converge on YAP/TAZ regulation (Meng et al., 2016).

In addition to evidence that MST1/MST2 proteins undergo autophosphorylation (Praskova et al., 2004), MST1/MST2 have also been shown to be activated through direct phosphorylation by TAO kinases 1/2/3 (Boggiano et al., 2011; Poon et al., 2011). Independently of canonical Hippo Pathway regulation by MST1/MST2, LATS1/LATS2 can be phosphorylated by other protein kinases. MAP4K proteins have been shown to directly phosphorylate and activate LATS1/LATS2 under certain conditions, thereby inhibiting downstream YAP/TAZ activity (Meng et al., 2015; Zheng et al., 2015). Additionally, our lab helped collaborators show that in *Drosophila*, MAP4K4 homolog Msn can directly phosphorylate Wts to negatively regulate Yki in enteroblasts in the fly gut (Li et al., 2014a). Therefore, there is evidence that MAP4K protein kinases can impact Hippo Pathway members through direct phosphorylation of both LATS1/2 as well as YAP/TAZ.

Cell-cell contact and cell density have also been shown to regulate Hippo Pathway signaling (Nishioka et al., 2009; Ota and Sasaki, 2008; Zhao et al., 2007). When cells are seeded at very high density, the Hippo Pathway is activated to phosphorylate

YAP/TAZ through LATS1/LATS2. At conditions of low cell density, the Hippo Pathway is inactivated and the inhibitory phosphorylation on YAP/TAZ is released to allow YAP/TAZ to translocate to the nucleus and upregulate target genes for proliferation and growth. Contact-mediated YAP inhibition has been shown to be required for different developmental processes, including the specification of the trophectoderm during early mouse development (Nishioka et al., 2009).

Related to cell-cell contact, YAP/TAZ have also been shown to be responsive to mechanical forces generated by the extracellular matrix. When cells are plated on ECM with varying stiffness, YAP/TAZ subcellular localization changes as a result of the tension forces placed on the actin cytoskeleton by the ECM (Dupont et al., 2011). Recently, it has been shown that stretch forces transferred to the nuclear envelope via the cytoskeleton is critical for YAP/TAZ activation in mesenchymal stem cells (Driscoll et al., 2015). Regulation of nuclear YAP after cell attachment to ECM is through activation of Rho-GTPases (Yu et al., 2012). Additionally, LATS1/LATS2 have been shown to be activated through cell detachment, resulting in inhibition of YAP/TAZ (Zhao et al., 2012). These data suggest the possibility that the mechanical forces that exist within growing tissues may be a mechanism through which the Hippo Pathway senses overall organ size.

The Hippo Pathway could also be sensing external stimuli through direct interactions with the actin cytoskeleton. This interaction has been shown to be mediated in part by interactions with the angiomotin protein family. Angiomotin family proteins localize to tight junctions, are known to be important for cell-cell contact, and also

interact with the F-actin cytoskeleton. Angiomotin family proteins have been shown to inhibit YAP/TAZ. The angiomotin family protein AMOTL2 is a scaffolding protein that binds both LATS2 and YAP/TAZ, which promotes LATS-mediated YAP/TAZ phosphorylation (Paramasivam et al., 2011; Zhao et al., 2011). Angiomotins are also direct phosphorylation targets of LATS1/LATS2 (Dai et al., 2013). Once phosphorylated by LATS1/LATS2, the interaction between AMOT and actin fibers is disrupted, resulting in reduction of focal adhesions and the inhibition of cell migration. These data provide further evidence that cell-cell contact and organization of the actin cytoskeleton, in this case through the angiomotions, represents a critical upstream regulation of Hippo Pathway signaling.

In addition to cell-cell contact, the Hippo Pathway can be regulated by G-protein coupled receptors (GPCRs) and Rho-GTPases. Secreted factors, such as hormones, have been shown to regulate YAP/TAZ activity through GPCRs. GPCRs can both inhibit and activate YAP/TAZ, dependent on context and GPCR receptor type. For example, LATS1/LATS2 are inhibited, and YAP/TAZ subsequently activated, by G12/13-coupled receptors when HEK293A and MCF10A cells are stimulated with either lysophosphatidic acid (LPA) or sphingosine 1-phosphophate (S1P) (Yu et al., 2012). In contrast, when Gs-coupled receptors are stimulated with the hormones glucagon or epinephrine, LATS1/LATS2 are activated, and YAP/TAZ subsequently inhibited (Yu et al., 2012). Since this critical finding, other groups have confirmed GPCR regulation of YAP/TAZ to be universally conserved mechanisms to both positively and negatively regulate Hippo

Pathway signaling (Miller et al., 2012; Mo et al., 2012; Yu et al., 2013; Zhou et al., 2015b).

Finally, the Hippo Pathway has recently been shown to regulate itself through a YAP/TAZ feedback regulation mechanism (Dai et al., 2015; Meng et al., 2015; Moroishi et al., 2015b). Knockout of YAP in either liver or intestinal epithelia results in the accumulation of TAZ. YAP directly activates its own upstream inhibitors LATS2 and AMOTL2 through transcriptional upregulation (Dai et al., 2015). Additionally, both YAP and TAZ directly activate LATS1/LATS2 in MCF10A and HEK293A cells (Moroishi et al., 2015b). YAP/TAZ feedback regulation also explains the phenotype observed in Yap^{S112A} transgenic mice. In these mice, animals appear normal and healthy unless stressed with the carcinogen diethylnitrosoamine (DEN), and animals then develop hepatocellular carcinoma (Chen et al., 2015). This overall phenotypic normality in Yap^{S112A} transgenic mice may be due to phosphorylation at other LATS1/LATS2 phosphorylation sites other than S112. However, this phenotype could also be caused in part by feedback regulation; TAZ is downregulated in response to the increase in YAP protein.

Upstream regulation of YAP/TAZ by canonical Hippo Pathway as well as non-canonical regulation is both multi-faceted and context-specific. Continued exploration into how this complex regulation functions during development and homeostasis will shed insight into how aberrant Hippo Pathway activation contributes to tumorigenesis.

Hippo Pathway in Cancer

The Hippo Pathway plays a critical role in organ size control during development and regeneration. Through strict regulation of YAP/TAZ, upstream kinases maintain homeostasis and prevent uncontrolled cell growth. However, nuclear YAP/TAZ is associated with many human cancers. Thus, similar to the aberrant activation of the Wnt and Hedgehog developmental signaling pathways, constitutive YAP/TAZ activity is also considered to be tumorigenic.

Many human cancers display elevated levels of nuclear YAP/TAZ protein (Chan et al., 2008; Fernandez et al., 2009; Harvey et al., 2013; Johnson and Halder, 2014; Mo et al., 2014; Moroishi et al., 2015a; Overholtzer et al., 2006; Steinhardt et al., 2008; Xu et al., 2009; Zender et al., 2006). In MCF10A cells, YAP overexpression is sufficient to induce epithelial-to-mesenchymal transition (EMT) and increase proliferation (Overholtzer et al., 2006). In hepatocellular carcinoma cells, YAP overexpression increases tumor growth after xenograft (Zender et al., 2006). TAZ protein has also been shown to be overexpressed in highly invasive breast cancer cell lines and knockdown of TAZ is sufficient to reduce invasion (Chan et al., 2008). Overall, these studies indicate that YAP/TAZ activity is oncogenic in solid tumors.

Recent studies have continued to investigate whether nuclear YAP/TAZ can be considered a bona fide prognostic marker for overall survival in human patients. In human hepatocellular carcinoma (HCC) tumor samples, one group reported 62% of HCC cases examined displayed nuclear YAP staining patterns and found a positive correlation

between nuclear YAP and more undifferentiated tumor cells (Xu et al., 2009). Another group investigated non-small cell lung cancer (NSCLC) and found nuclear YAP expressed in 66.3% (61/92) patient cases, and identified a correlation between nuclear YAP expression and shorter overall patient survival (Wang et al., 2010). A similar study in NSCLC patients showed a similar correlation between upregulation of TAZ protein in 66.8% (121/181) patients and shorter overall survival (Xie et al., 2012b). Finally, in breast cancer cell lines, higher levels of TAZ protein expression indicated greater resistance to the chemotherapeutic agent taxol through activation of downstream target genes *CTGF* and *CYR61*, and knockdown of TAZ was sufficient to sensitize cells to taxol treatment (Lai et al., 2011). These studies provide support that nuclear YAP/TAZ indicates a poor overall prognosis in a number of solid tumor cancers.

YAP/TAZ nuclear localization has also been shown to be a prognostic marker in gastrointestinal cancers. In esophageal and gastric adenocarcinomas, YAP protein levels were significantly increased in both cytoplasm and nucleus (Lam-Himlin et al., 2006). In gastric cancer, another group investigated gastric cancer patient samples and found high TAZ protein expression in 77.4% (113/146) of samples investigated, and when stratified by subtype of cancer, saw that signet cell carcinoma patient samples exhibited high TAZ protein expression in 85.7% of samples (Yue et al., 2014). Finally, in colorectal cancer patients, a survey of data from 522 patients revealed that patients with high levels of *Taz* or the YAP/TAZ target genes expression had significantly shorter overall survival (Yuen et al., 2013).

Overall, solid tumor samples from human patients showed a strong correlation between invasive cancer and nuclear YAP/TAZ levels in breast, lung, and colorectal cancer (Steinhardt et al., 2008). These data also provide evidence that inhibiting YAP/TAZ activity with targeted therapies may be a beneficial strategy to inhibit cancer cell growth.

When YAP/TAZ translocate to the nucleus, they bind to the TEAD family of transcription factors. YAP/TAZ are unable to bind DNA directly, and must instead interact with DNA-binding proteins in order to induce target gene expression (Vassilev et al., 2001). Therefore, nuclear accumulation of YAP/TAZ can be oncogenic due to YAP/TAZ functioning as transcriptional co-activators to upregulate genes involved with growth and proliferation. Additionally, in certain contexts YAP/TAZ/TEAD can associate with additional transcription factors to drive target gene expression and promote oncogenesis. For example, we and others have shown that AP-1 and TEAD cooperate to increase YAP/TAZ target gene expression in cancer cell lines, which results in increased migration and invasion (Liu et al., 2016; Zanconato et al., 2015).

While a correlation between accumulation of nuclear YAP/TAZ and poor prognosis for overall patient survival has been observed in many different types of solid tumors, it is surprising how few somatic or germline mutations have been identified in the Hippo Pathway in patients (Johnson and Halder, 2014; Moroishi et al., 2015a; Zanconato et al., 2016). One explanation for this observation could be an increase in gene copy number; genomic amplification of the *Yap* gene has been observed in mouse mammary tumors (Overholtzer et al., 2006). This could explain why YAP/TAZ proteins

are often observed to be overexpressed in human tumors despite the lack of genetic mutations. Another confounding observation is that despite playing a critical role in growth and differentiation, the list of canonical YAP/TAZ target genes is relatively short. This conundrum was recently addressed in the discovery by Zanconato et al. that YAP/TAZ can act as super enhancers to increase gene transcription of general growth and proliferation genes through chromatin looping (Zanconato et al., 2015). The finding that YAP/TAZ play a critical role in gene expression by acting at super enhancer regions was also confirmed by Galli et al., with an additional finding that YAP/TAZ directly recruit the Mediator complex to super enhancer sites (Galli et al., 2015). These data suggest that the YAP/TAZ can contribute to tumorigenesis through many mechanisms.

Conversely, it also been recently discovered that YAP/TAZ can act as tumor suppressors instead of oncogenes. Therefore, loss of YAP/TAZ might actually be tumor promoting in certain contexts. One study showed that a number of breast cancer patients had lost YAP protein expression in their tumors, as assayed by immunohistochemistry staining of biopsy sections, as well as through sequencing (Yuan et al., 2008). Another study showed that shRNA knockdown of YAP in MDA-MB-231 cells was sufficient to protect from anoikis and to increase anchorage-independent cell growth (Yuan et al., 2008). Finally, in contrast to earlier work showing that YAP acts as an oncogene in the intestinal epithelia, Barry et al. showed that intestinal epithelial knockout of *Yap* was sufficient to drive elevated Wnt signaling and expansion of the stem cell population after radiation-induced injury (Barry et al., 2013; Cai et al., 2010; Camargo et al., 2007).

Overall, these findings highlight the complexity of YAP/TAZ activity in tumorigenesis and the importance for further exploration into their context-specific roles.

Finally, while the general canon in the Hippo Pathway field has been that YAP/TAZ act as transcriptional co-activators to drive transcription of gene targets for growth and proliferation, it has recently been shown that under certain circumstances, YAP/TAZ can act as transcriptional co-repressors. Kim et al. showed that in MCF10A cells, YAP/TAZ-TEAD directly recruit and interact with the NURD complex to repress expression of the tumor suppression genes *DDIT4* and *TRAIL* (Kim et al., 2015b). When YAP/TAZ are removed from the system via siRNA knockdown, YAP/TAZ/NURD-repression of *DDIT4* and *TRAIL* is relieved, the promoters are acetylated, and the tumor suppressor genes are actively expressed. YAP/TAZ are still functioning as oncogenes; however, they are acting as transcriptional co-repressors of known tumor suppressor genes, rather than transcriptional co-activators of oncogenes.

Recent studies have elucidated the complex relationship between YAP/TAZ activity and tumorigenesis; it is no longer sufficient to assume that accumulation of nuclear YAP/TAZ is an oncogenic event in every situation. Further exploration into the context-specific function of nuclear YAP/TAZ and how it relates to tumorigenesis is desperately needed to help tease apart the intricacies of this complex pathway.

Molecular Compounds to Target the Hippo Pathway

While recent work has suggested that YAP/TAZ activity can be both tumor suppressive as well as oncogenic, there remains clinical interest in developing pharmacological agents to target Hippo Pathway members. Directly inhibiting YAP/TAZ activity or activating upstream Hippo Pathway members in human cancers with nuclear accumulation of YAP/TAZ is an attractive therapeutic strategy (Steinhardt et al., 2008). Many groups have shown that knockdown of either YAP/TAZ or TEAD proteins is sufficient to reduce growth and proliferation in cancer cell lines or mouse models of tumorigenesis (Ota and Sasaki, 2008; Wu et al., 2008; Zhang et al., 2009; Zhao et al., 2008). Therefore, an ideal candidate for YAP/TAZ inhibition could act through a number of mechanisms (e.g. inhibit YAP/TAZ protein, activate LATS1/LATS2, or inhibit TEAD proteins) to achieve the desired outcome: inhibition of YAP/TAZ-directed transcription of growth and survival genes in cancer cells.

Initial efforts to generate drugs to inhibit YAP/TAZ in patients focused on identifying small molecule compounds that inhibit YAP activity. A small molecule screen identified verteporfin, a member of the porphyrin molecule family, as a direct YAP protein inhibitor. Verteporfin binds to YAP directly and disrupts the YAP-TEAD interaction (Liu-Chittenden et al., 2012). Treatment of HEK293 cells with verteporfin increases trypsin-mediated degradation of YAP protein, and inhibited YAP-driven liver enlargement in mouse models with YAP overexpression (Liu-Chittenden et al., 2012). This identification of verteporfin as a YAP-inhibitor was particularly promising because

verteporfin had already been shown to be well-tolerated in human patients, and had been used in the clinic as a photodynamic therapy for macular degeneration (Michels and Schmidt-Erfurth, 2001). Verteporfin has since been shown to be effective in inhibiting retinoblastoma (Rb) cell growth *in vitro* through inhibition of YAP-TEAD (Brodowska et al., 2014). It has also been shown to inhibit growth of uveal melanoma cells carrying mutated copies of either *GNAQ* or *GNA11,* mutations that have been shown to activate YAP protein, both *in vitro* and *in vivo* in xenograft models (Yu et al., 2014). Overall, these *in vitro* studies suggested that verteporfin was a robust YAP inhibitor and could be a groundbreaking new drug for patients with cancer.

However, despite the excitement surrounding verteporfin as a promising cancer drug, clinical trials have proved to be less exciting. Verteporfin is a robust YAP inhibitor *in vitro*, but this inhibitory effect is not nearly as robust when the drug is administered systemically (Gibault et al., 2016). Indeed, while it has been shown that verteporfin is able to inhibit growth of colorectal cancer cells *in vitro*, this anti-tumorigenic effect was found to be due to a YAP-independent mechanism; verteporfin was found to inhibit p62 and STAT3 to inhibit colorectal cancer cell growth (Zhang et al., 2015). Additionally, verteporfin has been shown to significantly inhibit growth of cancer cell lines derived from other solid tumors that have been depleted of YAP protein through shRNA knockdown (Zhang et al., 2015). Based on these findings, verteporfin remains an important tool for *in vitro* experiments studying YAP inhibition, but is not an ideal drug for systemic treatment *in vivo* in mice or humans (Gibault et al., 2016).

More recent efforts to generate YAP/TAZ inhibitors have been focused on developing a drug capable of interfering with the YAP-TEAD binding interaction (Liu-Chittenden et al., 2012; Pobbati and Hong, 2013). When TEAD1 protein is mutated at just a single site, Y406H, in BEL-7404 hepatocellular carcinoma cells, the YAP-TEAD interaction is disrupted and growth of xenograft tumors is inhibited (Zhou et al., 2015c). Additionally, one group developed 17-mer peptides that bind directly to TEAD proteins at the YAP-TEAD binding interface to inhibit YAP activity (Zhou et al., 2015c). These studies suggest that small molecule inhibition of YAP-TEAD binding could provide therapeutic benefit for patients with constitutive YAP activation.

Endogenous proteins that inhibit the YAP-TEAD interaction *in vivo* are also ideal candidates for targeted therapies. It may be therapeutically challenging to develop a drug that inhibits YAP activity directly at a concentration that has minimal side effects in humans. However, it may be easier to activate an endogenous protein with YAP-inhibitory function. One such protein, Vestigial-like protein 4 (VGLL4), was recently shown to disrupt the YAP-TEAD interaction through competitive TEAD binding (Zhang et al., 2014). In both lung and gastric cancer, VGLL4 is downregulated in a significant number of patient cases, and when VGLL4 protein is added back to the system, tumor growth is inhibited (Chen et al., 2004; Jiao et al., 2014; Jin et al., 2011; Zhang et al., 2014). Additionally, a peptide that mimics the structure of VGLL4 binding site to the TEAD proteins also inhibits the YAP-TEAD interaction and results in growth inhibition of cancer cells both *in vitro* and *in vivo* (Jiao et al., 2014). Therefore, activation of an

endogenous YAP inhibitor such as VGLL4 represents a potential therapeutic strategy for cancer patients.

Overall, developing YAP/TAZ inhibitors is important for future research as well as eventual treatment strategies for patients. However, recent work has shown that YAP/TAZ activity and function is context-specific. Therefore, it is imperative that we understand YAP/TAZ activity during development and homeostasis in a range of tissues. This knowledge will ultimately improve our insight into how this pathway contributes to tumorigenesis.

Hippo Pathway in Mammalian Development

The Hippo Pathway is a major contributor to mammalian development, even as early as the pre-implantation stages of embryogenesis. YAP/TAZ nuclear localization is required in the patterning of the mouse embryonic trophectoderm. Cells in the outer layer of the developing mouse embryonic blastocyst, which eventually gives rise to the trophectoderm, display nuclear YAP/TAZ subcellular localization. By contrast, cells in the inner cell mass (ICM) display cytoplasmic YAP/TAZ localization (Nishioka et al., 2009). Similarly, siRNA knockdown of both LATS1/LATS2 early in development also results in severe developmental defects; mutant tissue is unable to differentiate into either epiblast or primitive endoderm tissues (Lorthongpanich et al., 2013). Finally, it was shown that the Hippo-regulated determination of cell polarity, which is critical for differentiation at pre-implantation development stages, is acting through the Angiomotin

proteins. This Amot-LATS interaction is critical for regulation of YAP/TAZ (Hirate et al., 2013). These findings illustrate that Hippo Pathway signaling is involved with a number of complex developmental processes during early embryogenesis.

Further evidence that Hippo Pathway activity is critical in early stages of embryogenesis comes from *in vivo* mouse model experiments. Simultaneous homozygous knockout of both *Yap* and *Taz* is embryonic lethal; mouse embryos lacking both YAP and TAZ die early in development at the morula stage due to cell fate defects (Nishioka et al., 2009). However, animals lacking just *Yap* or *Taz* survive past this early developmental stage. This indicates that YAP/TAZ can compensate for each other in this developmental instance through functional redundancy.

While YAP/TAZ exhibit functional redundancy at very early developmental stages, this relationship does not continue through embryogenesis. Whole body knockout of *Taz* is tolerated by animals and embryos survive through to birth, but adult $Taz^{-/-}$ animals develop renal cysts as they age (Hossain et al., 2007). This indicates that TAZ is dispensable for mammalian development and YAP is able to functionally compensate for loss of TAZ throughout the entirety of embryogenesis as well as most of adulthood. However, the reverse relationship is not true. Whole body knockout of *Yap* is embryonic lethal by E8.5 and $Yap^{-/-}$ embryos exhibited severe defects in yolk sac vasculogenesis (Morin-Kensicki et al., 2006). This indicates that TAZ is only able to compensate for YAP loss until E8.5, when YAP activity is critical for vasculogenesis.

YAP activity is required for embryonic development. However, too much YAP activity is similarly incompatible with development; YAP/TAZ function is tightly

regulated by upstream Hippo Pathway kinases. Whole body *Lats1* knockout animals survive through to adulthood, although *Lats1*$^{-/-}$ animals are prone to developing ovarian tumors and sarcomas (St et al., 1999). By contrast, *Lats2*$^{-/--}$ is embryonic lethal due to a cardiac development defect phenotype (McPherson et al., 2004). The important balance between YAP activity and upstream kinases during development is highlighted by an experiment that investigated MST1/MST2 activity during embryogenesis. Knockout of MST1/MST2 causes embryos to die around E8.5, and interestingly, mutant embryos exhibit similar phenotype to *Yap*$^{-/-}$ mutants. Specifically, *Mst1*$^{-/-}$*Mst2*$^{-/-}$ embryos display impaired vasculogenesis in both the yolk sac and embryo (Oh et al., 2009). Therefore, there exists a critical balance between YAP/TAZ activation and inhibition at this developmental timepoint, which is tightly controlled by canonical Hippo Pathway signaling.

Hippo Pathway signaling is also required in the lung epithelia during development. Genetic knockout of both *Mst1* and *Mst2* in the developing lung endoderm results in a lung differentiation defect phenotype. *Shh*^{Cre}*Mst1*$^{flox/flox}$*Mst2*$^{flox/flox}$ lung tissue exhibits elevated proliferation as well as accumulation of nuclear YAP protein (Lin et al., 2015). This phenotype is ameliorated when *Yap* is also knocked out in the MST1/MST2 null background. Additionally, knockout of YAP alone in the developing lung endoderm also results in a lethal phenotype; mutant embryos die at birth and exhibit a profound lung branching defect (Mahoney et al., 2014). Together, this suggests that canonical Hippo Pathway kinase activity is regulating YAP activity in the developing lung endoderm.

In addition to the previously discussed developmental requirements, Hippo Pathway signaling is also essential in the mammalian heart during embryonic development. When the conditional Cre driver *Nkx2.5*^{Cre} is used to knock out *Sav1* in the developing cardiac crescent in the heart, mutant animals develop cardiomegaly and eventually die after birth (Moses et al., 2001). Both genetic knockouts of *Lats1/Lats2* and *Mst1/Mst2* using the same *Nkx2.5*^{Cre} allele yield similar enlarged heart phenotypes due to increased cardiomyocyte proliferation (Heallen et al., 2011a). Additionally, overexpression of YAP using a Tet-On system for expression of a mutant *Yap-S127A* allele starting from E8.5 resulted in embryonic lethality by E15.5 due to heart failure caused by cardiomegaly (von Gisea et al., 2012). Together, these genetic experiments suggest that too much YAP activity is detrimental to proper heart development. Conversely, too little YAP activity is also incompatible to successful cardiac development. Genetic YAP knockout using *Tnnt2*^{Cre} to delete *Yap*^{flox} alleles at E12.5 in cardiomyocytes is sufficient to cause lethality in all mutant embryos by E16.5 due to heart failure (von Gisea et al., 2012).

Similar to what was observed with tightly regulated Hippo Pathway signaling contributing to differentiation during pre-implantation stages in the mouse embryo, YAP levels must also be balanced for successful cardiac development. Overall, tightly regulated control of YAP/TAZ by Hippo Pathway signaling is essential to maintain the optimal balance of nuclear YAP/TAZ in multiple key developmental processes.

Hippo Pathway in GI Homeostasis and Regeneration

The Hippo Pathway has also been shown to have critical functions in the mammalian intestine during both development and regeneration. The intestinal epithelia is highly proliferative in the crypt, where intestinal stem cells constantly divide and differentiate into different classes of epithelial cells to continually repopulate the epithelial compartment. These diverse classes include the paneth cells and endocrine cells, which reside in the crypt, as well as the enterocyte and goblet cells that leave the crypt to populate the surface of the villi. The cells on the surface of the villi are important for nutrient absorption (Barker et al., 2007; Paxton et al., 2003; Radtke and Clevers, 2005; Shaker and Rubin, 2010). The subcellular localization of YAP/TAZ protein has been well characterized in normal intestinal epithelial tissues; YAP/TAZ protein is predominantly cytoplasmic in the epithelial cells lining the villi and is predominantly nuclear in the crypt cells, where the ISCs reside (Barry et al., 2013).

Several groups have shown that constitutive YAP overexpression and/or accumulation of nuclear YAP in the mouse intestinal epithelia is sufficient to drive proliferation of undifferentiated ISCs (Azzolin et al., 2014; Cai et al., 2010; Camargo et al., 2007; Zhou et al., 2011). In 2007, Camargo et al. generated a Tet-On YAPS127A gain of function mouse allele which has the critical phosphorylation site for 14-3-3 binding mutated from serine to alanine. They showed that upregulated expression of the YAPS127A allele induces rapid proliferation of undifferentiated cells and tissue dysplasia in the intestinal epithelia, which is quickly reversed once expression of the

YAP transgene is turned off (Camargo et al., 2007). In 2010, Cai et al. reported that when the upstream Hippo Pathway component Salvador is knocked out in the intestinal epithelia, *Villin*^{Cre}*Sav1*$^{flox/flox}$ animals exhibited elevated epithelial cell proliferation, crypt hyperplasia, and eventually develop sessile serrated colonic polyps by 13 months of age. Polyp onset was greatly accelerated if animals are treated with dextran sodium sulfate (DSS) to induce chemical colitis (Cai et al., 2010). Additionally, when MST1/MST2 are knocked out in the intestinal epithelia, *Villin*^{Cre}*Mst1*$^{flox/flox}$*Mst2*$^{flox/flox}$ mutants exhibit reduced survival with a median survival of 13 weeks, in addition to highly proliferative, dysplastic crypts and polyps in the cecum (Zhou et al., 2011). These genetic experiments highlighted the link between YAP/TAZ activation and tumorigenesis in the intestinal epithelia.

While the observation that accumulation of nuclear YAP is sufficient to drive proliferation of undifferentiated ISCs and eventually lead to tumorigenesis is relatively straightforward, the opposite experiment, genetic ablation of YAP protein, has revealed a far more complex relationship between YAP protein and intestinal homeostasis/regeneration. Surprisingly, genetic knockout of YAP in *Villin*^{Cre}*Yap*$^{flox/flox}$ mutants yielded no observable phenotype. Mutant animals appear healthy and phenotypically normal with no differentiation defects (Barry et al., 2013; Cai et al., 2010). As the *Villin*^{Cre} transgene expression induces Cre-mediated recombination of floxed alleles in all intestinal epithelial cells starting from E12.5 (Madison et al., 2002), this indicates that YAP protein is completely dispensable for normal intestinal homeostasis starting at E12.5 and through postnatal stages. One hypothesis to explain

why YAP knockout yields no phenotype in the developing intestinal epithelia is that endogenous TAZ protein could be functionally compensating for YAP. However it has recently been shown by a number of groups that knockout of both YAP and TAZ in the intestinal epithelia does not cause any phenotype in double knockout animals under normal intestinal homeostasis conditions (Azzolin et al., 2014; Cai et al., 2015; Zanconato et al., 2015). These data show that YAP/TAZ are completely dispensable in the intestinal epithelia during development, which is in contrast to many other tissues such as the lung and heart. Further research is needed to fully understand why YAP/TAZ are not required in the intestinal epithelia during development and homeostasis.

While not required under normal conditions, a severe regeneration phenotype has been observed when the YAP-deficient intestinal epithelium is injured. As briefly discussed in an earlier section (see *Hippo Pathway in Cancer*), Cai et al. first showed that an intestinal epithelial knockout of *Yap* was phenotypically normal under conditions of homeostasis but generated a severe regeneration phenotype following injury (Cai et al., 2010). When $Villin^{Cre}Yap^{flox/flox}$ animals were treated with DSS, mutant animals experienced increased mortality and complete loss of intestinal crypts, in addition to a significant reduction in epithelial cell proliferation and an increase in apoptotic cells. Cai et al. concluded that this severe phenotype indicated that YAP protein was dispensable for intestinal epithelial development but becomes essential during tissue regeneration (Cai et al., 2010).

In 2013, Barry et al. published work that directly contradicted Cai et al.'s findings. Barry et al. claimed that YAP is acting as a tumor suppressor, rather than an

oncogene, in the intestinal epithelia to inhibit growth and regeneration (Barry et al., 2013). Using the same genetic knockout strategy, $Villin^{Cre}Yap^{flox/flox}$, Barry et al. induced intestinal tissue damage using whole-body irradiation instead of treatment with DSS. Instead of observing the complete loss of intestinal crypt cells due to apoptosis, they observed expansion of undifferentiated $Lgr5^{+}$ stem cells in the intestinal stem cell niche (Barry et al., 2013). From these data, they concluded that epithelial YAP restricts ISC growth during regeneration.

However, it must be noted that the two methods of injury-induction could be the cause for the contrasting observations. DSS treatment acts directly on the intestinal epithelia by increasing epithelial cell permeability and inducing acute colitis (Tamaru et al., 1993). Whole-body irradiation damages rapidly dividing cells, such as intestinal epithelial cells. Unlike DSS, irradiation also affects non-epithelial cells in the gastrointestinal tract such as adjacent mesenchymal cells (Potten, 1990). One hypothesis to explain the differing phenotypes is that the irradiation affects the signaling crosstalk between tissue compartments in the intestinal epithelia. Regardless, these two findings highlight the complexity that exists in Hippo Pathway regulation in the intestinal epithelia as well as the importance of context-specificity.

Intersection between Hippo Pathway and Wnt Pathway

One complexity is the intersection between Hippo and Wnt Pathway signaling in the intestinal epithelia. In certain contexts, nuclear YAP/TAZ activates Wnt pathway in

the intestinal epithelia. Imajo et al. reported that the phosphorylated YAP/TAZ directly binds to phosphorylated β-catenin (Imajo et al., 2012). The authors concluded that Hippo Pathway kinases inhibit Wnt target gene expression through this protein-protein interaction. Therefore, when upstream Hippo Pathway kinase activity is lost and YAP/TAZ translocate to the nucleus to drive gene expression, the phosphoYAP/TAZ-mediated inhibition of β-catenin is also relieved.

YAP/TAZ have also been shown to inhibit Wnt pathway signaling in the intestinal epithelia. One group found that YAP acts to inhibit Wnt pathway signaling independently of the APC-Axin-GSK3β destruction complex through inhibition of the Wnt effector, DVL (Barry et al., 2013). They observed that Wnt pathway signaling was upregulated, and undifferentiated stem cells were expanded in YAP-deficient tissue following injury caused by either irradiation or treatment with the Wnt agonist R-spondin. These observations supported a similar previous finding, when Varelas et al. reported that TAZ acts to inhibit Wnt/β-catenin pathway signaling through direct binding to, and inhibition of Dishevelled in the mouse kidney (Varelas et al., 2010). More recently, another group also showed that during intestinal regeneration, YAP inhibits Wnt pathway signaling to prevent differentiation while allowing for $Lgr5^{+}$ stem cells to be reprogrammed (Gregorieff et al., 2015). Collectively, these data suggest that YAP inhibits Wnt pathway in the intestinal epithelia during regeneration.

Reciprocal regulation, with Wnt signaling regulating YAP/TAZ independently of canonical Hippo Pathway kinases, has also been proposed. Azzolin et al. showed that YAP/TAZ were critical components of the β-catenin destruction complex during Wnt

signal transduction (Azzolin et al., 2014; Azzolin et al., 2012). The authors reported that TAZ acts in a non-canonical, Hippo Pathway-independent manner as part of activated Wnt pathway signaling. In that study, they observed that when Wnt signaling is inactive, phosphoryled β-catenin and phosphorylated TAZ both interact via the destruction complex. This interaction aids in ubiquitin-mediated degradation of phosphorylated TAZ (Azzolin et al., 2012). When Wnt signaling is activated, APC-Axin-GSK3β-mediated degradation of both β-catenin and TAZ is inhibited, and both proteins are able to accumulate, translocate to the nucleus, and drive transcription of downstream target genes (Azzolin et al., 2012). A few years later, the same group also reported that YAP functions in a similar manner as TAZ. Cytoplasmic YAP/TAZ bind to Axin when Wnt signaling is inactive. Through the interaction with Axin in the destruction complex, YAP/TAZ act to help inhibit β-catenin (Azzolin et al., 2014). When Wnt signaling is activated, YAP/TAZ and β-catenin dissociate from the destruction complex and are able to translocate to the nucleus, where they associate with the TEAD and TCF4 proteins, respectively, to drive gene target transcription (Azzolin et al., 2014). According to this model, Wnt pathway directly regulates YAP/TAZ independently of canonical Hippo Pathway signaling.

The relationship between YAP/TAZ and Wnt pathway signaling is complex in the intestinal epithelia. Conflicting molecular models have been proposed to explain the mechanistic interactions between the two pathways. However, one pair of observations has been observed by multiple groups: APC-mutant polyps exhibit elevated YAP/TAZ nuclear protein levels, and a genetic knockout of both *Yap* and *Taz* in the intestinal

epithelia is sufficient to inhibit APC-driven tumorigenesis in *Apc* knockout animals (Azzolin et al., 2014; Cai et al., 2015; Gregorieff et al., 2015). However, there is a lack of consensus in the field about the exact molecular interactions between YAP/TAZ and Wnt signaling, and more insight is needed to provide clarification for this important mechanism.

Hippo Pathway interactions with Hedgehog Pathway

In addition to the significant overlap with the Wnt pathway, the Hippo Pathway also crosstalks with other developmental signaling pathways in a myriad of contexts. One group reported that YAP/TAZ interacts with the mTOR growth pathway through regulation of the PTEN tumor suppressor via miR-29 (Tumaneng et al., 2012). Another group recently showed that YAP/TAZ regulates TGFβ signaling to control liver cell differentiation and proliferation (Lee et al., 2016). YAP/TAZ has been linked to the Hedgehog signaling pathway as well, although the exact interaction between the two pathways appears to be highly context-specific.

A number of groups have reported that Hedgehog signaling acts upstream of YAP and regulates activity in differentiation. One such study showed that in *Drosophila melanogaster* ovarian follicle stem cells, Hedgehog signaling activated Yki by upregulating *yki* transcription (Huang and Kalderon, 2014). Another group showed that Hedgehog activates YAP in hepatic stem cells (HSCs) to drive proliferation and differentiation into myofibroblast cells during liver regeneration (Swiderska-Syn et al.,

2014; Swiderska-Syn et al., 2016). In cerebellar granuale neuron precursor cells (CGNPs), the Hedgehog ligand Sonic Hedgehog (Shh) upregulates YAP. This suggests that YAP is a downstream effector of Hedgehog Pathway signaling in medulloblastoma, a type of brain cancer commonly associated with mutations in the Hedgehog Pathway (Fernandez et al., 2009).

The reciprocal relationship has also been observed, wherein YAP acts upstream of Hedgehog Pathway signaling in stem cell maintenance and differentiation. One recent publication showed that in both mouse embryonic fibroblasts (MEFs) and in NIH-3T3 cells, YAP acts upstream of the Hedgehog pathway by activating Shh pathway signaling to prevent differentiation of neuronal progenitor cells (Lin et al., 2012). Interestingly, yet another group also showed that YAP acts upstream of Hedgehog signaling, but conversely showed that YAP inhibits Shh signaling in MEFs and pancreatic stellate cells. They reconciled these contrary views by suggesting that Hedgehog activates YAP in a negative feedback loop (Tariki et al., 2014).

To reconcile the numerous examples of signaling crosstalk observed, it stands to reason that the Hippo Pathway interacts with a multitude of other developmental signaling pathways in both a temporal and spatial-dependent manner. Context specificity is integral to understanding how the Hippo Pathway interacts with other developmental signaling pathways, like Wnt and Hedgehog, in the gastrointestinal tract. To fully clarify this crosstalk, it is important to assess individual pathway function in a tissue-compartment specific manner during gastrointestinal development.

Mesenchyme in Development and Cancer

Mammalian Gastrointestinal Development

The mammalian gastrointestinal tract is comprised of two distinct tissue layers: the endoderm-derived epithelia and mesoderm-derived mesenchyme (Kedinger et al., 1998). During embryonic development, gastrointestinal development occurs as a finely timed sequence of events. First, the visceral endoderm recruits adjacent splanchnic mesoderm to form a primitive gut tube. This will eventually give rise to the organs in the gastrointestinal tract such as lung, stomach, pancreas, and small and large intestine. Following gastrulation, the E7.25 mouse embryo is shaped like a cup and has the endoderm lining the outside of the embryo structure, with the mesoderm located immediately underneath the endoderm. By E8.0, the endodermal tissue begins to invaginate at the anterior and posterior ends, forming the anterior intestinal portal (AIP) and caudal intestine portal (CIP). Both the AIP and CIP invaginate and eventually meet to create the fused gut tube. This process is occurring during the mouse turning process; therefore by E9.0 the embryo has completed turning and the gut tube is fused (Lawson et al., 1986; Spence et al., 2011).

Mesodermal-Endodermal Signaling in GI Development

While the gut tube is forming, signaling from the gut mesoderm to the endoderm is critical for establishing the GI anterior-posterior axis by E8.0 (Kiefer, 2003; Lewis and Tam, 2006; Wells and Melton, 1999, 2000; Zorn and Wells, 2007; Zorn and Wells, 2009). To pattern the A-P axis, the gut mesoderm expresses secretion factors such as FGF, Wnt, and BMP, as well as Wnt and BMP inhibitors, along expression gradients (Dessimoz et al., 2006; Kiefer, 2003; Kim et al., 2005; Kumar et al., 2003; McLin et al., 2007; Tiso et al., 2002). Mesodermally secreted ligands and inhibitors form an overlapping signal gradient that relays positional information to the adjacent epithelia, thereby establishing the GI axis.

In addition to the its role in A-P patterning, mesenchymal-epithelial signaling also plays a critical role in organogenesis. The lateral plate mesoderm has been shown to induce differentiation of pancreatic endoderm by inducing pancreatic-specific transcription factors such as *p48* and *Pdx1* (Kumar et al., 2003). One pathway important for mesenchymal-epithelial signaling is the Wnt pathway. Mesodermally-secreted Wnt antagonists inhibit Wnt pathway activity in the foregut endoderm, allowing for differentiation into pancreas tissue. Conversely, activation of Wnt pathway in the hindgut endoderm by mesodermally-secreted Wnt ligands allows for differentiation into intestinal tissue (McLin et al., 2007).

Mesenchymal-epithelial signaling is also involved with patterning and differentiation of adjacent intestinal epithelial cells. The forkhead transcription factor

Fkh6 is expressed specifically in the mesenchyme of the gastrointestinal tract to regulate intestinal cell proliferation. When *Fkh6* is genetically ablated in *Fkh6*$^{-/-}$ animals, the GI epithelia displays an increase in proliferation in addition to abnormal architecture and elongated villi (Kaestner et al., 1997). Additionally, Hedgehog-responsive mesenchymal cells in the gastrointestinal mesenchyme are involved with intestinal villi patterning (Walton et al., 2012).

Another mesenchymal-specific transcription factor, *Barx1*, has been well characterized for its role in stomach differentiation and patterning. Mesenchymal Barx1 protein induces differentiation of endoderm into stomach epithelia, while also inhibiting differentiation into intestinal epithelia through regulation of epithelial Wnt signaling. Genetic ablation of Barx1 in a whole body *Barx1* knockout is embryonic lethal by E13.0, and *Barx1*$^{-/-}$ embryos exhibit a significantly smaller and deformed stomach (Kim et al., 2005).

Mechanistically, Barx1 has been shown to inhibit Wnt signaling in the stomach epithelia through upregulation of the Wnt antagonist proteins, sFRPs. These antagonists, when secreted by the mesenchyme to the overlying endoderm, inhibit Wnt/β-catenin activity and thus allow differentiation into stomach epithelia instead of intestinal epithelia (Kim et al., 2005). Gastric epithelial differentiation, driven by mesodermal signals to the endoderm, begins by E15.5 in the mouse; the forestomach endoderm differentiates into stratified squamous epithelia and the hindstomach endoderm differentiates into glandular epithelia. The glandular stomach contains the gastric glands, which are somewhat

analogous to the crypt structures in the intestine and colon (Karam et al., 1997; van den Brink, 2007).

Like mesodermal-endodermal signaling, reciprocal signaling from the endoderm to the mesoderm is also critical during gastrointestinal differentiation (Buller et al., 2012; Huang & Cotton et al., 2013; Li et al., 2007; Mao et al., 2010; Zacharias et al., 2011). Secreted factors from the mouse stomach epithelium are involved with induction of smooth muscle differentiation in the underlying mesenchyme. This is detected by the upregulation of α-smooth muscle actin in mesenchymal progenitor cells starting from E12.0 (McHugh, 1995; Takahashi et al., 1998). This was first shown *in vitro* when it was observed that a mixture of epithelia and mesenchymal cells isolated from E11.0 embryos induced upregulation of the differentiation marker α-smooth muscle actin after a few days in culture. However, when E11.0 mesenchyme is cultured independently of the overlying epithelia, the mesenchyme is unable to differentiate (Takahashi et al., 1998). It was later discovered that the Hedgehog ligands Shh and Ihh are secreted by the stomach epithelium during development, with the Shh expression gradient highest in forestomach and Ihh expression gradient highest in the hindstomach. In this way, the stomach epithelia regulates differentiation of the underlying mesenchyme through the secretion of Ihh/Shh ligands (Ramalho-Santos et al., 2000).

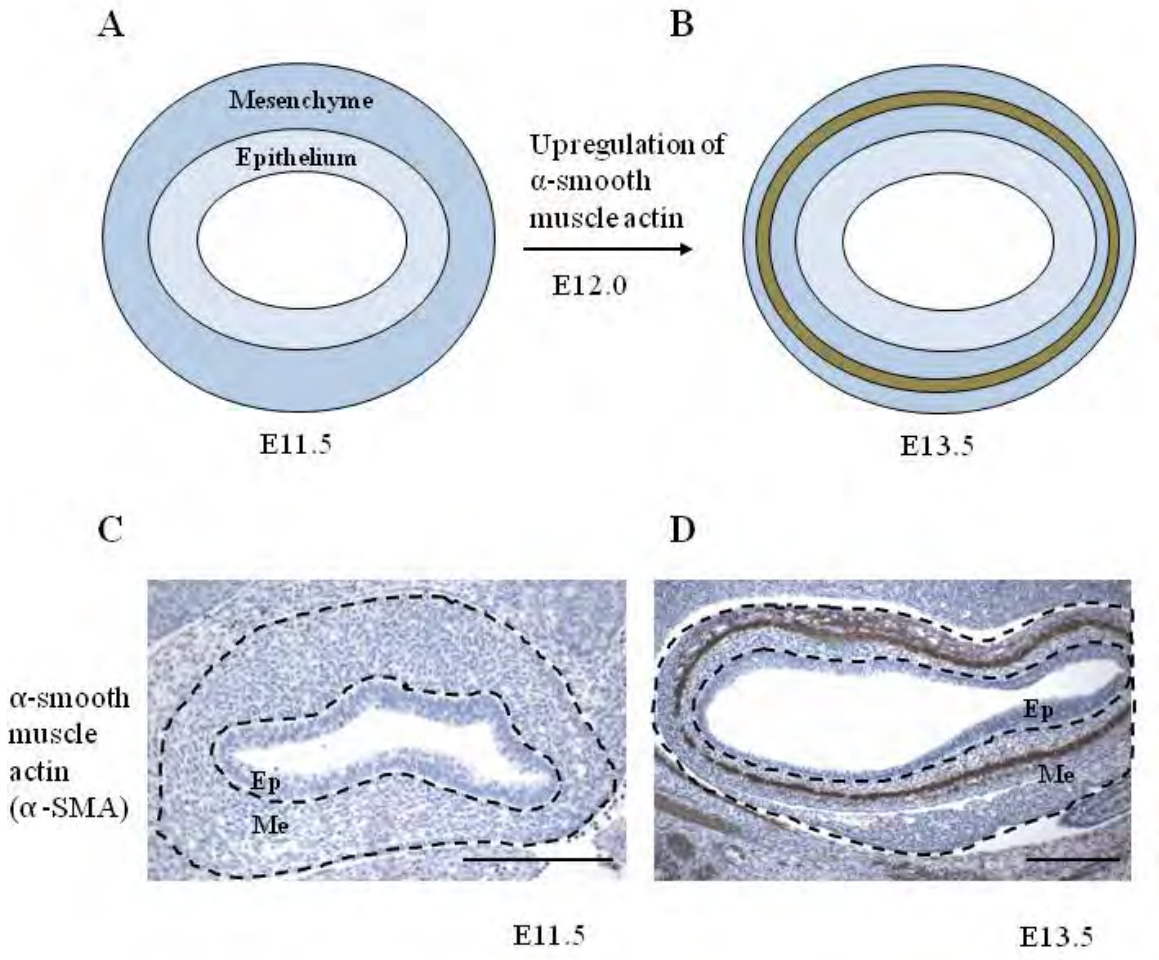


Figure 1.3. Differentiation in the gastrointestinal mesenchyme.
(A, C) In the gastrointestinal mesenchyme, the first marker for differentiation that can be easily detected by IHC/IF is the upregulation of α-smooth muscle actin (α -SMA), which is first detected at E12.0. *(B, D)* By E13.5, α-SMA staining is robustly detected in a tight band of differentiated smooth muscle progenitor cells in the gastrointestinal mesenchyme.

Hedgehog Pathway Signaling in GI Mesenchymal Differentiation

Paracrine Hedgehog pathway signaling is tightly linked to mesenchymal differentiation in the mammalian gastrointestinal tract (Huang & Cotton et al., 2013; Kolterud et al., 2009; Madison et al., 2005; Mao et al., 2010; van den Brink, 2007; Walton et al., 2012; Zacharias et al., 2011). In mammals, when the Hedgehog pathway is inactive, the transmembrane receptor Patched (Ptch) inhibits the transmembrane protein Smoothened (Smo). Hedgehog pathway signaling is activated when Hedgehog ligand binds to Ptch, thereby relieving the Ptch-mediated Smo-inhibition. Activated Smo relieves the Suppressor of Fused (Sufu)-mediated inhibition of Gli proteins, resulting in nuclear Gli translocation. Once in the nucleus, Gli directly binds to the DNA to drive transcription of downstream target gene expression (Varjosalo and Taipale, 2008). Paracrine Hedgehog pathway signaling provides a signaling bridge through which adjacent but dissimilar cells can communicate.

Hedgehog ligands can be secreted by both epithelial and mesenchymal cells. However, in the mammalian gastrointestinal tract only the GI epithelium secretes Hedgehog ligands, which bind to Ptch receptors expressed by mesenchymal cells (Kolterud et al., 2009). Both Shh and Ihh expression can be detected in the developing gastrointestinal endoderm as early as E8.5, and their expression is critical for both gastrointestinal development and proper development of the muscularis externa (Bitgood and McMahon, 1995; Mao et al., 2010).

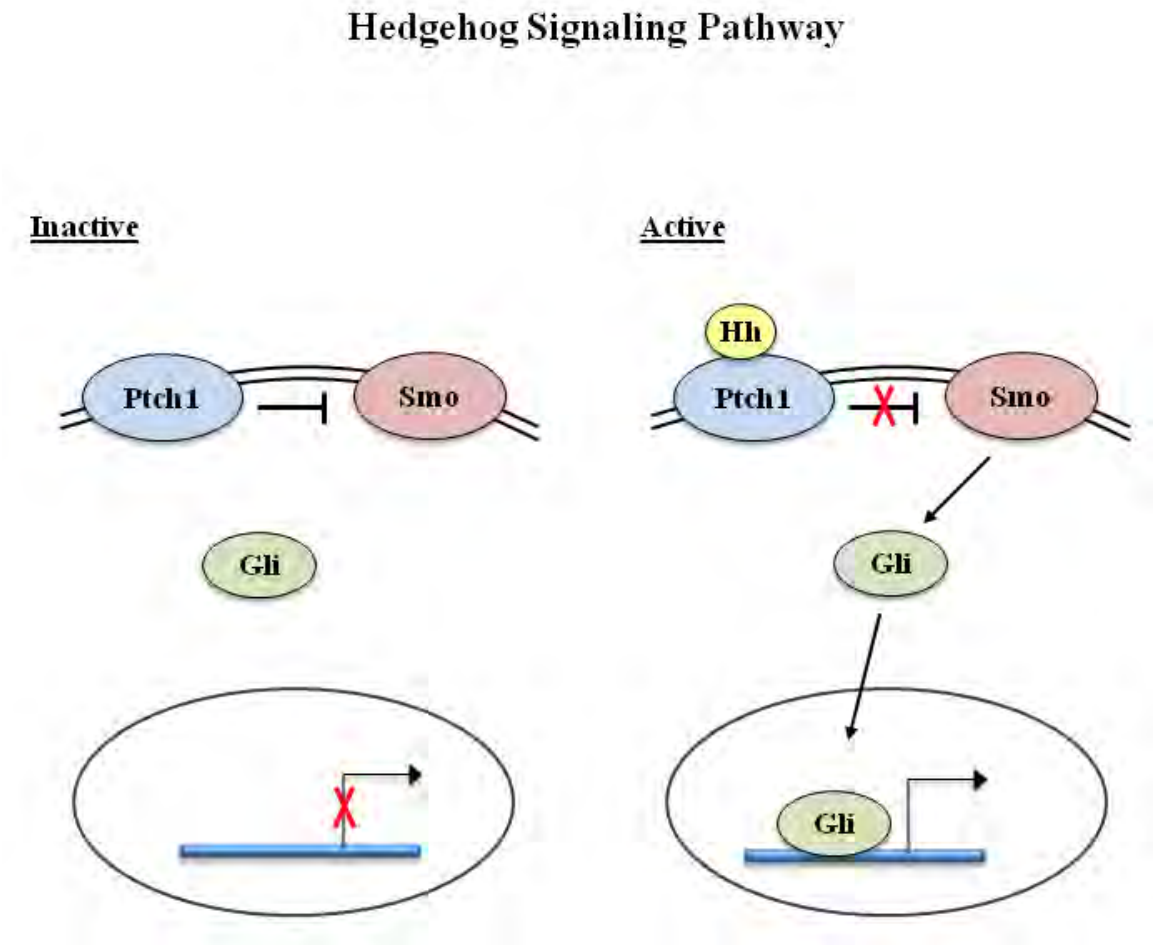


Figure 1.4. The Mammalian Hedgehog Pathway.
Canonical Hedgehog Pathway is dependent on secreted Hedgehog ligand for activation. When Hedgehog Pathway is inactive, the transmembrane receptor Patched (Ptch1) inhibits the transmembrane protein Smoothened (Smo). When Hedgehog ligand (Hh) binds to Ptch1, inhibition of Smo is released. Activated Smo activates Gli proteins, which then translocate to the nucleus, where they bind Gli-binding sites to activate transcription of Hedgehog target genes.

In a previous study, our lab knocked out both *Shh* and *Ihh* specifically in the developing gastrointestinal endoderm using Shh^{Cre}. We observed a severely diminished mesenchymal compartment by E18.5, due to a drastic reduction in overall proliferation (Mao et al., 2010). Additionally, the gastrointestinal mesenchyme failed to induce the smooth muscle progenitor differentiation, as noted by a lack of α-smooth muscle actin^{+} mesenchymal cells in $Shh^{Cre/flox}Ihh^{flox/flox}$ mutant embryos (Mao et al., 2010). Overall, this indicated that the gastrointestinal epithelium has a critical role in mesenchymal development through activation of Hedgehog signaling in the mesenchyme by epithelial secretion of Shh and Ihh ligand.

Recently, we further investigated the role of Hedgehog signaling in the developing mesenchyme of the gastrointestinal tract. We first knocked out the transmembrane protein Smoothened in the developing mesoderm using the $Nkx3.2^{Cre}$ allele. In these mutant $Nkx3.2^{Cre}Smo^{flox/flox}$ embryos, we observed diminished mesenchymal compartments as well as complete inhibition of epithelial villi development (Huang & Cotton et al., 2013). Conversely, when we activated Hedgehog pathway signaling by expressing a Smoothened gain of function allele in the developing mesoderm, $Nkx3.2^{Cre}R26^{SmoM2/+}$ mutant embryos exhibited a profound expansion of the gastrointestinal mesenchymal compartment. Hedgehog gain-of-function mutant mesenchymal cells were strongly positive for α-smooth muscle actin, indicating the expansion of a differentiated mesenchymal cell population (Huang & Cotton et al., 2013; Mao et al., 2010). Additionally, we showed that activated Hedgehog pathway signaling in the developing gastrointestinal mesenchyme is converging on Gli2, not Gli3, to drive

downstream gene transcription for mesenchymal differentiation (Huang & Cotton et al., 2013). Together, these data reveal that Hedgehog Pathway signaling between the epithelia and mesenchyme in the GI tract is a carefully controlled system to drive differentiation during development.

Mechanistically, it is believed that tight regulation of Hedgehog signaling during mesenchymal development is what initiates the Myocardin master-regulatory complex for smooth muscle differentiation in the gastrointestinal tract (Zacharias et al., 2011). In smooth muscle progenitor cells, Myocardin associates with serum response factor (SRF) form the Myocardin-SRF master regulatory complex. This complex induces differentiation through transcription of genes such as α-smooth muscle actin (α-SMA), Smooth Muscle Protein 22-Alpha (SM22α), and smooth muscle myosin heavy chain (SMMHC) (Du et al., 2003). Hedgehog signaling promotes differentiation of mesenchymal progenitor cells through direct regulation of *Myocd.* When Hedgehog pathway is activated during mesenchymal development, Gli proteins may bind to several conserved Gli-binding sites within the *Myocd* gene, thereby driving *Myocd* transcription and allowing for the formation of the Myocardin-SRF master regulatory complex (Zacharias et al., 2011).

How Hedgehog Pathway signaling activates Myocardin-SRF to induce mesenchymal differentiation has been well characterized. However, what remains unclear is what happens before Hedgehog initiates differentiation. Further investigation into the biology of the gastrointestinal mesenchymal stem cell is needed to understand this process.

Stromal Contribution to Colorectal Cancer

Expanding our understanding of gastrointestinal mesenchymal progenitor cell differentiation is also critical to advancing our understanding of colorectal cancer. Colorectal cancer arises from aberrant proliferation of gastrointestinal epithelial cells with stem cell characteristics and expression patterns (Barker, 2014). Consequently, CRC research has focused on the biology of epithelial cell transformation for many years. However, the underlying intestinal mesenchyme maintains the intestinal stem cell niche as well as contributes to the tumor cell microenvironment (Gerling et al., 2016; Kabiri et al., 2014; Kosinski et al., 2010; Li et al., 2014b; Shaker and Rubin, 2010). Mesenchymal cells regulate intestinal stem cell quiescence, proliferation, and renewal through the secretion of factors such as Wnt, BMP, Notch, and EGFR ligands (Clevers, 2013; Kabiri et al., 2014; Sailaja et al., 2016). This critical role of the mesenchyme helps to maintain the dedifferentiated state of the intestinal stem cells and support growth within the intestinal stem cell niche (Barker, 2014).

However, the mesenchymally-secreted pro-growth signals that are so integral to stem cell maintenance during normal intestinal epithelial homeostasis can also confer a survival advantage to epithelial cancer cells during tumorigenesis by maintaining a supportive, pro-growth microenvironment (Barker, 2014). Myofibroblasts are α-smooth muscle actin^{+} mesenchymal cells involved in both structural support as part of the extracellular matrix (ECM) as well as secretion of growth factors. However, when present in solid tumors such as colorectal cancer, invasive ductal breast cancer, and oral

squamous cell carcinoma, myofibroblasts are correlated with an overall poor prognosis (Kellermann et al., 2007; Surowiak et al., 2007; Tsujino et al., 2007). Additionally, tumors with greater numbers of cancer associated fibroblasts (CAFs) are generally more invasive and yield a poorer prognosis for overall survival (Calon et al., 2015). Stromal-specific gene signatures in tumors have also been linked to a worse prognosis in colorectal cancer survival (Calon et al., 2015; Isella et al., 2015). Together, these data illustrate that mesenchymal tissue is integrally involved with tumorigenesis.

It is clear that the tumor-promoting environment created by the stromal secretion of pro-growth factors, primarily by CAFs, is critical to overall tumor prognosis and patient survival. Therefore, myofibroblasts in the tumor stroma represent an attractive therapeutic target for cancer treatment. However, a better understanding of how the gastrointestinal mesenchyme regulates normal homeostasis as well as its involvement in tumorigenesis is required.

Scope of Dissertation

Colorectal cancer is the third leading cause of cancer-related death in the United States and has a dismal 13% overall 5 year survival rate for patients diagnosed with metastatic disease. A deeper understanding of colorectal cancer initiation and progression, in addition to further elucidating the supportive role that the underlying mesenchyme provides to the tumor microenvironment, is desperately needed to understand disease etiology and develop novel drugs for targeted therapies.

The Hippo Pathway has emerged as a key regulator in organ size control and progenitor cell maintenance, and has also been shown to be aberrantly regulated in a wide spectrum of solid tumors. In particular, YAP/TAZ have been linked to intestinal epithelial regeneration, in addition to Wnt-driven colorectal tumorigenesis. However, many questions remain about the specifics of the interactions between the Hippo Pathway and the Wnt Pathway in intestinal homeostasis and tumorigenesis. Additionally, nothing is known about the role of the Hippo Pathway in the intestinal mesenchyme in either development or postnatal stages.

My dissertation broadly explores the role of developmental signaling pathways in gastrointestinal development and tumorigenesis. In this dissertation, I systematically dissect the roles of YAP/TAZ in both gastrointestinal development and homeostasis by genetically removing both proteins from the endoderm-derived gastrointestinal epithelia and mesoderm-derived gastrointestinal mesenchyme.

In Chapter II, I demonstrate an anterior endodermal requirement of YAP/TAZ during embryonic development. I find that YAP/TAZ are dispensable for gastrointestinal epithelial differentiation, as well as dispensable for Wnt signal transduction during normal development and homeostasis. I find that YAP/TAZ act as direct transcriptional targets of Wnt pathway signaling during epithelial cell transformation.

In Chapter III, I uncover a previously unknown requirement of YAP/TAZ function in gastrointestinal mesenchymal growth and differentiation. I describe a novel transgenic YAP gain of function mouse allele engineered to be spatially and temporally controlled via Cre recombinase to investigate Hippo Pathway signaling *in vivo*. Next, I identify a functional interaction between YAP/TAZ and Hedgehog signaling in the specification of the smooth muscle lineage, wherein YAP/TAZ act as a molecular gatekeeper to differentiation. Finally, I demonstrate for the first time *in vivo* that YAP/TAZ can act as transcriptional co-repressors; YAP/TAZ function as co-repressors to maintain a mesenchymal progenitor cell population in the developing gastrointestinal mesoderm.

In Appendix A, I explore the role of SMAD7 in the intestinal epithelia. I describe a unique role for SMAD7 in polyp initiation and progression, independent of either an *Apc* mutation or Wnt pathway activation, in a novel mouse model for serrated polyposis. I characterize the molecular signature in the SMAD7-mutant serrated polyps and find that significant gene expression overlap with human serrated polyps, indicating that my mouse model represents the first bona fide model for serrated polyp initiation and progression.

In Appendix B, I explore the role of developmental signaling pathways in the postnatal mesenchyme. I first identify a mesenchymal shared cell of origin for gastrointestinal hamartomatous polypsis syndromes. I generate three novel mouse models for gastrointestinal hamartomatous polyposis syndromes: (1) Peutz Jegher's Syndrome, (2) Juvenile Polyposis Syndrome, and (3) PTEN Hamartoma Syndrome/Cowden's Syndrome. Furthermore, I investigate whether the three gastrointestinal hamartomatous syndromes converge on a shared downstream signaling pathway to drive polyposis. I find that although the three syndromes share a common cell of origin, they diverge onto independent downstream signaling mechanisms to drive polyposis.

Overall, this dissertation both uncovers novel roles for the Hippo Pathway in the mammalian gastrointestinal tract as well as highlights the critical importance of spatial and temporal regulation of developmental signaling pathways in both gastrointestinal development and tumorigenesis.

CHAPTER II

The role of YAP and TAZ in the GI epithelium

Abstract

YAP and TAZ are the major intracellular mediators of Hippo Pathway signaling in mammals. However the precise function of YAP/TAZ in gastrointestinal development and homeostasis remains poorly understood. Here I use mouse genetics to systematically knock out both YAP and TAZ from the developing endodermal epithelia as well as intestinal epithelia during mammalian development. I find that YAP/TAZ are dispensable for Wnt pathway signaling and GI epithelial cell differentiation during development and homeostasis. However, I report that YAP/TAZ are required for Wnt-driven tumorigenesis in colorectal cancer cell lines as well as in APC-mutant mouse models. Finally, I show that YAP/TAZ are direct Wnt/TCF4 targets during epithelial cell transformation. Overall these findings highlight the complexity that exists between Hippo Pathway and Wnt Pathway signaling in the intestinal epithelia and uncovers an additional layer of YAP/TAZ regulation in tumorigenesis.

Results

Differential requirement of YAP/TAZ in endoderm-derived epithelia

We first decided to investigate the role of YAP/TAZ in the intestinal epithelia using mouse genetics to target the developing endoderm. We crossed *Yap* (Xin et al., 2011a) and *Taz* conditional alleles (Xin et al., 2013) to the Shh^{Cre} allele (Harfe et al., 2004), to restrict Cre recombination starting at embryonic day 8.5 (E8.5) to the embryonic endoderm, which eventually gives rise to epithelia of the lung, esophagus, stomach, and intestine. Genetic ablation of both YAP and TAZ from the early developing endoderm in $Shh^{Cre}Yap^{flox/flox}Taz^{flox/flox}$ mutants caused embryonic lethality at E18.5. Mutant embryos exhibited severe lung epithelial branching *(Fig. 2.1.A and insets)* and differentiation defects, assayed by Sox2 expression *(Fig. 2.1.D)*, and resemble the YAP knockout phenotype recently reported in the developing lung (Mahoney et al., 2014).

However, in contrast to the severe lung defect, genetic ablation of YAP/TAZ had no effect in the gastrointestinal epithelia; both the stomach and intestine appeared normal at E18.5 *(Fig.2.1.B)*. Despite having lost YAP protein *(Fig.2.1.C)*, proliferation (assayed by Ki67 immunohistochemical staining) *(Fig.2.1.E)* and parietal cell differentiation (assayed by H/K-ATPase immunohistochemical staining) *(Fig.2.1.F)* in $Shh^{Cre}Yap^{flox/flox}Taz^{flox/flox}$ mutants appears indistinguishable from control staining. We observed no obvious defect in mesenchymal differentiation *(Fig.2.2.A,B)* or enteric neuron innervation *(Fig.2.2.C)* in $Shh^{Cre}Yap^{flox/flox}Taz^{flox/flox}$ mutants as compared to control. Finally, we examined whether Wnt signaling was affected in intestinal crypts

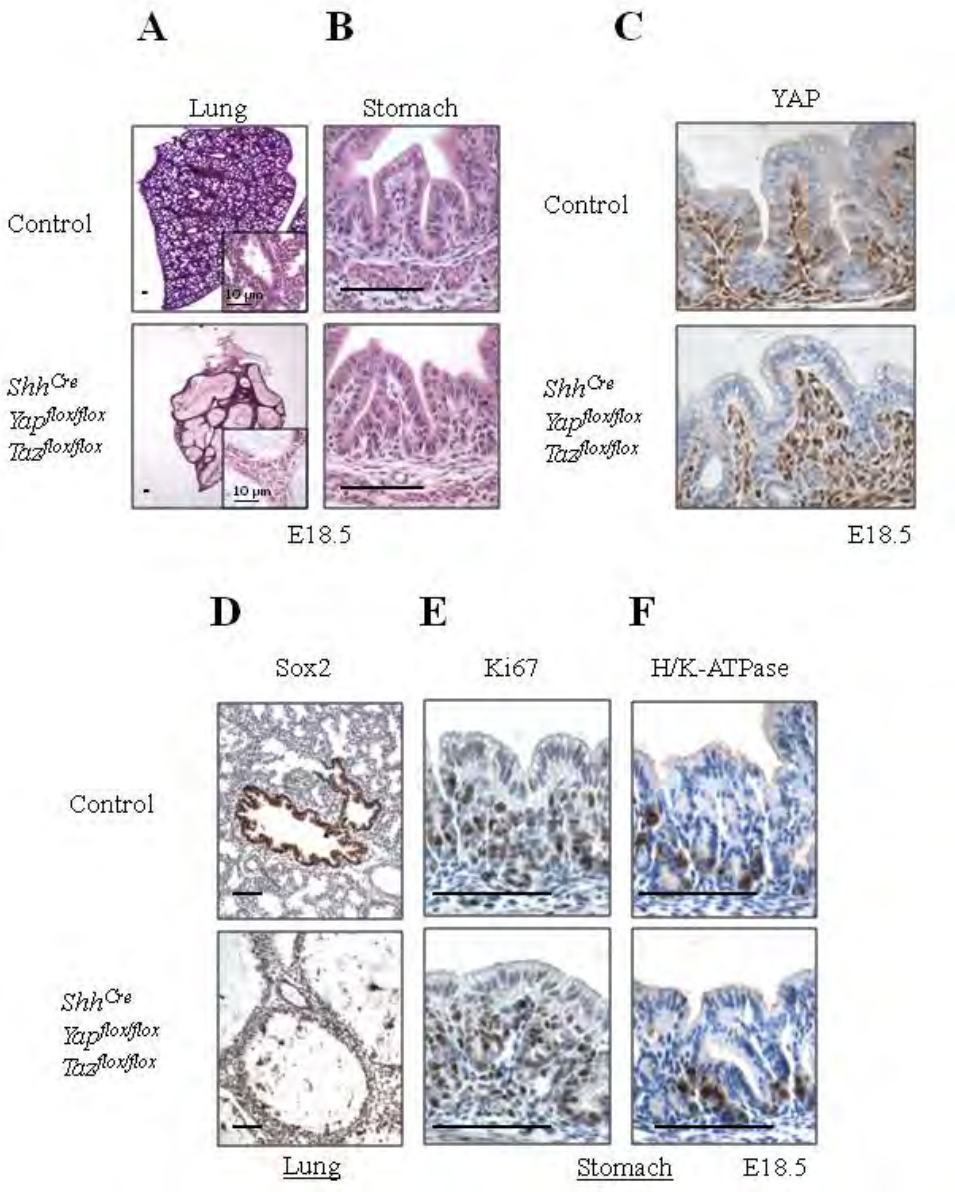


Figure 2.1. Compartmental requirement of YAP/TAZ in the endodermal epithelia. Histology of *(A)* lung (inset at 40x magnification, scale at 10 μm) and *(B)* stomach in control and YAP/TAZ deficient endoderm ($Shh^{Cre}Yap^{flox/flox}Taz^{flox/flox}$) animals at E18.5. *(C)* Immunohistochemical YAP staining in control and YAP/TAZ deficient intestinal epithelia. *(D)* Sox2 (lung epithelial marker), *(E)* Ki67 (proliferation marker), and *(F)* H/K-ATPase (gastric parietal cell marker) immunohistochemical staining in lung and stomach in control and $Shh^{Cre}Yap^{flox/flox}Taz^{flox/flox}$ animals at E18.5. Scale bar = 20 μm.

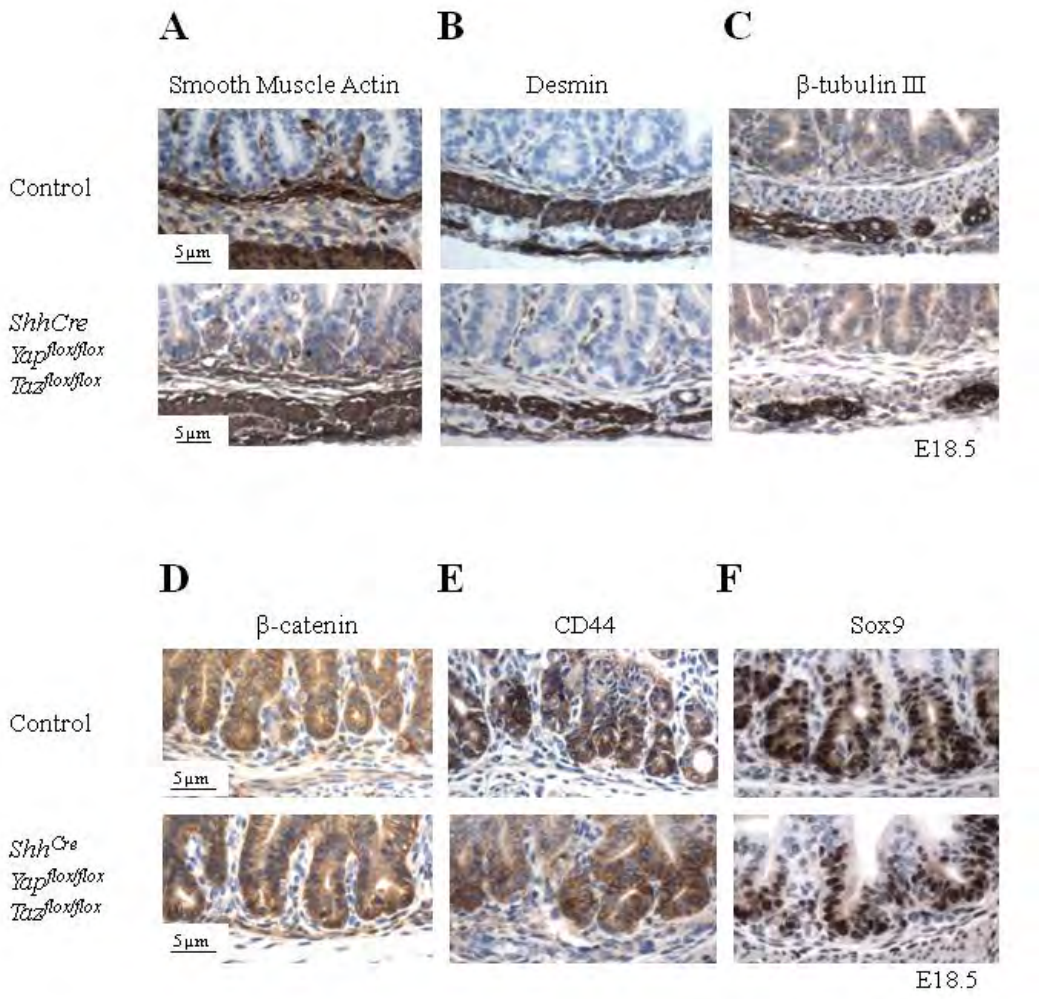


Figure 2.2. Loss of YAP/TAZ in the developing endoderm does not affect mesenchymal differentiation or Wnt/β-signaling in intestinal crypts.
(A-C) Immunohistochemical staining for mesenchymal, smooth muscle, and neuronal cells, *(A)* smooth muscle actin, *(B)* desmin, *(C)* β-tubulin III. Immunohistochemical staining for Wnt/β-signaling *(D-E)* in intestinal crypt cells *(F)*, *(D)* β-catenin, *(E)* CD44, *(F)* Sox9. Scale bar = 5 μm.

null for YAP/TAZ in *Shh*^{Cre}*Yap*$^{flox/flox}$*Taz*$^{flox/flox}$ animals and observed no difference in β-catenin, CD44, or Sox9 staining patterns in mutants as compared to control at E18.5 *(Fig.2.2.D-F)*.

We next decided to restrict the epithelial knockout of YAP/TAZ specifically to the gastrointestinal epithelia using *Villin*^{Cre} (Madison et al., 2002), which drives Cre-mediated recombination in the intestinal epithelia starting from E12.5. Consistent with the results observed in *Shh*^{Cre}*Yap*$^{flox/flox}$*Taz*$^{flox/flox}$ mutant embryos at E18.5, we observed that removal of both *Yap* and *Taz (Fig.2.3.B,C)* in the intestinal epithelia in *Villin*^{Cre}*Yap*$^{flox/flox}$*Taz*$^{flox/flox}$ animals generated normal crypt-villi architecture *(Fig.2.3.A)*. Additionally, we observed no discernible patterning defects in either proliferation or differentiation, including the goblet cell and Paneth cell lineage, in *Villin*^{Cre}*Yap*$^{flox/flox}$*Taz*$^{flox/flox}$ animals *(Fig.2.3.D-F).*

We again decided to investigate Wnt signaling in YAP/TAZ null intestinal crypts in *Villin*^{Cre}*Yap*$^{flox/flox}$*Taz*$^{flox/flox}$ animals. We observed that nuclear localization of β-catenin *(Fig.2.4.A)* in YAP/TAZ null intestinal crypts maintained wild-type patterning. Additionally, expression of Wnt target genes CD44 and Sox9 *(Fig.2.4.B,C),* as well as *Axin2* and *Lgr5 (Fig.2.4.D)* was unaffected in mutants as compared to control.

Together, our analyses revealed that YAP/TAZ are required for lung development in developing embryonic endoderm. However, YAP/TAZ are dispensable for epithelial proliferation and differentiation in the gastrointestinal tract, as well as for Wnt pathway signal transduction during normal intestinal crypt development and homeostasis.

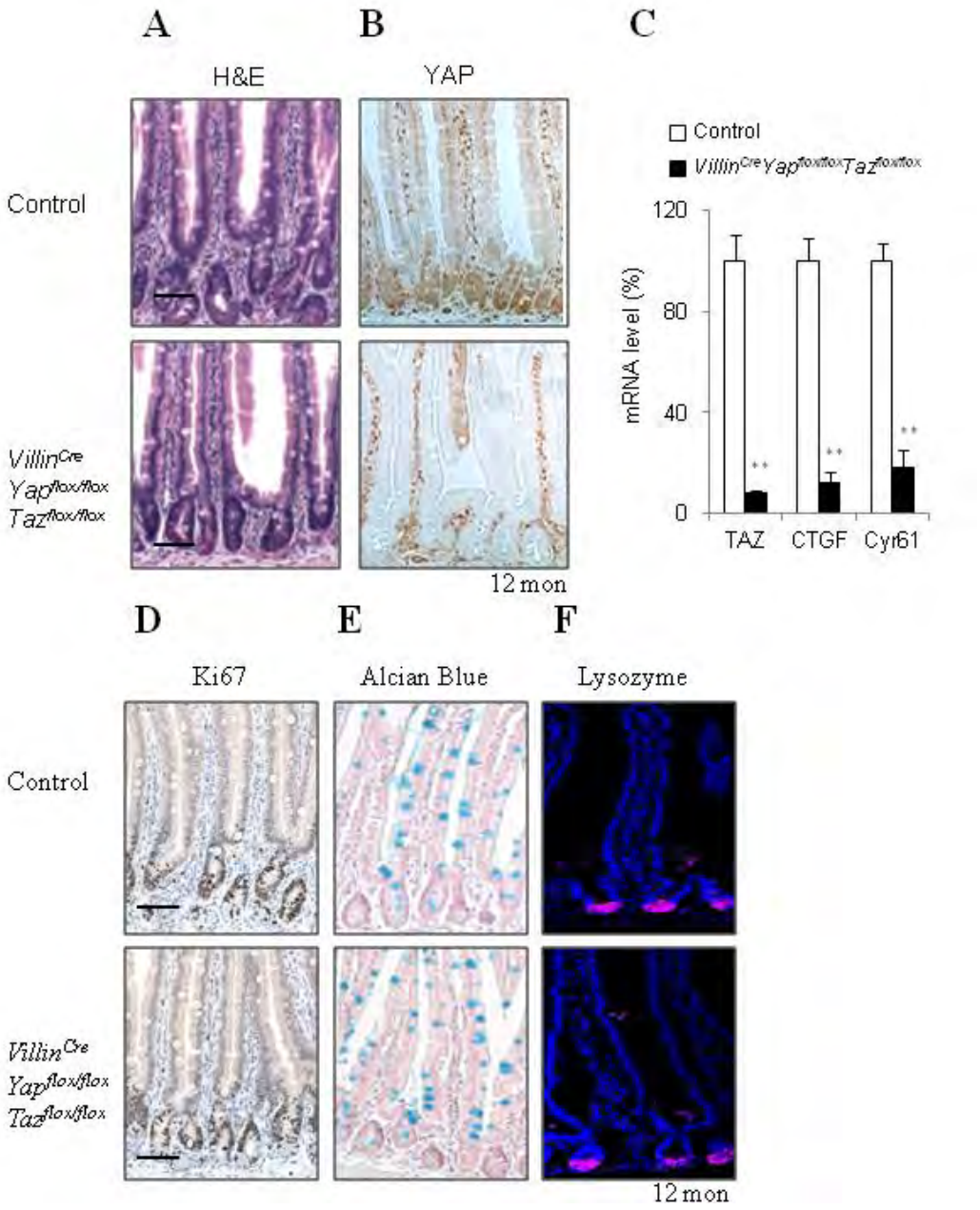


Figure 2.3. YAP and TAZ are dispensable for intestinal epithelial homeostasis.
(A) Histology of intestine and *(B)* immunohistochemical YAP staining in control and YAP/TAZ deficient intestinal epithelia *(Villin^{Cre}Yap$^{flox/flox}$Taz$^{flox/flox}$)* animals at 12 months old. *(C)* Real-time qPCR analysis of mRNA levels of *TAZ* and Hippo targets, *Ctgf* and *Cyr61*, in intestinal epithelia of control and *Villin^{Cre}Yap$^{flox/flox}$Taz$^{flox/flox}$* animals at 12 months old. *(D)* Ki67 immunohistochemical, *(E)* Alcian Blue (Goblet cell), and *(F)* lysozyme (Paneth cells) immunofluorescence staining in control and *Villin^{Cre}Yap$^{flox/flox}$Taz$^{flox/flox}$* animals at 12 months old. Scale bar = 20 μM. Data are mean ± S.D., * = p value ≤ 0.05; ** = p value ≤ 0.01.

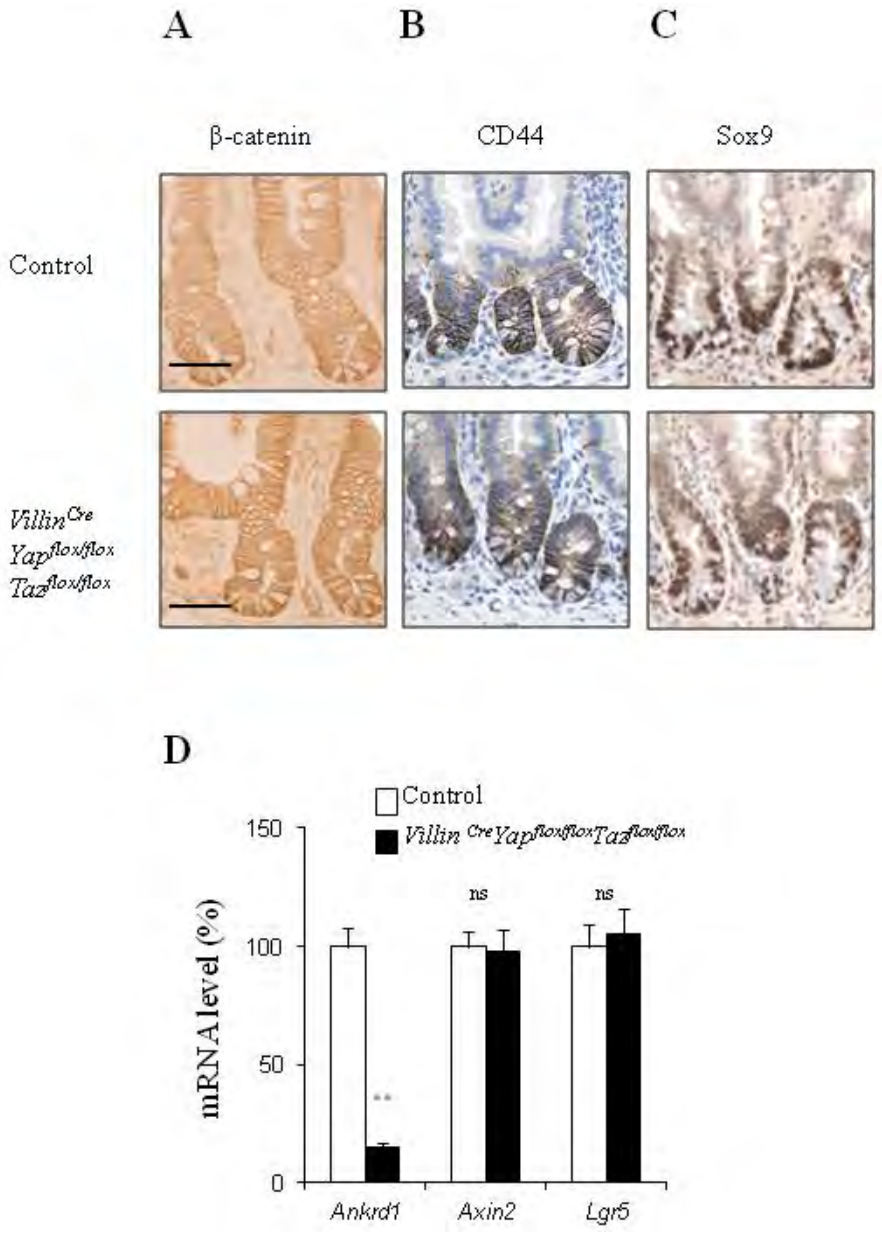


Figure 2.4. YAP/TAZ deletion does not affect Wnt/β-catenin signal transduction in the intestinal epithelium during normal homeostasis.
Immunohistochemical staining of *(A)* β-catenin, *(B)* CD44, and *(C)* Sox9 in intestinal epithelia of control and *Villin*^{Cre}*Yap*$^{flox/flox}$*Taz*$^{flox/flox}$ animals at 12 months old. *(D)* Real-time qPCR analysis of mRNA levels of canonical YAP/TAZ target, *Ankrd1*, and canonical Wnt targets, *Axin2* and *Lgr5* in intestinal epithelia of control and *Villin*^{Cre}*Yap*$^{flox/flox}$*Taz*$^{flox/flox}$ animals. Scale bar = 20 μm. Data are mean ± S.D., ns = p value > 0.05, * = p value ≤ 0.05; ** = p value ≤ 0.01.

YAP/TAZ are critical Wnt/TCF4 transcriptional targets during transformation.

Our lab has previously published a study showing that YAP can be regulated by Wnt signaling at both transcriptional and posttranscriptional levels in liver cancer cells (Wang et al., 2013). Therefore, although we and others have shown that YAP/TAZ are likely not the primary Wnt signaling transducers in gastrointestinal epithelial cells during normal development and homeostasis (Azzolin et al., 2014; Barry et al., 2013; Cai et al., 2010; Gregorieff et al., 2015), we wondered whether YAP/TAZ could be downstream targets of Wnt signaling during pathway hyperaction in colon cancer cells.

To address this possibility, we first performed genome-wide analysis of publically available TCF4 ChIPseq datasets (http://genome.ucsc.edu/ENCODE/downloads.html) in HCT116 cells, a cell line carrying a β-catenin mutation which leads to constitutive Wnt pathway hyperactivation. Interestingly, our analysis revealed TCF4 occupancy at both the *YAP* and *TAZ* loci *(Fig.2.5.A,B*). We intersected the TCF4 ChipSeq data with HCT116 genome ChIPseq datasets of histone modification, including H3K4me1 (markers of active enhancers) and H3K4me3 (markers of active promoters). We found that TCF4 was able to bind to the active promoter region of the *YAP* gene and occupied an active enhancer site 5' upstream of the transcriptional start site of the *TAZ* gene. We next performed chromatin immunoprecipitation (ChIP) using TCF4 antibody in HCT116 cells and further confirmed via ChIP-qPCR that TCF4 binds to *cis*-regulatory elements at both the *YAP* and *TAZ* loci *in vitro (Fig.2.5.C).*

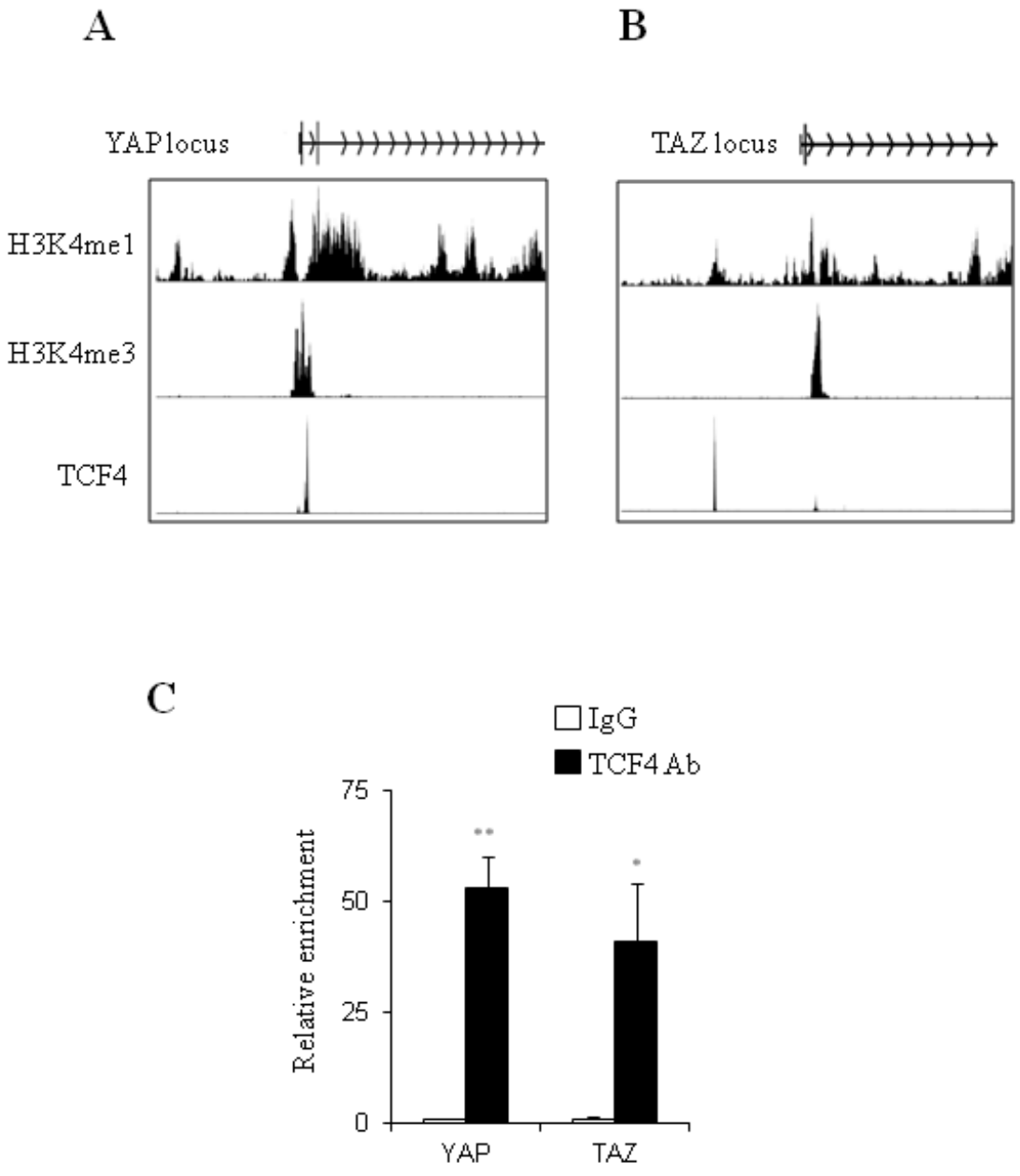


Figure 2.5. TCF4 binds directly to *cis* regulatory elements of both *Yap* and *Taz* loci in cancer cells with Wnt/β-catenin hyperactivation.
Track views of H3K4me1 (markers of active enhancers), H3K4me3 (markers of active promoters), and TCF4 peaks in the *(A) Yap* and *(B) Taz* loci from the HCT116 genome. *(C)* ChIP-qPCR in HCT116 was performed with control IgG or TCF4 antibody (Ab), and enrichment of the TCF4 enhancer regions from the *YAP* and *TAZ* loci was measured by qPCR. Data are mean ± S.D., * = p value ≤ 0.05; ** = p value ≤ 0.01.

Moreover, we found that blocking Wnt pathway activation with DN-TCF4, a TCF4 repressor lacking the β-catenin interacting domain (van de Wetering et al., 2002), significantly inhibited *YAP* and *TAZ* transcription in both HCT116 (β-catenin gain-of-function mutation, loss of S45 phosphorylation site) and DLD1 (APC loss-of-function mutation, truncated protein) colon cancer cells *(Fig.2.6.A)*. In agreement with our hypothesis that *Yap* and *Taz* are Wnt transcriptional targets during pathway hyperactivation, *Yap* mRNA levels were also elevated *ex vivo* in wild-type mouse intestinal organoids treated with the Wnt3A ligand for 6 hours *(Fig.2.6.B)*.

To test whether the increased YAP/TAZ levels are critical for Wnt/β-catenin oncogenic activity during pathway hyperactivation, we first generated organoid cultures from isolated intestinal crypts of $Ubc^{CreER}Yap^{flox/flox}Taz^{flox/flox}$ mice. We then introduced DA-β-catenin, a dominantly active form of β-catenin that lacks the GSK3β phosphorylation sites (Barth et al., 1997), into the cultured organoids via lentiviral infection. Ubc^{CreER} (Ruzankina et al., 2007) is a general inducible *Cre* allele allowing Tamoxifen-induced Cre recombination and subsequent *Yap/Taz* knock-out in $Ubc^{CreER}Yap^{flox/flox}Taz^{flox/flox}$ intestinal organoids. We found that removal of *Yap/Taz* resulted in the collapse of both Yap/Taz-null organoids as well as Yap/Taz-null + constitutive expression of DA-β-catenin as compared to control organoids *(Fig.2.6.C,D)*. Next we expressed lentiviral-based shRNA constructs against *YAP* and *TAZ* in HCT116 and DLD1 colon cancer cells and found that YAP/TAZ knockdown inhibited the

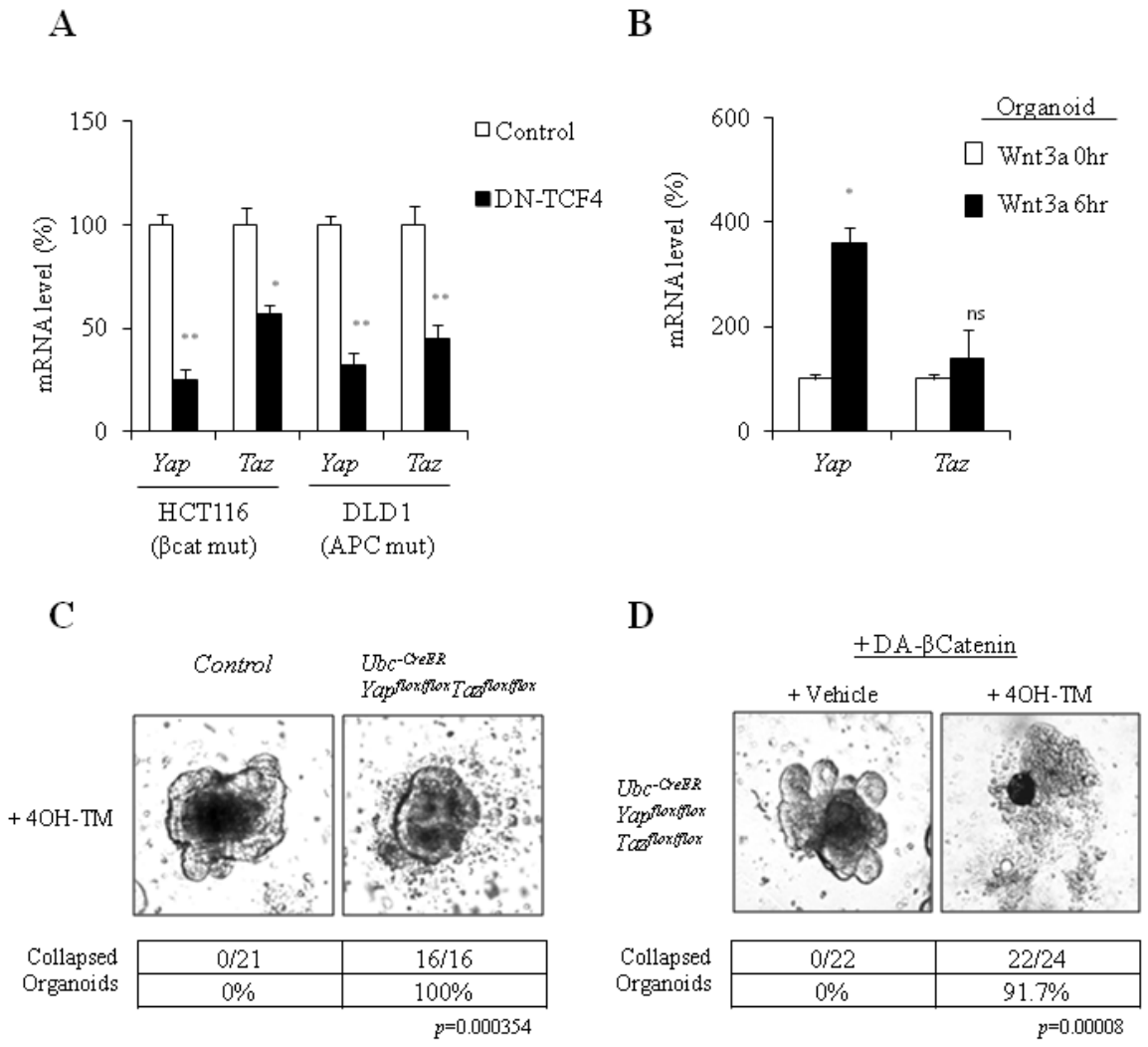


Figure 2.6. YAP/TAZ are TCF4 transcriptional targets during Wnt/β-catenin pathway hyperactivation.
(A) Real-time qPCR analysis of *Yap* and *Taz* mRNA levels in HCT116 (*β-catenin* mutant) and DLD1 (*Apc* mutant) colon cancer cells with or without DN-TCF4 expression. *(B)* Real-time qPCR analysis of *Yap* and *Taz* mRNA levels in mouse intestinal organoids with or without Wnt3a treatment for 6 hours. *(C)* Images of control and $Ubc^{-CreER}Yap^{flox/flox}Taz^{flox/flox}$ organoids following 4-hydroxytamoxifen treatment for 48 hours. *(D)* Images of $Ubc^{-CreER}Yap^{flox/flox}Taz^{flox/flox}$ intestinal organoids expression DA-β-catenin with or without 4-hydroxytamoxifen treatment. Data are mean ± S.D., ns = p value > 0.05, * = p value ≤ 0.05; ** = p value ≤ 0.01.

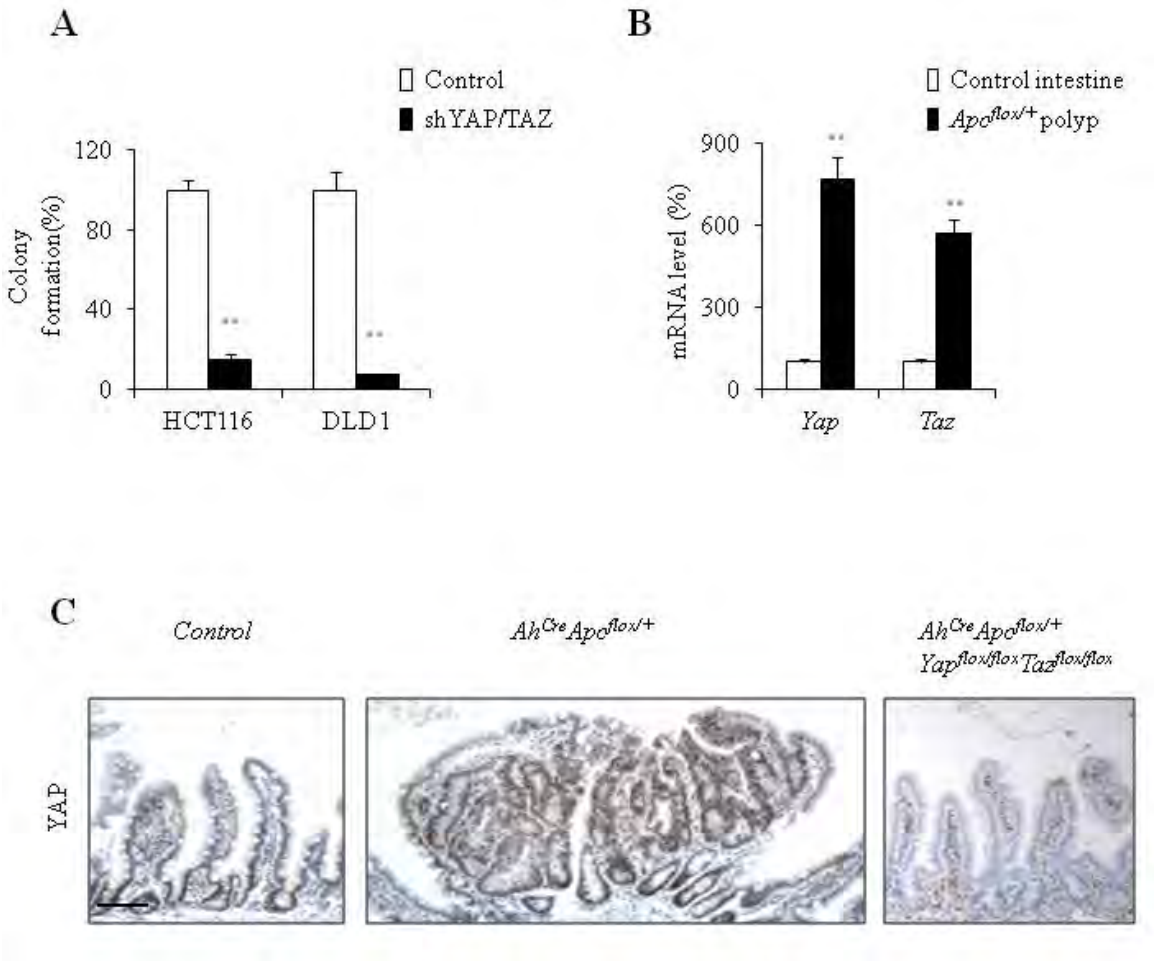


Figure 2.7. Double homozygous knockout of YAP/TAZ is sufficient to inhibit Wnt-driven polyposis.
(A) Relative soft agar colony formation in HCT116 and DLD1 cells with or without lentiviral expression of shRNAs against *YAP* and *TAZ*. *(B)* Real-time qPCR analysis of mRNA levels of *Yap* and *Taz* in mouse control intestine and APC-deficient intestinal polyps. *(C)* Immunohistochemical YAP staining in control, *Apc* mutant ($Ah^{Cre}Apc^{flox/+}$), and *Apc* mutant-*Yap/Taz* deficient $Ah^{Cre}Apc^{flox/+}Yap^{flox/flox}Taz^{flox/flox}$) animals. Scale bar = 10 μm. Data are mean ± S.D., * = p value ≤ 0.05; ** = p value ≤ 0.01.

tumorigenicity of both colon cancer cell lines, as measured by anchorage-independent soft agar colony formation *(Fig.2.7.A)*.

Finally, we examined the requirement of YAP and TAZ in Wnt-induced intestinal tumorigenesis *in vivo*. We first observed that *in vivo*, polyps driven by *Apc* deficiency in mouse intestinal epithelial cells exhibit elevated levels of both *Yap* and *Taz* mRNA as compared to control intestine *(Fig.2.7.B)*. Lastly, we used the β-napthoflavone-inducible Ah^{Cre} allele (Ireland et al., 2004) to target the intestinal epithelia in $Ah^{Cre}Apc^{flox/+}Yap^{flox/flox}Taz^{flox/flox}$ animals, and specifically investigate the postnatal requirement for YAP/TAZ in a Wnt pathway hyperactivated background. In conjunction with heterozygous loss of APC, postnatal removal of both YAP and TAZ from the intestinal epithelia effectively blocked *Apc* mutation-induced polyposis in $Ah^{Cre}Apc^{flox/+}Yap^{flox/flox}Taz^{flox/flox}$ mice *(Fig.2.7.C)*. Taken together, our data provide further evidence that *Yap* and *Taz* are dispensable for normal intestinal epithelial development and homeostasis, but are direct TCF4 transcriptional targets only during conditions when Wnt signaling is hyperactivated, thereby mediating, at least in part, Wnt pathway driven transformation in gastrointestinal epithelia.

Materials and Methods

Mouse Genetics

Shh^{Cre} (Harfe et al., 2004), *Villin*^{Cre} (Madison et al., 2002), *Ubc*^{CreER} (Ruzankina et al., 2007), *Apc*^{flox} (Cheung et al., 2010) and *R26*$^{mT/mG}$ (Muzumdar et al., 2007) mice were obtained from the Jackson laboratory. *Ah*^{Cre} (Ireland et al., 2004), *Yap*^{flox} (Xin et al., 2011d) and *Taz*^{flox} (Xin et al., 2013) mice were kindly provided by Drs. WE Zimmer, OJ Sansom, DJ Winton, and EN Olson. The University of Massachusetts Medical School Transgenic Animal Core performed ES cell targeting and blastocyst injection to generate chimeric animals. To target the endodermal and gastrointestinal epithelia, *Shh*^{Cre}, *Villin*^{Cre}, *Ah*^{Cre} animals were bred with *Yap*^{flox}, *Taz*^{flox}, *Apc*^{flox} mice. Postnatal *Ah*^{Cre} activation was achieved by intraperitoneal injection of 80 mg/kg β-napthoflavone at postnatal day 30, and intestinal tissue were collected 5 months later. All mouse experiments were conducted according to the University of Massachusetts Medical School IACUC guidelines.

Tissue Collection and Histology

Following dissection, mouse tissue was fixed in 10% Neutral Buffered Formalin (NBF) at 4°C overnight. Tissues for paraffin sectioning were dehydrated, embedded in paraffin wax, and sectioned at 6 μm. Tissues for frozen sectioning were dehydrated in 30% sucrose overnight at 4°C, embedded in OCT, and sectioned at 12 μm. Paraffin sections were stained using standard hematoxylin & eosin reagents.

Immunohistochemistry and Immunofluorescence

For immunohistochemistry(IHC), 6 μm tissue sections were deparaffinized, rehydrated, and then subjected to heat-induced antigen retrieval in 10 mM sodium citrate buffer (pH 6.0) for 30 minutes. Sections were first blocked for endogenous peroxidase for 20 minutes at room temperature then blocked in blocking buffer (5% BSA, 1% goat serum, 0.1% Tween-20 buffer) in PBS for 1 hour at room temperature. Sections were incubated overnight in primary antibody diluted in blocking buffer or SignalStain® Antibody Diluent (Cell Signaling) at 4°C. Sections were incubated for 1 hour at room temperature in biotinylated secondary antibody and Vectastain Elite ABC kit (Vector Laboratories) was used to detect signal.

For immunofluorescence (IF), 12 μm tissue sections were blocked for 1 hour at room temperature in blocking buffer and then incubated in primary antibody diluted in blocking buffer overnight at 4°C. Sections were incubated in Alexa Fluor-conjugated secondary antibodies (Invitrogen) at 1:500 dilution in blocking buffer for 1 hour at room temperature then mounted with mounting media with DAPI (EMS). Primary antibodies used for IHC/IF are listed in Table 2.1.

Mouse intestinal crypt organoid culture.

The intestinal crypt organoid culture was established according to the Sato/Clevers protocol(Sato and Clevers, 2013). Briefly, the intestinal crypts from the $Ubc^{CreER}Yap^{flox/flox}Taz^{flox/flox}$ mice were isolated following EDTA treatment and centrifugation, and then maintained in a matrigel mixture containing recombinant

Noggin, EGF and R-spondin (R&D systems). For RNA isolation, organoids were treated with 1ug/ml recombinant Wnt3a (R&D systems) for 6 hours. For YAP/TAZ functional assay, $Ubc^{CreER}Yap^{flox/flox}$ $Taz^{flox/flox}$ organoids were infected with a lentiviral construct expressing DA-β-catenin (pLV-beta-catenin deltaN90 was a gift from Bob Weinberg (Addgene # 36985)) for 7 days, and Cre-mediated recombination was induced with addition of 2ug/ml 4-OH-Tamoxifen (Sigma).

Cell culture, lentiviral infection and soft agar colony formation

HCT116, DLD1 and HEK293T cells were obtained from ATCC and cultured in DMEM supplemented with 10% FBS. For lentiviral infection, pGIPZ or pLKO based constructs expressing DN-TCF4 or shRNAs against human YAP and TAZ were transfected along with the packaging plasmids into growing HEK293T cells. shRNA primer sequences are listed in Table 2.3. Viral supernatants were collected 48 hours after transfection, and target cells were infected and underwent selection with puromycin for 3-4 days, prior to RNA isolation or subsequent anchorage-independent soft-agar colony formation assay, which was described previously (Rajurkar et al., 2012)**.**

TCF4, H3K4me1 and H3K4me3 ChIP-seq analysis

TCF4, H3K4me1, and H3K4me3 ChIP-seq data in HCT116 cells were downloaded from the ENCODE (http://genome.ucsc.edu/ENCODE/downloads.html) project website. The following antibodies were used for chromatin immunoprecipitation: TCF4 (Cell Signaling, 2569), H3K4me1 (Abcam, ab8895) and H3K4me3 (Abcam,

ab8580). The TCF4, H3K4me1, and H3K4me3 ChIP peaks were annotated using R/Bioconductor package ChIPpeakAnno (Zhu et al., 2010). BigWig tracks were plotted with TrackViewer (https://bioconductor.org/packages/devel/bioc/html/trackViewer.html).

Chromatin immunoprecipitation-qPCR analysis

ChIP assays were performed according to the manufacturer's instructions (Active Motif, CA). Briefly, 2×10^7 cells were fixed with 1% formaldehyde, washed with cold PBS and lysed in lysis buffer. After sonication, protein-DNA complexes were incubated with TCF4 antibody (Cell Signaling, 2569)-coupled protein G beads at 4℃ overnight. After elution and reverse cross-link, DNA was purified for subsequent PCR analysis. The primers used for real-time PCR of TCF4 cis-regulatory elements are listed in Table 2.4.

Quantitative Real-Time PCR

Trizol reagent (Invitrogen) was used to isolated RNA from both animal tissues and cultures cells, and SuperscriptII Reverse Transcriptase (Invitrogen) was used for reverse-transcription. Sybr Mastermix (Kapa Bioscience) was used for quantitative real-time PCR. Primer sequences used for real-time PCR are listed in Table 2.2. All qPCR experiments were conducted in biological triplicates, error bars represent mean ± standard deviation, and Student's t-test was used to generate p-values (* = p value ≤0.05; ** = p value ≤0.01; *** = p value ≤0.001).

Table 2.1: Antibodies for IHC/IF

Antibody	**Dilution**	**Company**
YAP	1:200	Cell Signaling
Sox2	1:2000	Seven Hills Bioreagents
Ki67	1:10000	Abcam
H/K-ATPase	1:500	MBL
Lysozyme	1:1000	Novus Biologicals
β-catenin	1:500	BD Biosciences
CD44	1:400	eBioscience
Sox9	1:200	Abcam
Desmin	1:400	ThermoFisher
α-SMA	1:5000	Abcam
β-tubulin III	1:800	Covance

Table 2.2: qPCR Primers

qPCR Primers	**Sequence**
mouse YAP forward	ACCCTCGTTTTGCCATGAAC
mouse YAP reverse	TGTGCTGGGATTGATATTCCGTA
mouse TAZ forward	GAAAATCACCACATGGCAAGACCC
mouse TAZ reverse	TTACAGCCAGGTTAGAAAGGGCTC
mouse CTGF forward	TGTGCACTGCCAAAGATGGTGCAC
mouse CTGF reverse	TGGGCAGGCGCACGTCCATG
mouse Cyr61 forward	GAGGCTTCCTGTCTTTGGCAC
mouse Cyr61 reverse	ACTCTGGGTTGTCATTGGTAAC
mouse ANKRD1 forward	GGAACAACGGAAAAGCGAGAA
mouse ANKRD1 reverse	GAAACCTCGGCACATCCACA
mouse Axin2 forward	AAGCCTGGCTCCAGAAGATCACAA
mouse Axin2 reverse	TTTGAGCCTTCAGCATCCTCCTGT
mouse LRG5 forward	TGCAGAATGAGCGCCAACCT
mouse LGR5 reverse	CACCCTGAGCAGCATCCTG
mouse GAPDH forward	GTGAAGGTCGGTGTGAACG
mouse GAPDH reverse	ATTTGATGTTAGTGGGGTCTCG

Table 2.3: shRNA Primers

shRNA Primers	**Sequence**
human YAP shRNA	AAGCTTTGAGTTCTGACATCC
human TAZ shRNA	AGAGGTACTTCCTCAATCA

Table 2.4: ChIP-qPCR Primers

ChIP-qPCR Primers	**Sequence**
human YAP forward	CATCAATGCCGGCTCACGGTATCTA
human YAP reverse	CTTTGGTCTCCGACAGGAGACTATA
human TAZ forward	AATTACTGTGCAGCTAGAGTGGATG
human TAZ reverse	CAGGACCTTGGAAAGTTCCCATGAA
human intergenic region forward	CTCATGTACTCTATTGGCTTC
human intergenic region reverse	GCATCTTCACCTGCCCAAGAA

CHAPTER III

The role of YAP/TAZ in the developing GI mesenchyme

Abstract

We and others showed that the Hippo Pathway members YAP and TAZ are dispensable in the gastrointestinal epithelia during development and homeostasis. However, the role of YAP and TAZ in the gastrointestinal mesenchyme is unknown. Here I use mouse genetics to systematically explore the YAP/TAZ requirement in the developing gastrointestinal mesenchyme during mammalian embryogenesis. Surprisingly, I find that both YAP/TAZ loss of function as well as gain of function in the GI mesenchyme is embryonic lethal, indicating a Goldilocks Effect for YAP/TAZ activity in this tissue during development. I find that YAP/TAZ functions as a molecular gatekeeper in a mesenchymal progenitor cell population prior to Hedgehog-mediated differentiation. Finally, I demonstrate that YAP/TAZ function as transcriptional co-repressors in the developing GI mesenchyme. Overall these findings uncover a novel mechanism for Hippo Pathway activity in the developing GI tract.

Results

YAP and TAZ are highly expressed in the gastrointestinal mesenchyme

The increased levels of YAP/TAZ and the correlated functional requirement during epithelial transformation prompted us to more closely examine YAP/TAZ protein expression in the developing endoderm and gastrointestinal tract. Immunohistochemical YAP staining showed that, in the embryonic lung, YAP is highly expressed in the nuclei of both epithelial and mesenchymal cells *(Fig. 3.1.A)*. Interestingly, we found that YAP expression was much higher in the mesenchyme than the epithelia in the stomach and intestine *(Fig. 3.1.A)*. This dichotomy in expression levels of YAP/TAZ continued at perinatal and adult stages, with significantly higher mesenchymal expression *(Fig. 3.1.B, C)*. The higher expression and nuclear localization of YAP/TAZ in lung epithelia as compared to gastrointestinal epithelia may explain the disparate requirement of YAP/TAZ in the developing endoderm while the differential expression levels of YAP/TAZ in gastrointestinal epithelia and mesenchyme raises an intriguing possibility that YAP/TAZ may have a role in the gastrointestinal mesenchyme.

YAP and TAZ are essential for gastrointestinal mesenchymal development

To investigate the role of YAP/TAZ in the gastrointestinal mesenchyme, we used *Nkx3.2*^{Cre} (Verzi et al., 2009) mice to specifically target the gastrointestinal mesoderm during development. *Nkx3.2*^{Cre} is expressed from E8.5 in the lateral plate mesoderm which gives rise to the gastrointestinal mesenchyme (Verzi et al., 2009). Mesenchymal

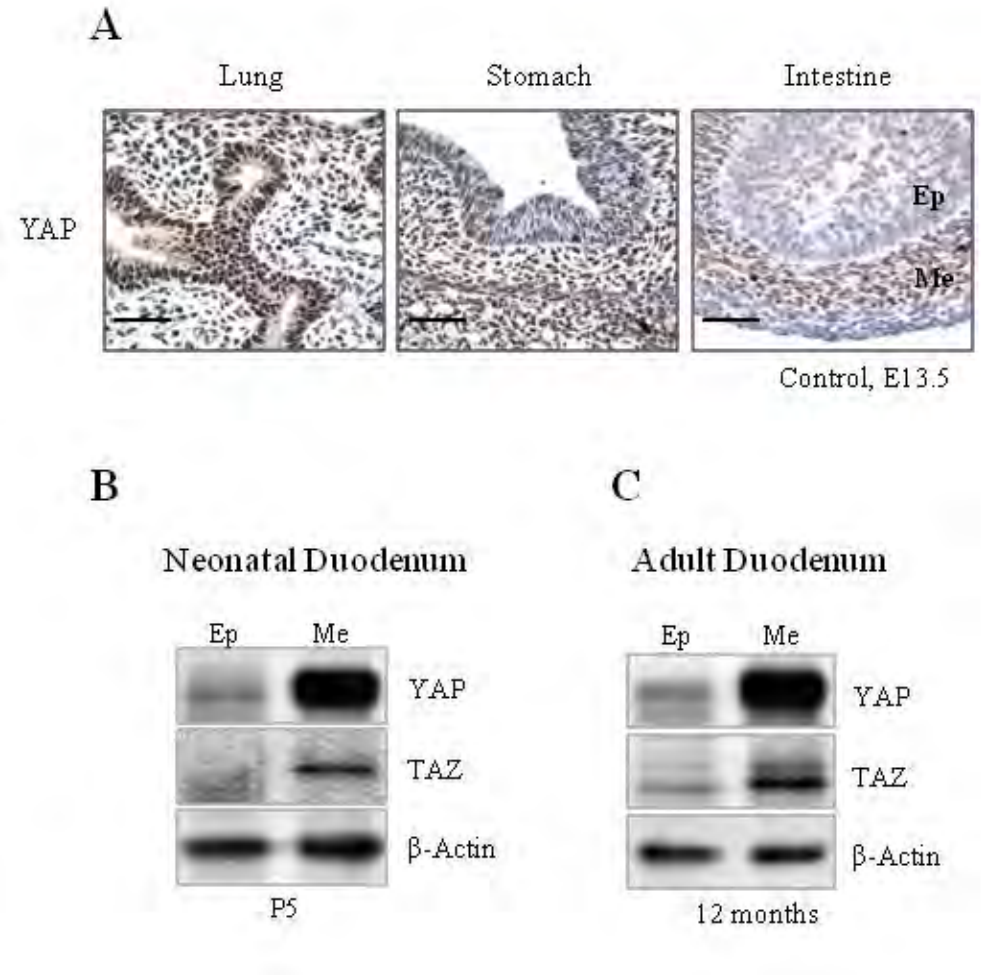


Figure 3.1. Expression of YAP/TAZ in endodermal epithelia and mesenchyme of embryonic lung and stomach.
(A) Immunohistochemical YAP staining in wild-type lung, stomach, and small intestine tissue at E13.5. *(B-C)* Western blot analysis. Protein lysates from intestinal epithelia and intestinal mesenchyme of wild-type postnatal day 5 (P5) mice (B) and 12 month old mice (C) were probed with YAP and TAZ antibodies. Scale Bar = 10μM.

homozygous knockout of both YAP and TAZ resulted in perinatal embryonic lethality *(Fig. 3.2.A)* and a dramatic mesenchymal growth defect *(Fig. 3.2.B)*. Consistent with a caudal-rostral time-dependent expression gradient for Nkx3.2, gastrointestinal mesenchymal reduction in the $Nkx3.2^{Cre}Yap^{flox/flox}Taz^{flox/flox}$ animals was most pronounced in the stomach and less so in the intestine *(Fig. 3.2.B, C)*. As such, we focused our analysis on the stomach of mutant animals. Although YAP/TAZ double mutant animals *(Fig 3.3.A)* exhibited a significant loss of overall gastrointestinal mesenchyme *(Fig. 3.2.C)*, the different mesenchymal cell populations such as smooth muscle cells, myofibroblasts, and enteric neurons were still present, albeit greatly reduced *(Fig. 3.3. E-G)*. Gastric epithelia adjacent to YAP/TAZ deficient mesenchyme exhibited apparently normal Wnt signaling activity as assayed by β-catenin and CD44 expression (*Fig. 3.3. B,C*) and normal epithelial differentiation, including H/K-ATPase^{+} parietal cells (*Fig. 3.3 D*).

Further analysis of YAP/TAZ mutant animals at an earlier embryonic time point, E14.5, revealed similar phenotypic characteristics *(Fig 3.4)*. YAP/TAZ mutant embryos exhibited a smaller mesenchymal compartment as compared to controls yet the induction of the mesenchymal lineages, including smooth muscle cells, enteric neurons, and endothelial cells, still occurred *(Fig 3.4. A-C)*. Moreover, the mutant mesenchyme did not exhibit increased apoptosis *(Fig 3.4. D)*, but did display a proliferation defect, as measured by Ki67 staining *(Fig 3.4. E, F)*. Together, our genetic analyses reveal an essential requirement of YAP/TAZ in gastrointestinal mesenchymal development, and suggest an interesting hypothesis that YAP/TAZ might be involved in the expansion of

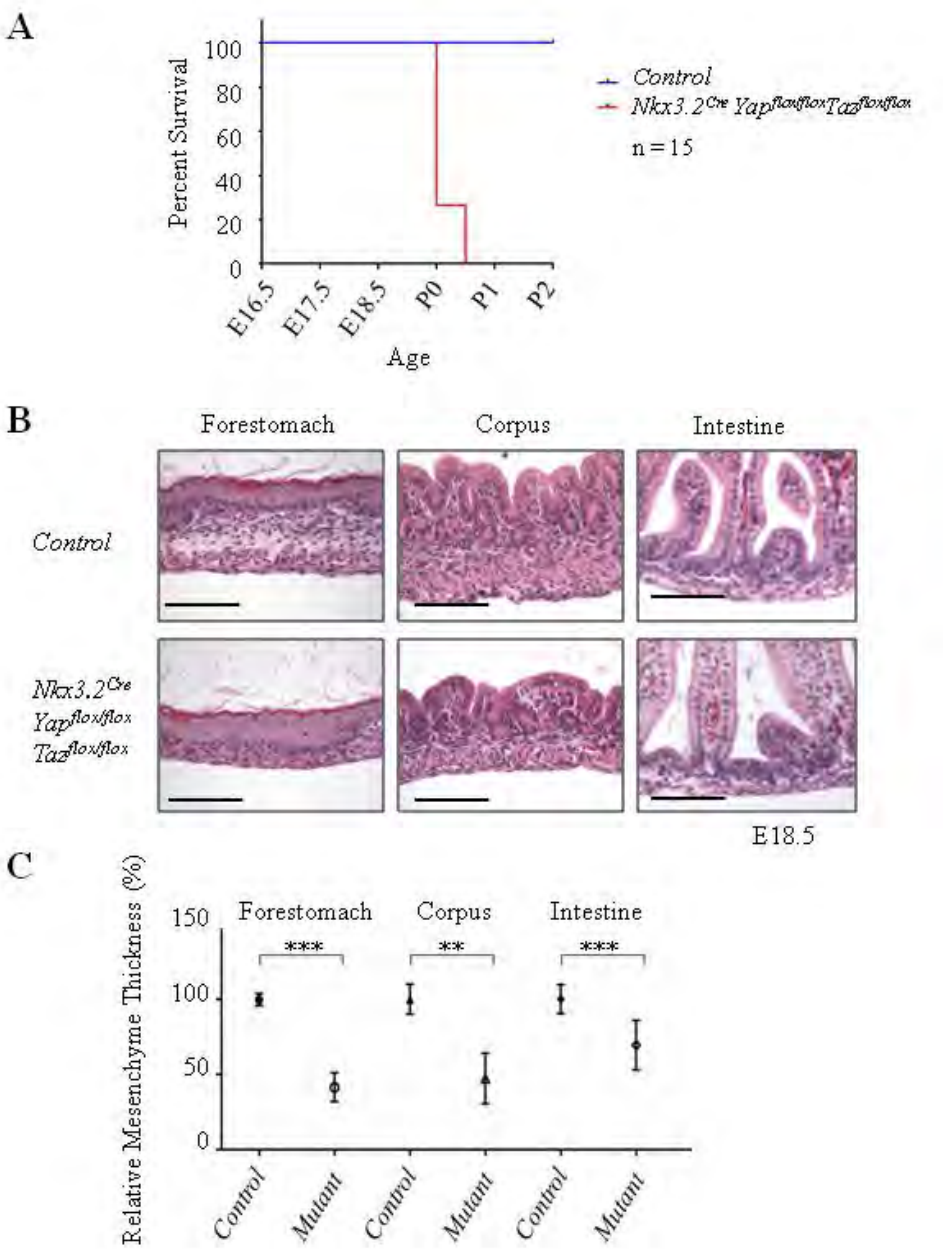


Figure 3.2. YAP and TAZ are required for gastrointestinal mesenchymal development.
(A) Mesodermal loss of *Yap* and *Taz* is perinatal lethal. *(B)* Histology of forestomach, corpus, and intestine of control and YAP/TAZ deficient mesenchyme (*Nkx3.2*^{Cre}*Yap*$^{flox/flox}$*Taz*$^{flox/flox}$) animals at E18.5. *(C)* Relative mesenchyme width quantification. Scale bar = 20μM. Data are mean ± S.D., n = 3, * = p value ≤0.05; ** = p value ≤0.01; *** = p value ≤0.001, Student's *t*-test.

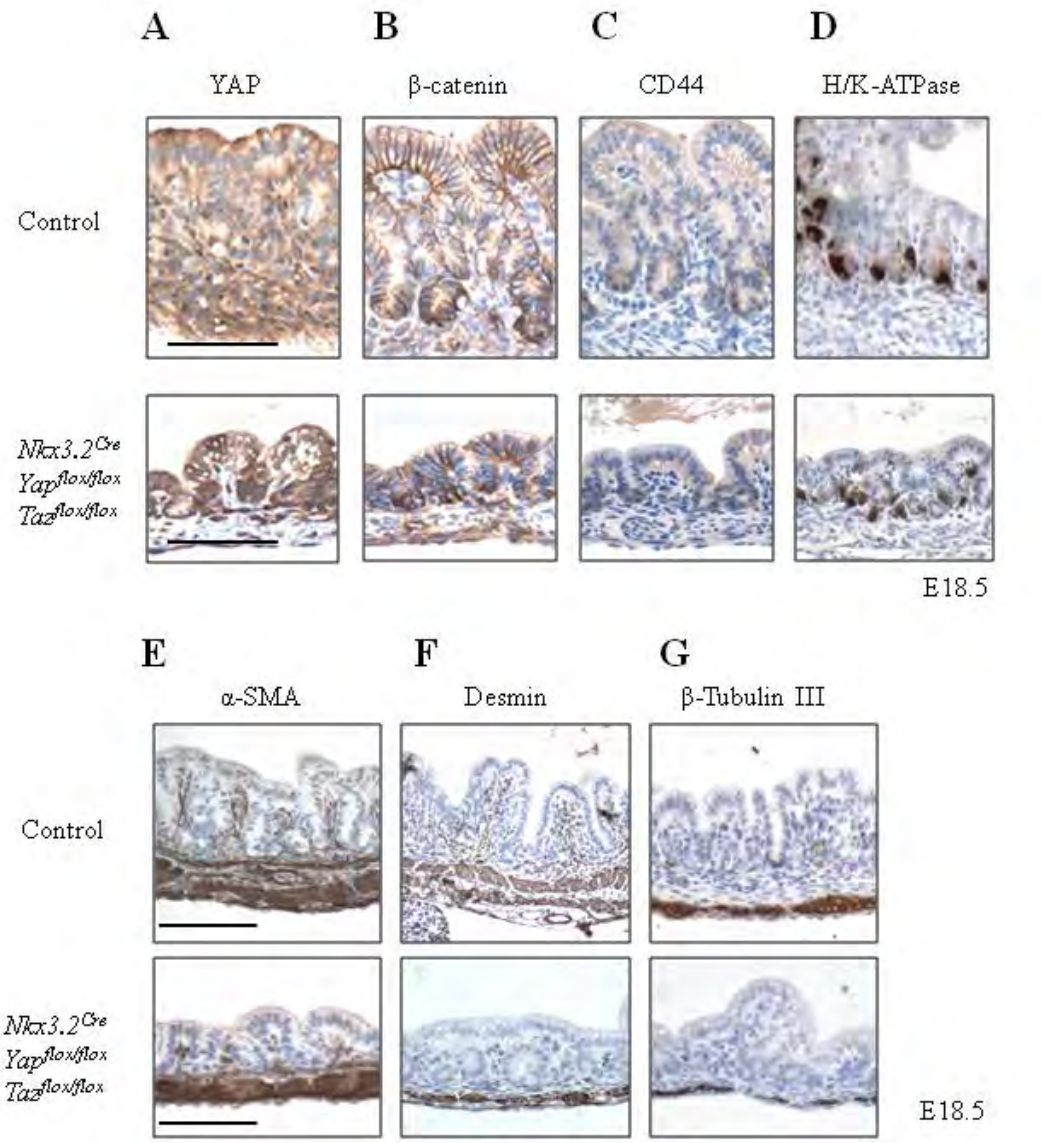


Figure 3.3. YAP/TAZ knockout in GI mesenchyme does not affect intestinal epithelia Wnt target expression or intestinal cell differentiation at E18.5.
YAP/TAZ deficient mesenchyme differentiates into muscle with innervation by enteric neurons. Immunohistochemical *(A)* YAP, *(B)* β-catenin, *(C)* CD44, *(D)* H/K-ATPase, *(E)* α-smooth muscle actin (α-SMA), *(F)* desmin, and *(G)* β-TubulinIII staining in stomach mesenchyme of control and *Nkx3.2*^{Cre}*Yap*$^{flox/flox}$*Taz*$^{flox/flox}$ animals at E18.5. Scale bar = 20μM.

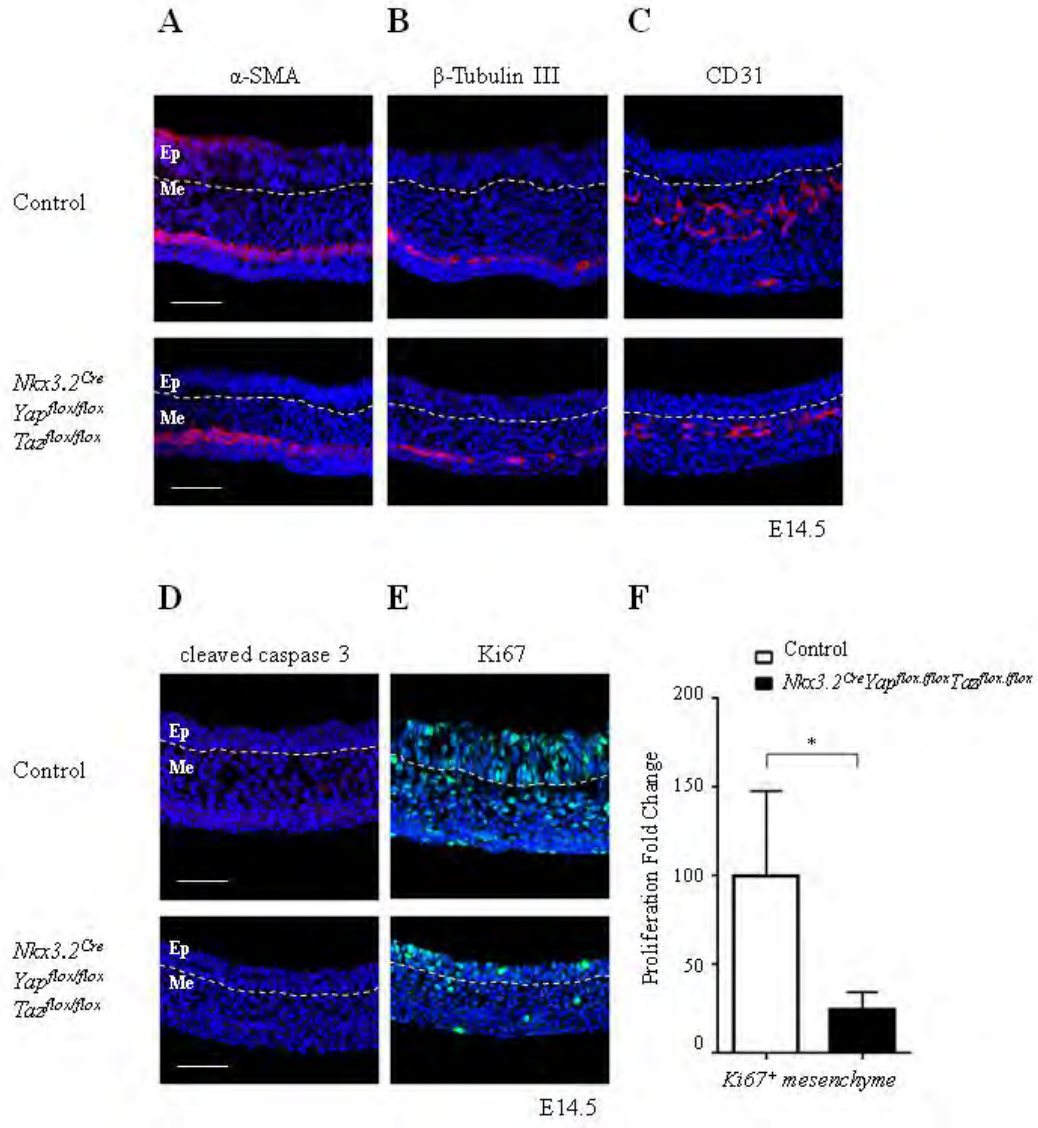


Figure 3.4. YAP/TAZ knockout in GI mesenchyme at E14.5 exhibits significantly reduced mesenchymal proliferation.
(A) a-SMA, *(B)* β-TubulinIII, *(C)* CD31, *(D)* cleaved caspase 3, and *(E)* Ki67 immunofluorescence staining and *(F)* Ki67 quantification in stomach mesenchyme of control and *Nkx3.2*^{Cre}*Yap*$^{flox/flox}$*Taz*$^{flox/flox}$ animals at E14.5. Data are mean ± S.D., n = 3, * = p value ≤0.05. Student's *t*-test. Scale bar = 20μM.

early mesenchymal progenitor populations, and the overall mesenchymal reduction in YAP/TAZ mutant animals is due to the failure of early progenitors to adequately proliferate before subsequent differentiation.

YAP activation promotes mesenchymal growth

To further examine the role of YAP in early gastrointestinal mesenchyme, we generated a conditional *Rosa26* allele, $R26^{YAP5SA}$, which enables the *in vivo* expression of YAP5SA, a constitutively active form of YAP *(Fig. 3.5.A)*. YAP5SA has five canonical LATS phosphorylation sites mutated from serine to alanine to prevent Hippo/Lats mediated inhibition and degradation (Zhao et al., 2007). The $R26^{YAP5SA}$ allele also carries a 1X-Flag tag, an N-terminal nuclear localization signal (NLS), and a C-terminal IRES-nuclear LacZ tag, under the control of a *CMV* enhancer/β-actin hybrid CAGGS promoter targeted into the *Rosa26* locus. Subcellular localization of $R26^{YAP5SA}$ protein product is strictly nuclear following Cre-mediated recombination *(Fig. 3.5.B)*.

To investigate the function of this active YAP in the gastrointestinal mesenchyme, the $R26^{YAP5SA}$ allele was crossed to the $Nkx3.2^{Cre}$ mice. $Nkx3.2^{Cre}$ $R26^{YAP5SA}$ animals were embryonic lethal by E14.5 *(Fig. 3.6.A)* with an enlarged abdomen *(Fig. 3.6.B)* and expanded somites *(Fig. 3.6.C)*. Upon dissection, we observed a striking enlargement of the gastrointestinal tract in mutant animals *(Fig. 3.7.A)*. Expression of the $R26^{YAP5SA}$ transgene in the mesenchyme was detected by nuclear lacZ expression *(Fig. 3.8.A)* and nuclear YAP staining *(Fig. 3.8.B)*. As expected, transcription of the YAP target genes, such as *Ctgf* and *Ankrd1*, were significantly up-regulated *(Fig. 3.7.B)*. We found that the

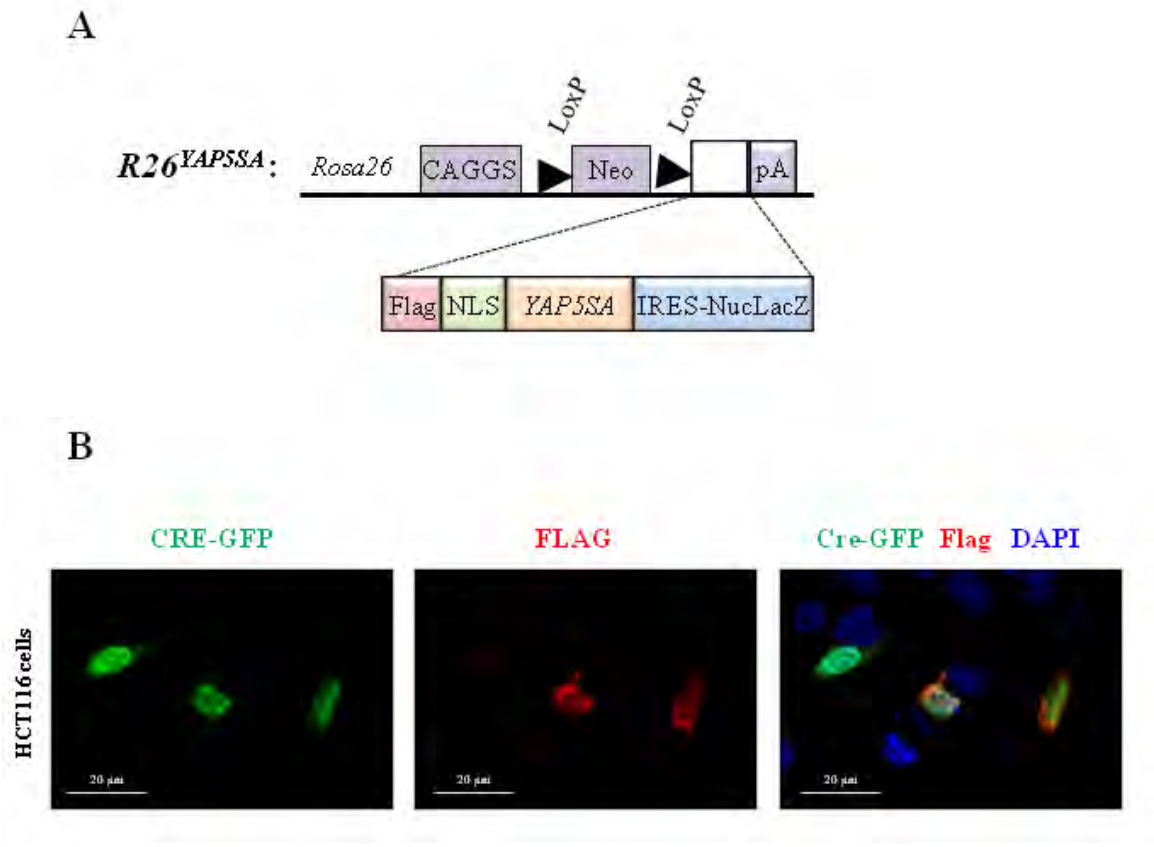


Figure 3.5. Generation of a novel YAP gain of function mouse allele.
(A) Diagram of *R26*^{YAP5SA} conditional mouse allele. *YAP5SA* cDNA, 1X-Flag tag, and IRES-NucLacZ under control of the CAGGS promoter were knocked into the *Rosa26* locus. Flanking Lox^{P} sites allow transgene expression through traditional Cre-Lox technology. *(B)* HCT116 cells transfected with *pCN-YAP5SA* plasmid and infected with lentiviral *pGIPZ-Cre-GFP* show exclusive nuclear localization of Flag-YAP5SA.

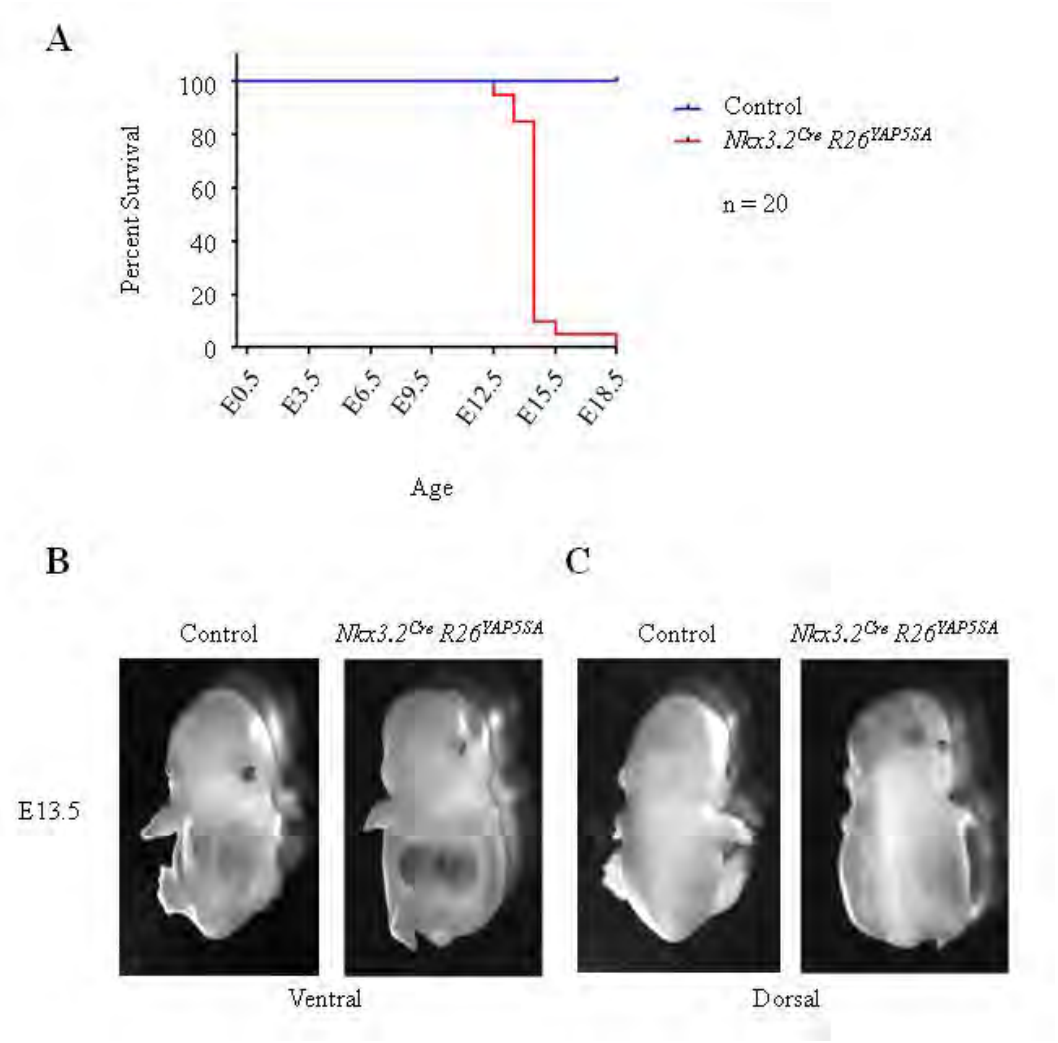


Figure 3.6. YAP gain of function in the developing mesoderm is embryonic lethal. *(A)* Mesodermal expression of $R26^{YAP5SA}$ transgene is embryonic lethal; the majority of mutant animals die by E14.5. *(B)*$Nkx3.2^{Cre}R26^{YAP5SA}$ embryos at E13.5 *(C)* exhibit a severe somite overgrowth phenotype.

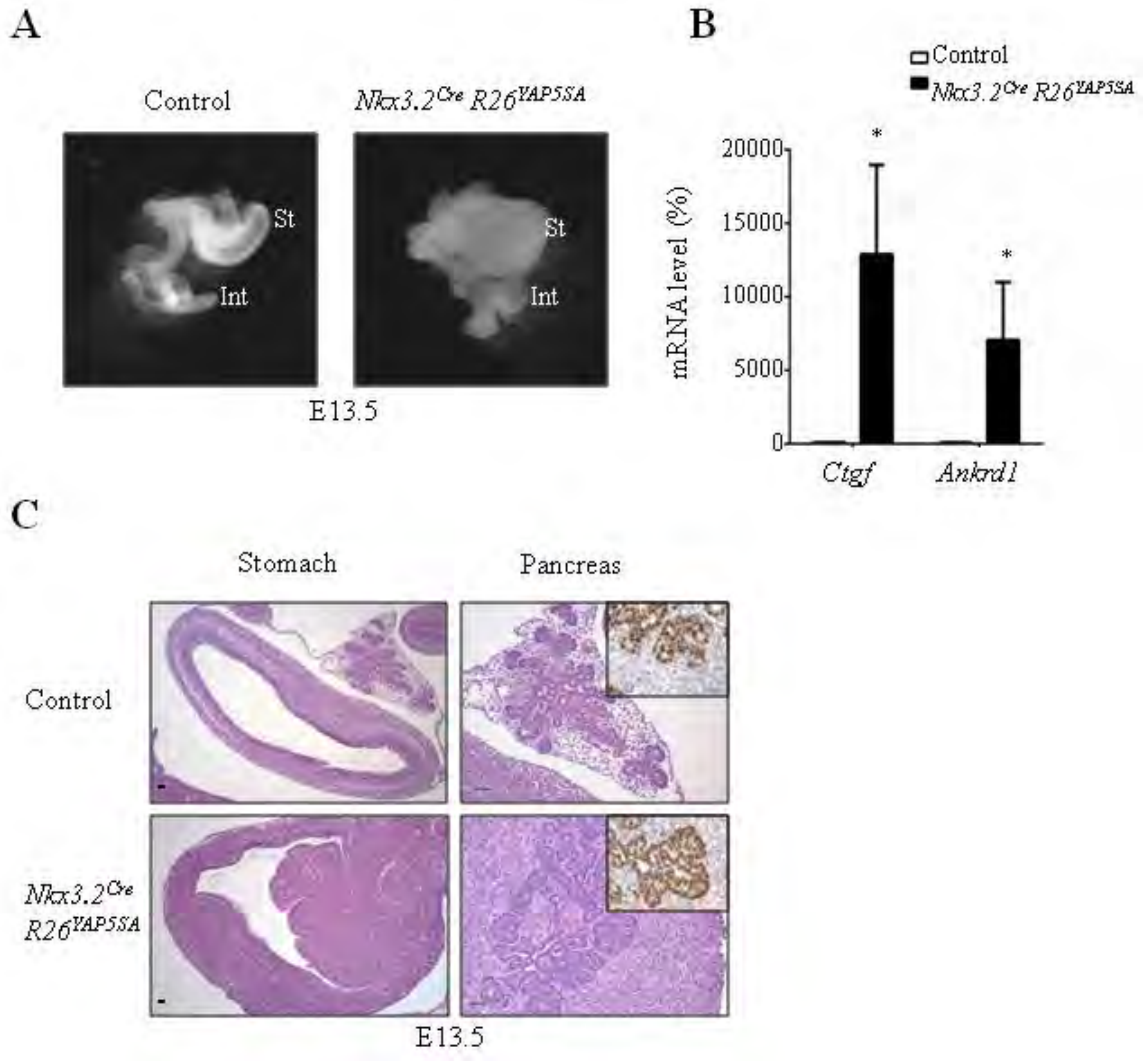


Figure 3.7. Constitutive *R26*^{YAP5SA} overexpression drives gastrointestinal mesenchymal expansion.
(A) Gastrointestinal tract from control and mesenchymal YAP gain-of-function (*Nkx3.2*^{Cre}*R26*^{YAP5SA}*)* mice at E13.5. *(B)* Real-time PCR analysis of *Ctgf* and *Ankrd1* mRNA levels in control and *Nkx3.2*^{Cre}*R26*^{YAP5SA}*)* mutant stomach at E13.5. *(C)* Histology of stomach and pancreas of control and mesenchymal *Nkx3.2*^{Cre}*R26*^{YAP5SA} mutant animals at E13.5. Inserts show Sox9 immunohistochemical staining in the branching epithelia of pancreatic buds. Data are mean ± S.D., n = 3, * = p value ≤0.05, Student's *t*-test.

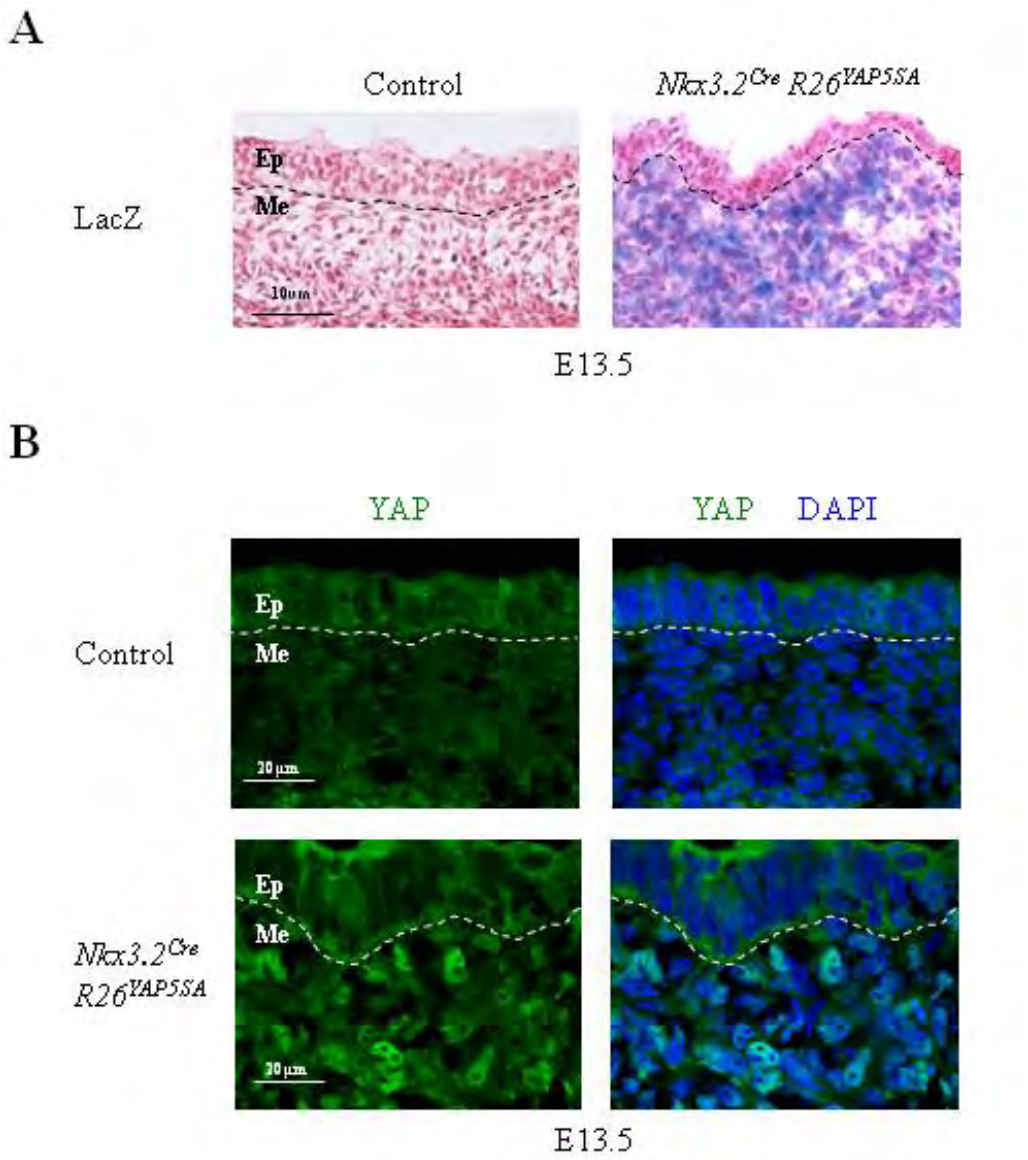


Figure 3.8. Cells in expanded GI compartment are derived from *R26*$^{YAP5SA\ +}$ cell population.
(A) Nuclear lacZ staining in the *Nkx3.2*^{Cre} *R26*^{YAP5SA} gastrointestinal tract is restricted to the mesenchyme. *(B)* YAP overexpression and nuclear localization in gastrointestinal mesenchymal cells in *Nkx3.2*^{Cre} *R26*^{YAP5SA} mutants as compared to control.

greatly expanded mesenchyme had even begun to engulf the developing pancreatic bud at E13.5 *(Fig. 3.7.C, insert)*. Enteric neurons and endothelial cells were present, although overall cell organization was disrupted *(Fig. 3.9.B-D)*. Not surprisingly, we find that mesenchymal cells with activated YAP were highly proliferative *(Fig. 3.9.A)*.

We next examined embryos at E11.5, a developmental time point prior to induction of differentiated mesenchymal lineages. At E11.5, YAP5SA-induced mesenchymal expansion was clearly evident *(Fig. 3.10.A)* and was likely due to elevated proliferation, as measured by Ki67 staining *(Fig. 3.10.B, C)*. Thus, consistent with the YAP/TAZ knockout studies, our $R26^{YAP5SA}$ analyses also suggest a critical role of YAP/TAZ in the expansion of early gastrointestinal mesenchyme, thereby controlling subsequent growth.

YAP/TAZ block smooth muscle differentiation induced by Hedgehog signaling

The Yap/TAZ loss- and gain-of-function phenotypes in the gastrointestinal mesenchyme reminded us of the phenotypes of Hedgehog pathway mutants. It has been demonstrated that paracrine Hedgehog signaling from gastrointestinal epithelia plays an important role in regulating mesenchymal growth and differentiation (Huang & Cotton et al., 2013; Kolterud et al., 2009; Mao et al., 2010; Zacharias et al., 2011). Our prior studies showed that loss of Shh and Ihh ligands resulted in significant reduction of mesenchymal populations (Mao et al., 2010), while Hedgehog pathway over-activation by $R26^{SmoM2}$ (Mao et al., 2006), a *Smoothened* gain-of-function allele, in the mesenchyme generated dramatic overgrowth (Mao et al., 2010). The similarity of these phenotypes

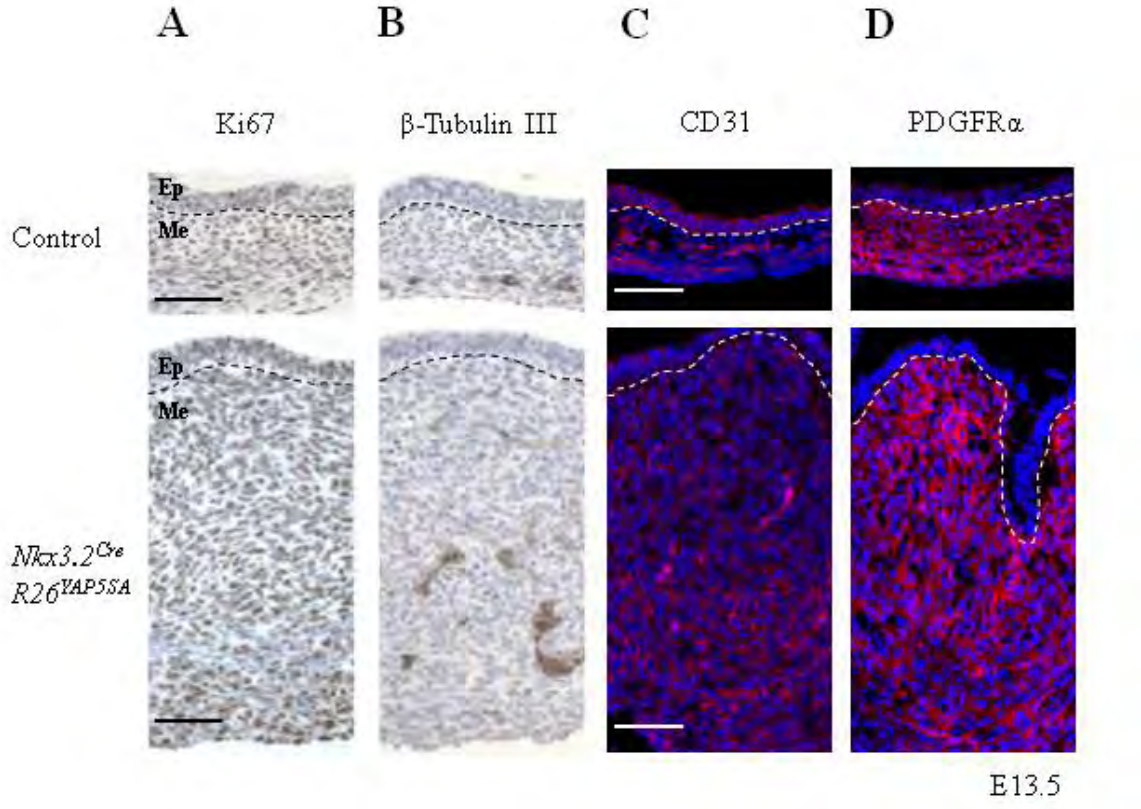


Figure 3.9. Highly proliferative GI compartment in *Nkx3.2^{Cre} R26^{YAP5SA}* mutants is mesenchymal in origin.
Expanded proliferative (*A.* Ki67) compartment in *Nkx3.2^{Cre} R26^{YAP5SA}* mutants at E13.5 is mesenchymal in origin (*D.* PDGFRα staining), with disrupted endothelial cell (*C.* CD31) and enteric neuron *(B.* β-TubulinIII) organization.

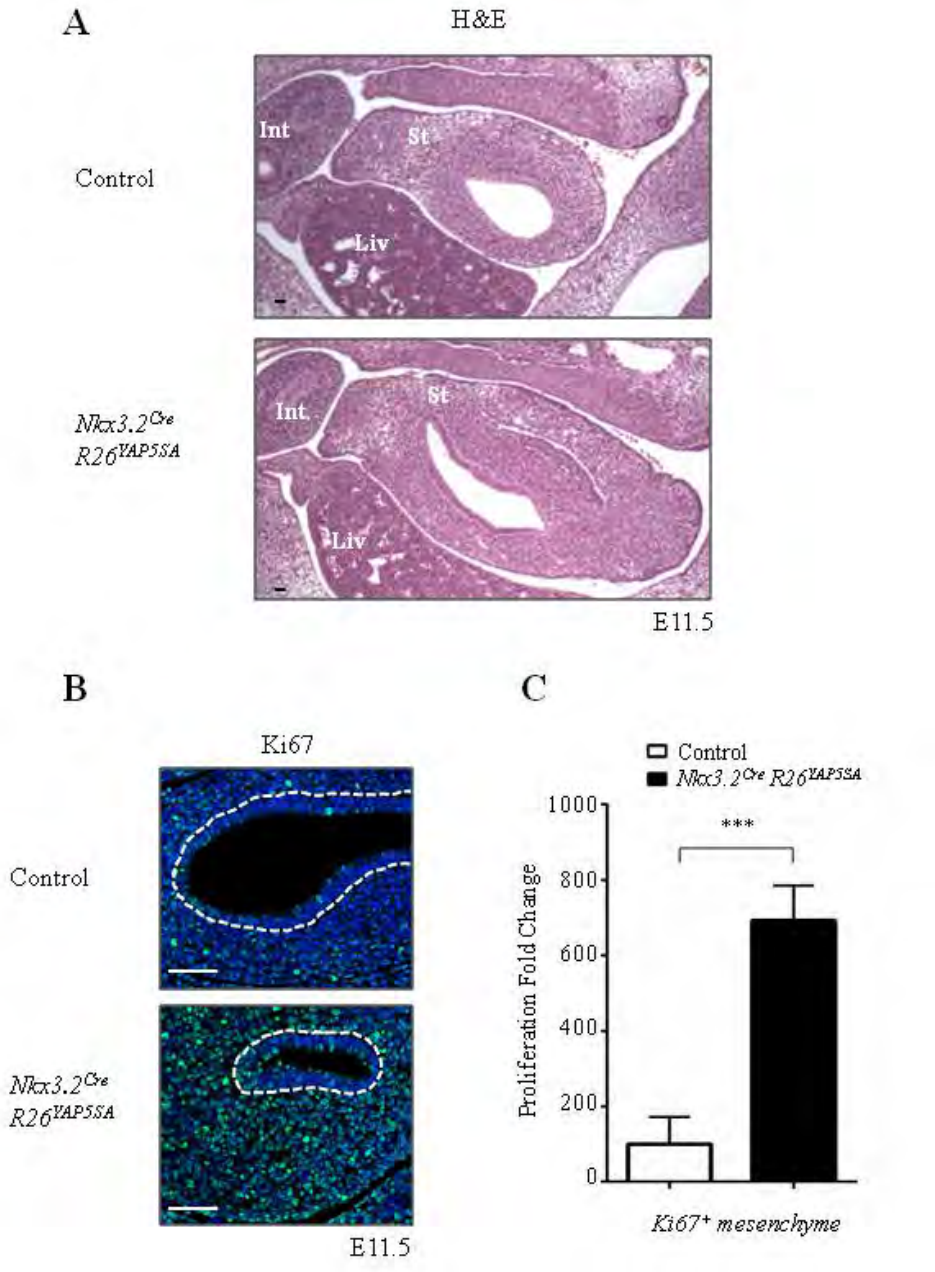


Figure 3.10. Expanded GI mesenchymal compartment exhibits elevated proliferation even at developmental timepoint E11.5
(A) Histology and *(B)* immunofluorescence Ki67 staining in control and $Nkx3.2^{Cre}R26^{Yap5SA}$ stomach at E11.5. *(C)* Quantification of fold change of $Ki67^{+}$ mesenchymal cells at E11.5. Data are mean ± S.D., n = 3, * = p value ≤0.05; ** = p value ≤0.01; *** = p value ≤0.001, Student's *t*-test.

prompted us to test whether there is a functional interaction between these two important signaling pathways during gastrointestinal development.

We next wanted to investigate whether YAP5SA overexpression impacted mesenchymal cell differentiation. Smooth muscle progenitor cells are first marked by the expression of α-smooth muscle actin (α-SMA) at E12.0 (McHugh, 1995; Takahashi et al., 1998). We first investigated expression of differentiation markers α-SMA and Desmin at E11.5 and observed no expression in either $Nkx3.2^{Cre}$ $R26^{YAP5SA}$ or control gastrointesintal tract, but did observe developmentally appropriate innervation by enteric neurons *(Fig. 3.11.A- D)* in both mutant and control tissues.

One of the key features in SmoM2-induced mesenchymal overgrowth is the expansion of the α-SMA-expressing progenitor cells *(Fig. 3.12.B)*, a mesenchymal lineage later giving rise to smooth muscle cells (SMC) and myofibroblasts. In wild-type gastrointestinal tract, the α-SMA-expressing smooth muscle progenitor population is detected as a tight band of cells located in the outer half of the mesenchyme at E13.5 *(Fig. 3.12.A)*. Surprisingly, we found that α-SMA and neuron-glia2 (NG2) expression was dramatically reduced in the $R26^{YAP5SA}$ mutants *(Fig. 3.12.C,D)*. In the YAP5SA mutant animals, expression of other mesenchymal markers, such as Vimentin, PDGRA, PDGRB, PDGFRα, and PDGFRβ, *(Fig. 3.9.D* and *Fig. 3.12.D)*, as well as the induction of enteric neuron and vasculature system *(Fig. 3.9.B,C)* were still present; this suggests that the effect is specific for the smooth muscle lineage and raises the possibility that YAP activation may actually inhibit smooth muscle cell differentiation.

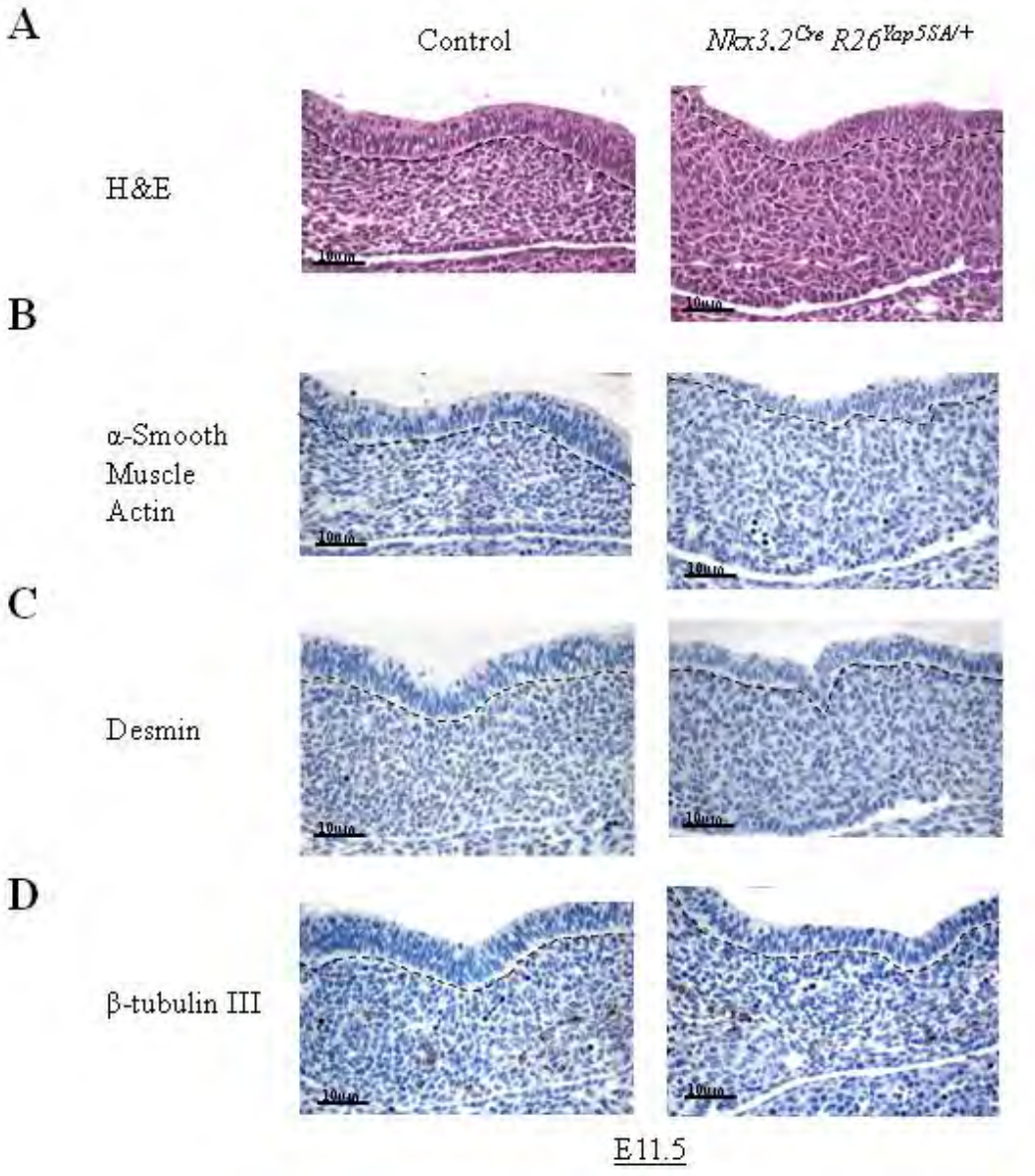


Figure 3.11. At E11.5, there is no difference in mesenchymal differentiation between *Nkx3.2^{Cre}R26^{YAP5SA}* and control tissue.
(A) Histology and *(B)* immunohistochemical staining for α-smooth muscle actin *(B)*, desmin *(C),* and β-tubulin III *(D)*.

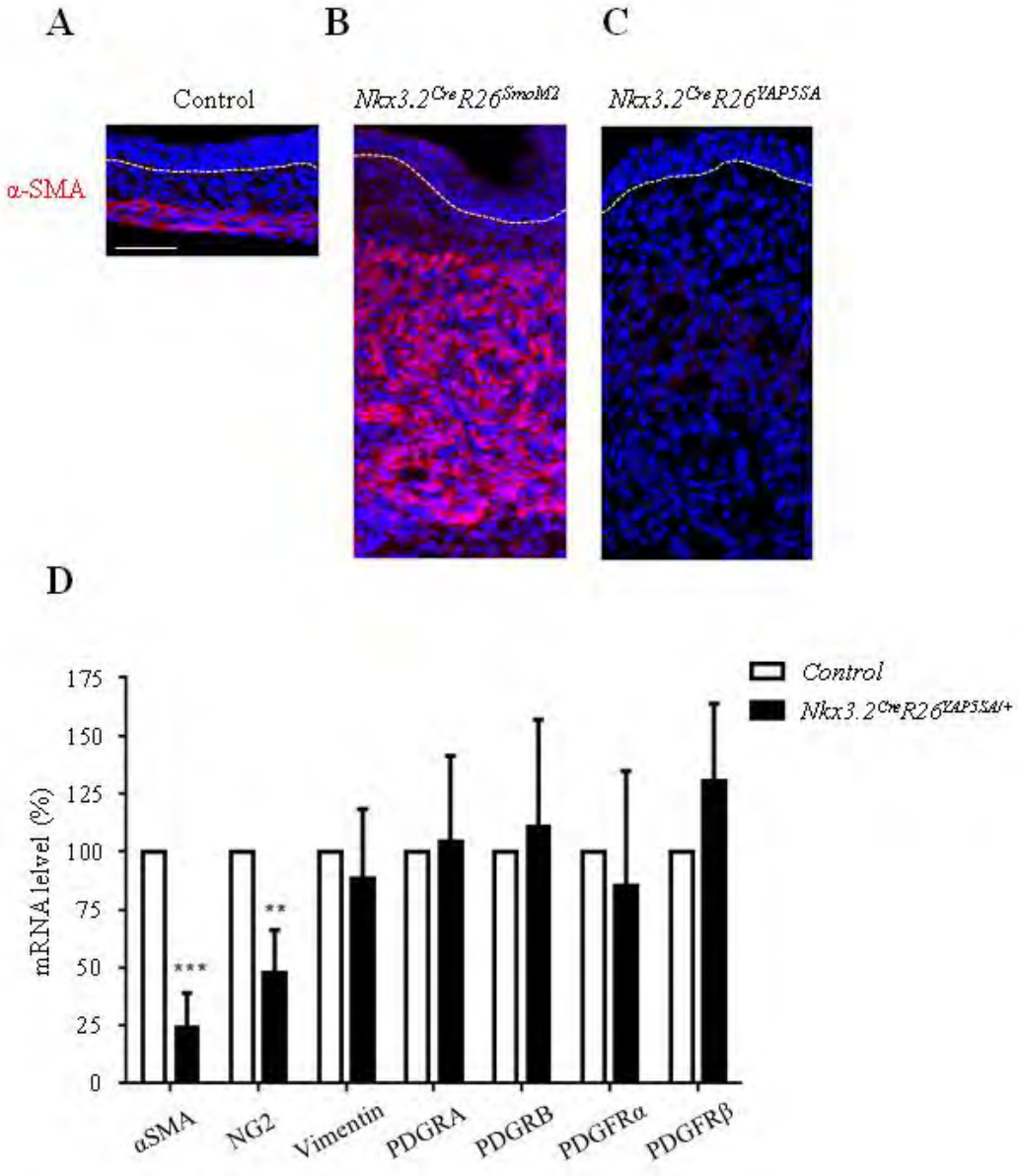


Figure 3.12. YAP activation blocks smooth muscle cell differentiation. Immunofluorescence a-SMA staining in *(A)* control, *(B)* Hedgehog gain-of-function (*Nkx3.2^{Cre}R26^{SmoM2})* and *(C)* YAP gain-of-function (*Nkx3.2^{Cre}R26^{YAP5SA})* stomach at E13.5. *(D)* Real-time PCR analysis. Expression of differentiation markers *α-SMA* and *NG2* is significantly less in *Nkx3.2^{Cre}R26^{YAP5SA}* mutant gut as compared to control, whereas there is no significant change in gene expression of general mesenchymal markers *Vimentin*, *PDGRA, PDGRB, PDGFRα,* and *PDGFRβ.* Data are mean ± S.D., n = 3, * = p value ≤0.05; ** = p value ≤0.01; *** = p value ≤0.001, Student's *t*-test.

To further elucidate the molecular mechanism underlying YAP activation in the gastrointestinal mesenchyme, we performed RNAseq analysis of wild-type and $R26^{YAP5SA}$ mutant stomach at E13.5, and intersected the data set with the Affymetrix microarray data we generated from E13.5 $R26^{SmoM2}$ mutant stomach (Huang & Cotton et al., 2013). Consistent with our phenotypic analysis, we found that transcription of the genes associated with smooth muscle differentiation, including *Acta2* (encoding α-SMA), *Actg2* (encoding γ-SMA), and *Myocd* (encoding myocardin), were up-regulated in $R26^{SmoM2}$ mutants, but down-regulated in $R26^{YAP5SA}$ mutants *(Fig. 3.13.A and B)*. It is not surprising that Hippo pathway targets, such as *Ankrd1, Ctgf,* and *Cyr61* were up-regulated in the $R26^{YAP5SA}$ mutants, while they were largely unaffected in the $R26^{SmoM2}$ mutants *(Fig. 3.13.A)*. Interestingly, in the $R26^{YAP5SA}$ mutants Hedgehog targets such as *Hhip1, Gli1*, and *Ptch1/2*, as well as Hedgehog pathway ligands *Shh* and *Ihh,* were downregulated *(Fig. 3.13. A and B)*, suggesting that YAP may inhibit SMC differentiation by regulating Hedgehog signaling activity, perhaps through ligand secretion.

To test this possibility, we further examined the $Nkx3.2^{Cre}$ $R26^{SmoM2}$ mutant embryos. Despite the wide-spread expression of SmoM2 in the gastrointestinal mesenchyme *(Fig. 3.14.C),* we noticed a mesenchymal cell population adjacent to the intestinal epithelia that remained negative for α-SMA expression and also displayed high endogenous nuclear YAP expression *(Fig. 3.14.D and E)*. These data suggest that Hedgehog activation by SmoM2 cannot override endogenous YAP activity and that Hedgehog signaling does not simply act downstream of YAP/TAZ to induce differentiation into SMC lineage. Consistent with this hypothesis, immunohistochemical

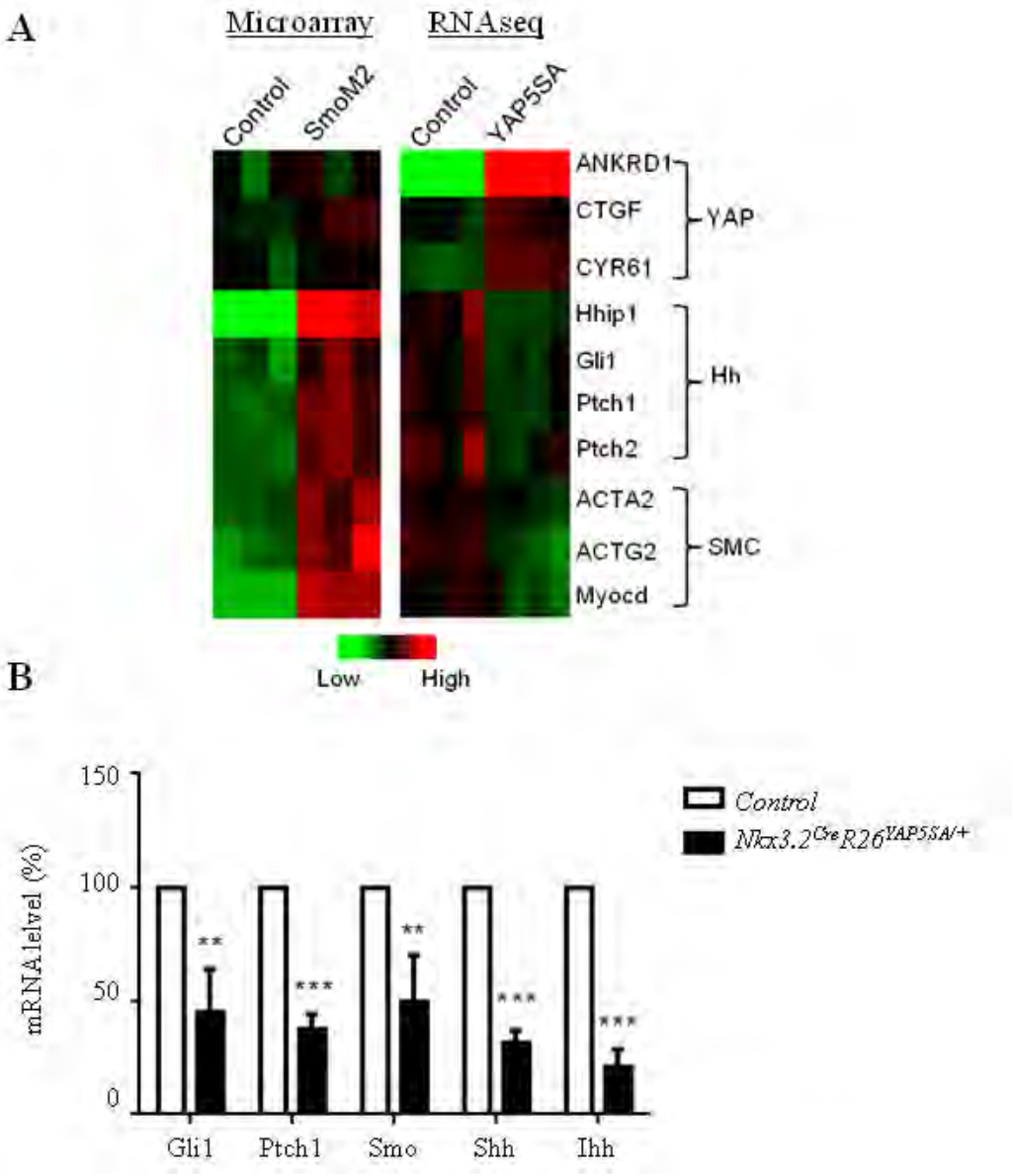


Figure 3.13. Gene expression profiling in GI tissues with Hedgehog and YAP activation.

(A) Heat map analysis comparing mRNA expression of YAP/TAZ targets (*Ankrd1, Ctgf*, and *Cyr61*), Hedgehog pathway targets (*Hhip1, Gli1, Ptch1* and *Ptch2*), and smooth muscle differentiation markers (*Acta2, Actg2*, and *Myocd*) in control, $Nkx3.2^{Cre}R26^{SmoM2}$, and $Nkx3.2^{Cre}R26^{YAP5SA}$ stomach at E13.5, using the data from RNAseq and Affymetrix array. *(B)* Real-time PCR analysis of Hedgehog Pathway components *Gli1, Ptch1, Smo, Shh,* and *Ihh* mRNA levels in control and $Nkx3.2^{Cre}R26^{Yap5SA}$ stomach at E13.5. Data are mean ± S.D., * = p value ≤0.05; ** = p value ≤0.01; *** = p value ≤0.001, Student's *t*-test.

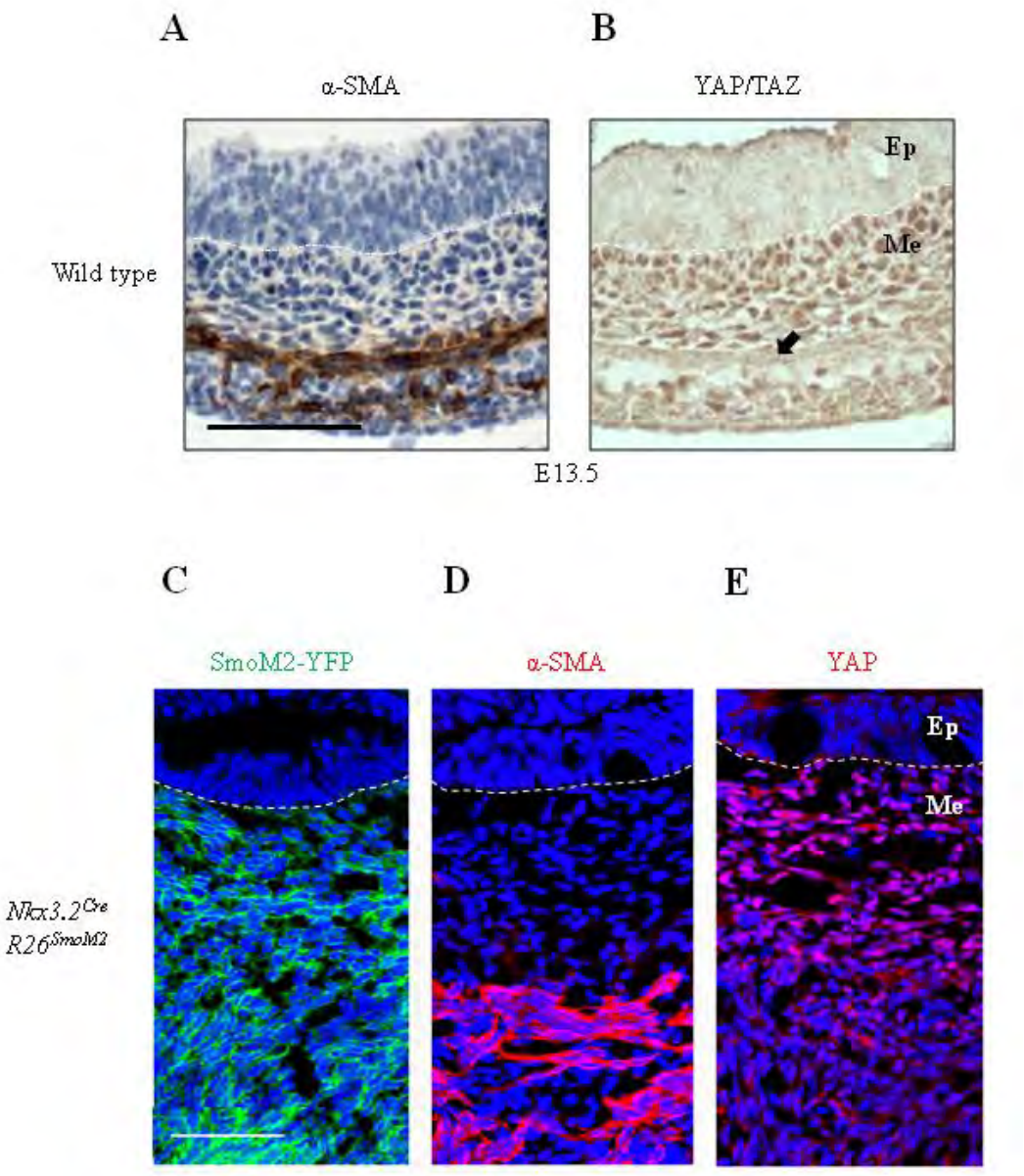


Figure 3.14. YAP and α-SMA expression in wild type and SmoM2-expressing gut mesenchyme.
Immunohistochemical staining of *(A)* α-SMA and *(B)* YAP/TAZ in wild-type stomach at E13.5. *(D)* a-SMA and *(E)* YAP immunoflourescence staining in *(C) SmoM2-YFP*-expressing gastrointestinal mesenchyme in $Nkx3.2^{Cre}R26^{SmoM2}$ animals at E13.5.

analysis showed that in the wild-type mesenchyme at E13.5, YAP/TAZ expression was significantly lower in the ring of the mesenchymal cells where α-SMA was expressed *(Fig. 3.14.A and B).*

To conclusively test whether endogenous YAP/TAZ was sufficient to inhibit Hedgehog-induced differentiation, we generated mesodermal-specific homozygous YAP knockout, homozygous TAZ knockout, and double homozygous YAP/TAZ knockouts in the SmoM2 gain of function background. We found that homozygous knockout of either YAP in $Nkx3.2^{Cre}$ $R26^{SmoM2}$ $Yap^{flox/flox}$ animals *(Fig. 3.15.A)* or TAZ in $Nkx3.2^{Cre}$ $R26^{SmoM2}$ $Yap^{flox/f+}Taz^{flox/flox}$ animals *(Fig. 3.15.B)* is not sufficient to expand the population of αSmooth Muscle $Actin^{+}$ mesenchymal cells in *Smoothened* gain of function mesenchyme. However, homozygous mesodermal knockout of both YAP and TAZ in conjunction with $R26^{SmoM2}$ overexpression in $Nkx3.2^{Cre}$ $R26^{SmoM2}$ $Yap^{flox/flox}$ $Taz^{flox/flox}$ *(Fig 3.15.C)* mutants is sufficient to induce the expression of αSmooth Muscle Actin in epithelial-adjacent mesenchymal cells at E13.5. These data provide further evidence that Hedgehog signaling is likely acting in parallel to, rather than downstream of, YAP/TAZ, to regulate mesenchymal progenitor cell differentiation.

We decided to further explore the relationship between YAP/TAZ and α-SMA in mesenchymal cells *in vitro* using the C3H10T1/2 cell line. C3H10T1/2 cells are an α-SMA^{-} mesenchymal progenitor mouse cell line that undergoes differentiation into α-SMA^{+} cells following Hedgehog pathway activation (Huang & Cotton et al., 2013; Reznikiff et al., 1973; Zacharias et al., 2011) Consistent with previously published reports, we also found that treating C3H10T1/2 cells for 24 hours with 0.1μm SAG

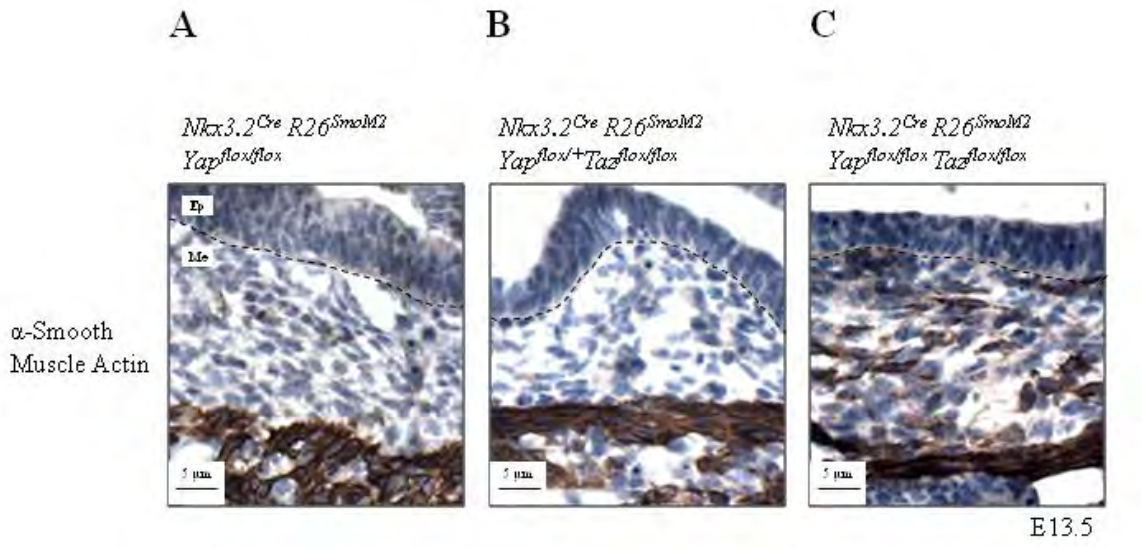


Figure 3.15. Loss of endogenous YAP/TAZ extends of αSMA^{+} cell compartment in Smoothened gain of function mutants.
Homozygous knockout of either YAP in *Nkx3.2^{Cre} R26^{SmoM2} Yap$^{flox/flox}$* animals *(A)* or TAZ in *Nkx3.2^{Cre} R26^{SmoM2} Yap$^{flox/f+}$Taz$^{flox/flox}$* animals *(B)* is not sufficient to expand the population of αSmooth Muscle Actin^{+} mesenchymal cells in *Smoothened* gain of function mesenchyme. Homozygous mesodermal knockout of both YAP and TAZ in conjunction with *R26^{SmoM2}* overexpression in *Nkx3.2^{Cre} R26^{SmoM2} Yap$^{flox/flox}$ Taz$^{flox/flox}$ (C)* mutants is sufficient to induce the expression of αSmooth Muscle Actin in epithelial-adjacent mesenchymal cells at E13.5.

(Smoothened Agonist) was sufficient to induce cell differentiation and α-SMA protein expression as compared to DMSO-treated cells *(Fig. 3.16.A and B)*. Importantly, we found that YAP overexpression through a lentiviral infection of *pGIPZ-3XFlag-YAP5SA* was sufficient to inhibit differentiation in C3H10T1/2 cells. We observed that following SAG treatment, compared to control cells, YAP5SA^{+} cells showed an inhibition of α-SMA protein *(Fig. 3.16.C and D)* and RNA expression, as well as mesenchymal differentiation markers *Myocd, SMMHC,* and *SM22α (Fig. 3.16.E)*.

Also, we demonstrated that YAP activation was sufficient to inhibit α-SMA expression in differentiated Smooth Muscle Myosin Heavy Chain^{+} (SMMHC^{+}) *in vivo*. We crossed $R26^{YAP5SA}$ to the inducible $Myh11^{CreER\text{-}T2}$ allele, which targets efficient Cre recombination in SMMHC^{+} cells following Tamoxifen injection at E12.5 *(Fig. 3.17.A)*. We found that YAP5SA expression in differentiated SMMHC^{+} mesenchymal cells effectively inhibited α-SMA expression *(Fig. 3.17.B)*.

Finally, we investigated both Hedgehog gain of function and YAP5SA gain of function in the same mesenchymal cell population. We were unable to generate $Nkx3.2^{Cre}R26^{SmoM2/YAP5SA}$ animals because single mutant animals are individually embryonic lethal and therefore could not be used as breeders in a genetic cross. We instead used the inducible $Myh11^{CreER}$ allele to specifically target the SMMHC^{+} mesenchymal cell population during development. We crossed the $R26^{YAP5SA}$ allele to the $Myh11^{CreER}R26^{SmoM2}$ allele and injected pregnant females intraperitoneally with tamoxifen at 12.5 dpc (days post coitus) to induce Cre-recombination, when embryos were estimated to be at stage E12.5, and dissected animals three days later at E15.5. We

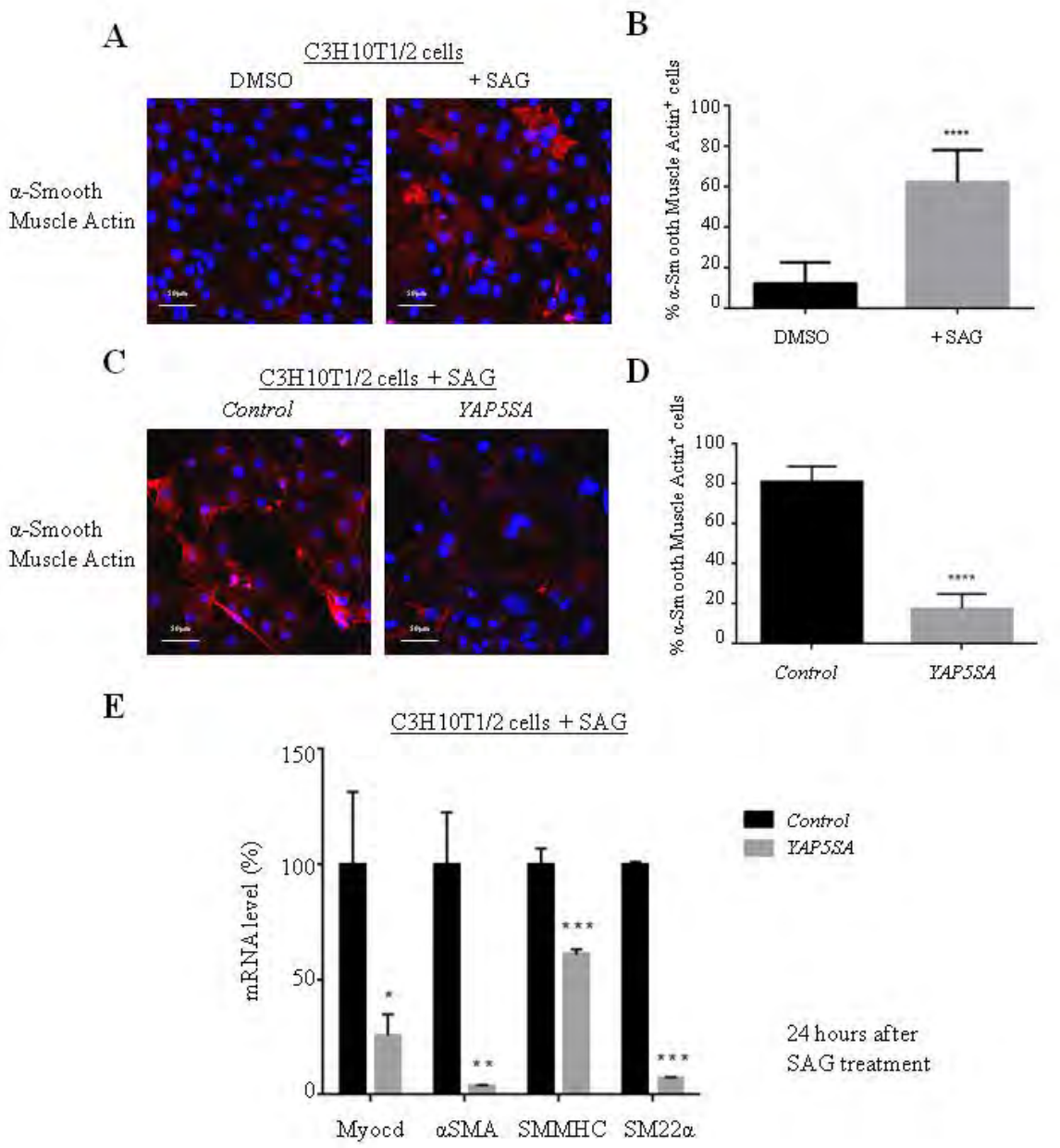


Figure 3.16. *YAP5SA* overexpression in mesenchymal progenitor cells blocks Hedgehog-induced differentiation *in vitro.*
C3H10T1/2 cells treated with Smoothened Agonist (SAG) undergo differentiation and upregulate expression of αSmooth Muscle Actin *(A,* immunofluoresence, and *B,* quantification). C3H10T1/2 cells infected with lentiviral *pGIPZ-3XFlag-YAP5SA* inhibit SAG-induced differentiation, as assayed by immunofluorescence *(C* and *D)* and mRNA expression of *Myocd*, *αSMA, SMMHC,* and *SM22α (E)*. Data are mean ± S.D., n = 3, * = p value ≤0.05; ** = p value ≤0.01; *** = p value ≤0.001, **** = p value ≤0.0001, Student's *t*-test.

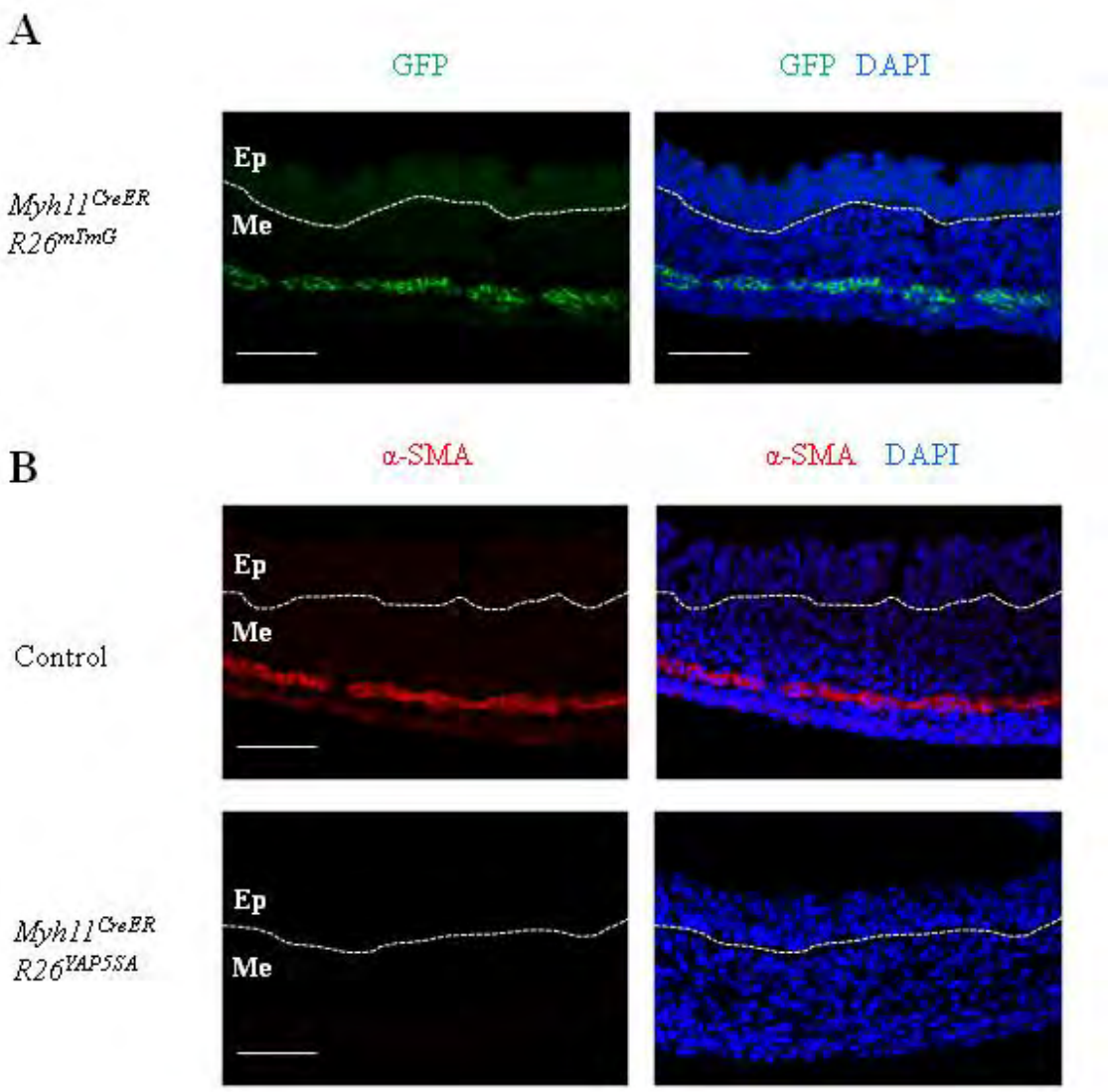


Figure 3.17. *YAP5SA* overexpression in a SMMHC^{+} mesenchymal cell population is sufficient to inhibit α-SMA expression *in vivo*.
(A) GFP images of the *Myh11*^{CreER}*R26*$^{mT/mG}$ stomach at E15.5, following intraperitoneal tamoxifen injection at E12.5. (*B*) α-SMA immunofluorescence staining in control and *Myh11*^{CreER}*R26*^{YAP5SA} stomach at E15.5, following intraperitoneal tamoxifen injection at E12.5.

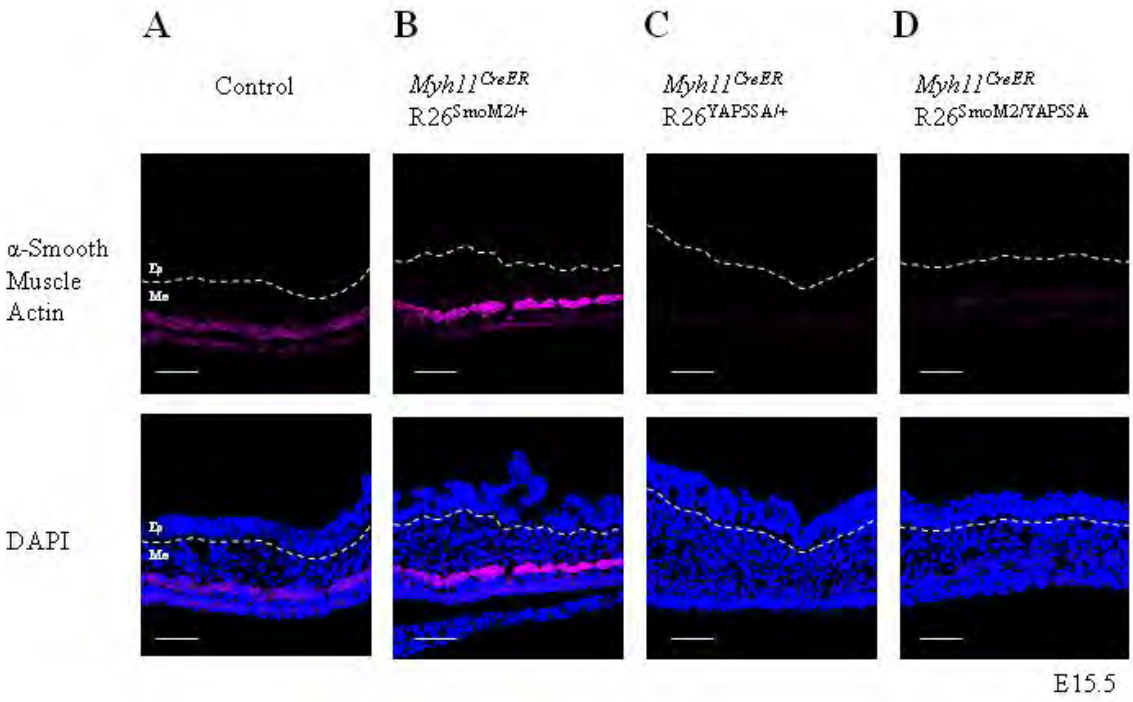


Figure 3.18. *SmoM2* overexpression is unable to rescue YAP5SA-mediated inhibition of smooth muscle differentiation *in vivo.*
Smoothened gain of function in SMMHC^{+} mesenchymal progenitor cells upregulates α-SMA expression *(B)* compared to control *(A)*. Smoothened gain of function in conjunction with YAP gain of function *(D)* does not rescue the YAP-mediated inhibition *(C)* of α-SMA protein expression in a differentiated SMMHC^{+} mesenchymal cell population at E15.5, following intraperitoneal tamoxifen injection at E12.5.

found that Hedgehog pathway gain of function in conjunction with YAP gain of function in *Myh11*^{CreER}*R26*$^{SmoM2/YAP5SA}$ mutants yields the same phenotype as *Myh11*^{CreER}*R26*$^{/YAP5SA}$; α-SMA protein expression is inhibited *(Fig. 3.18. C and D)* as compared to control and Hedgehog gain of function mutants *(Fig. 3.18.A and B)*.

Together, these data suggest that persistent YAP activation in either undifferentiated mesenchyme or specified smooth muscle progenitor cells is sufficient to block smooth muscle differentiation, and that down-regulation of YAP/TAZ expression is critical for Hedgehog-induced differentiation of the smooth muscle lineage.

Mesenchymal YAP/TAZ are regulated by canonical Lats1/2-mediated inhibition

We next investigated whether canonical Hippo Pathway was responsible for the downregulation and nuclear exclusion of YAP/TAZ in the gastrointestinal mesenchyme. To investigate this, we crossed mice carrying *Lats1*^{flox} and *Lats2*^{flox} alleles to mice carrying the *Nkx3.2*^{Cre} allele to genetically remove LATS1/LATS2 from the developing mesoderm. We found that animals tolerated single homozygous knockouts of either *Lats1* or *Lats2*, survived development through to adulthood, were fertile, and displayed normal architecture of the gastrointestinal tract as compared to control *(Fig. 3.19. A and B)*. However, we found that double homozygous knockout of both *Lats1* and *Lats2* in the developmental mesoderm faithfully recapitulated the YAP5SA gain of function phenotype; *Nkx3.2*^{Cre} *Lats1*$^{flox/flox}$ *Lats2*$^{flox/flox}$ was embryonic lethal by E14.5 and exhibited a dramatic gastrointestinal mesenchymal overgrowth phenotype *(Fig. 3.19.C)*.

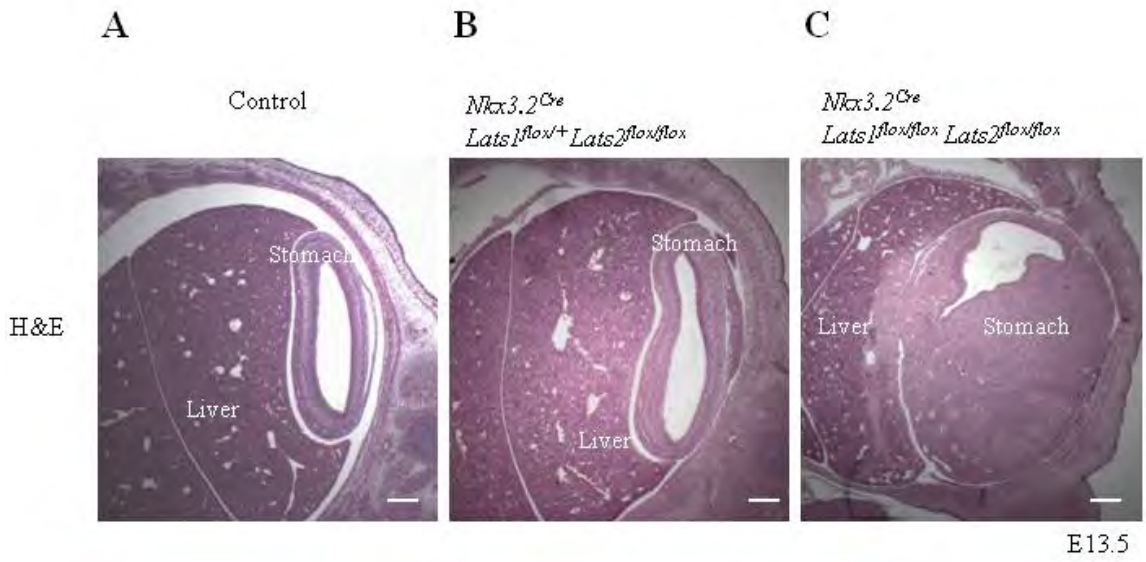


Figure 19. Genetic ablation of both *Lats1* and *Lats2* generates a gastrointestinal mesenchymal overgrowth phenotype.
(C) *Nkx3.2*^{Cre}*Lats1*$^{flox/flox}$*Lats2*$^{flox/flox}$ animals at E13.5 as compared to control *(A)*. Heterozygous loss of *Lats1* or *Lats2* is insufficient to drive overgrowth in *Nkx3.2*^{Cre}*Lats1*$^{flox/+}$*Lats2*$^{flox/flox}$ animals *(B)* or in *Nkx3.2*^{Cre}*Lats1*$^{flox/flox}$*Lats2*$^{flox/+}$animals (*data not shown*).

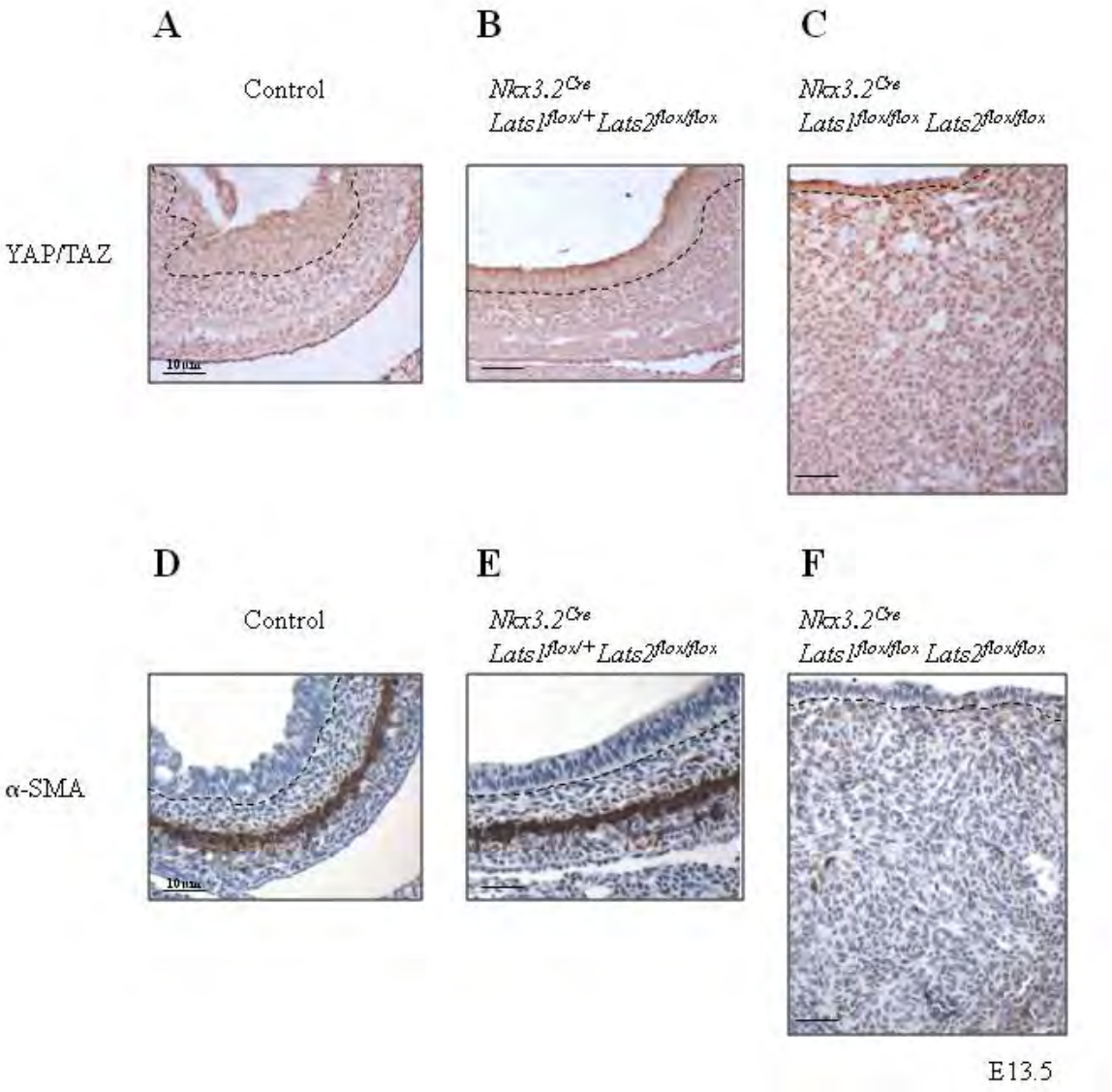


Figure 3.20. Lats1/2 deletion inhibits mesenchymal differentiation.
Mesodermal loss of both *Lats1* and *Lats2* is sufficient for nuclear localization of endogenous YAP protein *(C)* and inhibition of α-SMA expression *(F)* in mutant gut mesenchyme of *Nkx3.2*^{Cre}*Lats1*$^{flox/flox}$*Lats2*$^{flox/flox}$ animals at E13.5 as compared to control *(A, D)*. Heterozygous loss of *Lats1* or *Lats2* is insufficient to stabilize nuclear YAP *(B)* or inhibit expression of *α-SMA (E)* in mesenchyme of *Nkx3.2*^{Cre}*Lats1*$^{flox/+}$*Lats2*$^{flox/flox}$ animals or in *Nkx3.2*^{Cre}*Lats1*$^{flox/flox}$*Lats2*$^{flox/+}$animals (*data not shown*).

We observed nuclear accumulation of endogenous YAP/TAZ protein in LATS1/LATS2 deficient mesenchyme in *Nkx3.2*^{Cre} *Lats1*$^{flox/flox}$ *Lats2*$^{flox/flox}$ mutant animals as compared to control, but retaining just a single copy of either *Lats1* or *Lats2*, such as in *Nkx3.2*^{Cre} *Lats1*$^{flox/+}$ *Lats2*$^{flox/flox}$ animals, was sufficient to maintain normal mesenchymal YAP/TAZ expression patterning *(Fig. 3.20.A-C)*. Additionally, mesodermal loss of both LATS1/LATS2 was sufficient for inhibition of α-SMA protein expression in mutant gut mesenchyme. Again, retaining a single copy of either *Lats1* or *Lats2* was sufficient to maintain normal progenitor cell differentiation into α-SMA^{+} mesenchymal cells *(Fig. 3.20.D-F)*.

Together these data support our hypothesis that canonical Hippo pathway regulation of YAP/TAZ through LATS1/2-mediated inhibitory phosphorylation is responsible for exclusion of YAP/TAZ from the nucleus before the Hedgehog-mediated mesenchymal differentiation program is able to proceed.

YAP inhibits mesenchymal differentiation through direct regulation of Myocardin

Finally, we wanted to elucidate the molecular mechanism through which YAP is inhibiting gastrointestinal mesenchymal progenitor cell differentiation. We hypothesized that YAP is acting as a direct transcriptional co-repressor of *Myocd*. To investigate this, we performed multiple chromatin immunoprecipitation (ChIP) experiments in C3H10T1/2 cells expressing a lentiviral 3x-Flag-*YAP5SA* construct. We first performed chromatin immunoprecipitation with Flag antibody and determined that YAP binds directly to the promoter region of *Myocd*, as well as bona fide YAP target genes *Cyr61*

and *Ctgf (Fig. 3.21.A)*. Interestingly, we found no enrichment for YAP at the promoter region for *α-SMA*, indicating that the YAP-mediated inhibition of α-SMA is not happening through a direct regulation at the transcriptional level.

We then performed chromatin immunoprecipitation with H3K27ac antibody, a generic marker for active gene expression, to determine whether our genes of interest were being actively transcribed. We observed that the promoter regions of bona fide YAP target genes *Cyr61, Ctgf,* and *Ankrd1* were all strongly enriched for H3K27ac binding, indicating these genes were actively expressed. However, when compared to the bona fide YAP targets, the *Myocd* promoter region was not strongly enriched for H3K27ac, indicating that it was not actively expressed *(Fig. 3.21B)*, and supporting our data showing downregulation of *Myocd* mRNA following YAP5SA overexpression *(Fig. 3.13.A and Fig. 3.16.E)*. We also performed a chromatin immunoprecipitation for Chd4, the core component of the NURD complex known to be involved in epigenetic transcriptional repression (Kim et al., 2015b), and found only the *Myocd* promoter region was statistically enriched by CHD4 pulldown, whereas the bona fide YAP target gene promoter regions for *Cyr61, Ctgf,* and *Ankrd1* were not statistically enriched.

We next wanted to investigate whether this level of transcriptional regulation of *Myocd* was occurring *in vivo* in our $Nkx3.2^{Cre}$ $R26^{YAP5SA}$ animals. We dissected $Nkx3.2^{Cre}$ $R26^{YAP5SA}$ animals at E13.5 and performed chromatin immunoprecipitation using a highly concentrated YAP antibody. Similar to our results in C3H10T1/2 cells, we found statistically significant enrichment at the promoter regions of bona fide YAP targets *Ctgf* and *Cyr61*, as well as at the *Myocd* promoter region *(Fig. 3.22)*.

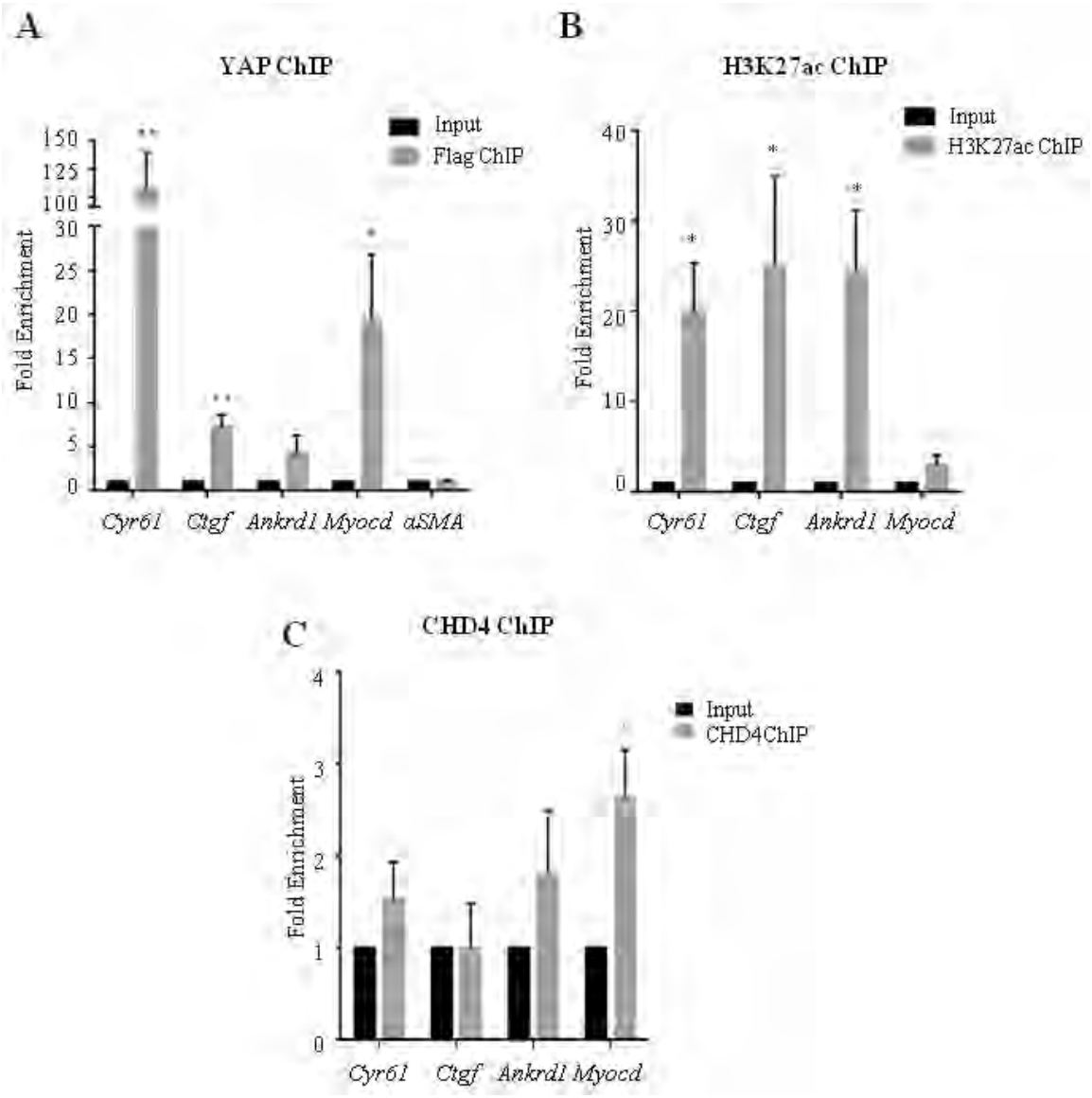


Figure 3.21. YAP directly binds to the promoter region of the *Myocd* gene *in vitro.* Chromatin immunoprecipitation in C3H10T1/2 cells infected with *pGIPZ-3XFlag-YAP5SA* and incubated with *(A)* Flag, *(B)* H3K27ac, and *(C)* Chd4 antibody. Data are mean ± S.D., n = 3, * = p value ≤0.05; ** = p value ≤0.01. Student's *t*-test.

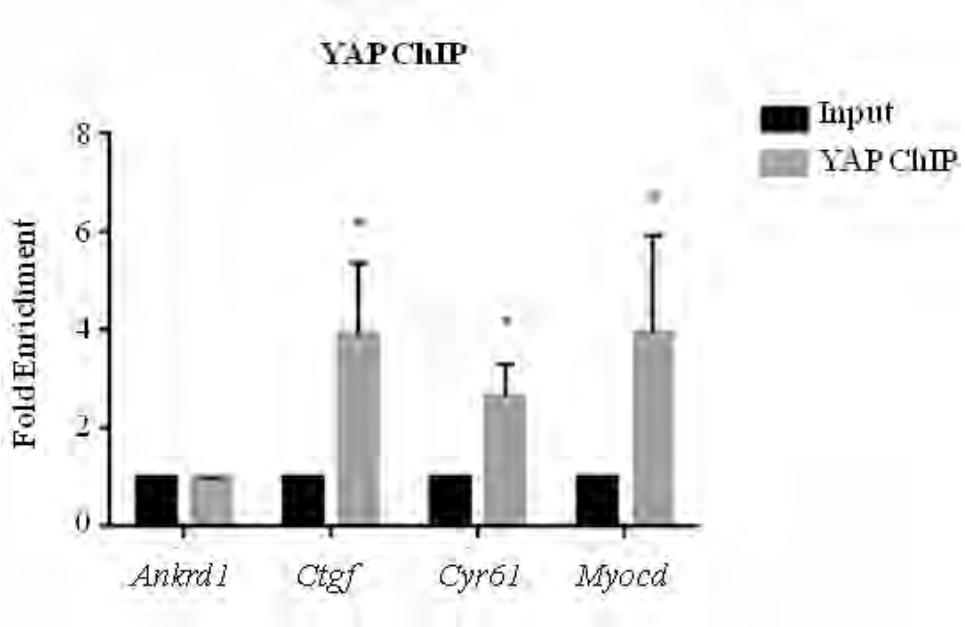


Figure 3.22. YAP binds to the promoter region of the *Myocd* gene *in vivo.*
YAP protein binds to the upstream *Myocd* promoter region, as well as the upstream promoter regions of bona fide target genes *Ctgf* and *Cyr61* in the gastrointestinal tracts of *Nkx3.2*^{Cre}*R26*^{YAP5SA} mutants at E13.5. ChIP-qPCR analysis following chromatin immunoprecipitation with YAP antibody. Data are mean ± S.D., n = 3, * = p value ≤0.05. Student's *t*-test.

We then analyzed sequence homology of the human *Myocd* promoter region and gene to identify putative TEAD4 binding sites. We found a TEAD4 binding site is present in the transcriptional start site (TSS) for *Myocd* in humans, and is highly conserved amongst a number of vertebrates, including mouse, rat, dog, pig, and cow *(Fig. 3.23.A)*, suggesting a highly conserved evolutionary mechanism. Finally, we investigated whether YAP protein binds directly to the TEAD4 binding site in the *Myocd* TSS. We again performed chromatin immunoprecipitation using Flag antibody in C3H10T1/2 cells expressing the lentiviral 3x-Flag-*YAP5SA* construct and determined that YAP binds directly to the TEAD4 binding site in the *Myocd* TSS *(Fig. 3.23.B)*. Together these data support our hypothesis that YAP is inhibiting differentiation of gastrointestinal mesenchyme by acting as a direct transcriptional co-repressor of *Myocd*, at the promoter region and/or the TSS, and is therefore inhibiting the Myocardin master regulatory complex program for mesenchymal differentiation.

From the data collected in this study, we have arrived at a novel model wherein YAP/TAZ act as an evolutionarily conserved molecular switch to control the balance between progenitor cell and differentiated cell in the developing gastrointestinal mesenchyme. YAP/TAZ associate with TEAD proteins to bind to TEAD binding sites within the *Myocd* promoter region, including a highly evolutionarily conserved site within the *Myocd* TSS, to prevent transcription. Even if Hedgehog Pathway signaling is activated (which has been shown to directly upregulate *Myocd* transcription), YAP/TAZ repress *Myocd* transcription while bound to the TEAD binding site. When differentiation is set to begin, LATS1/LATS2 inhibits YAP/TAZ through inhibitory phosphorylation

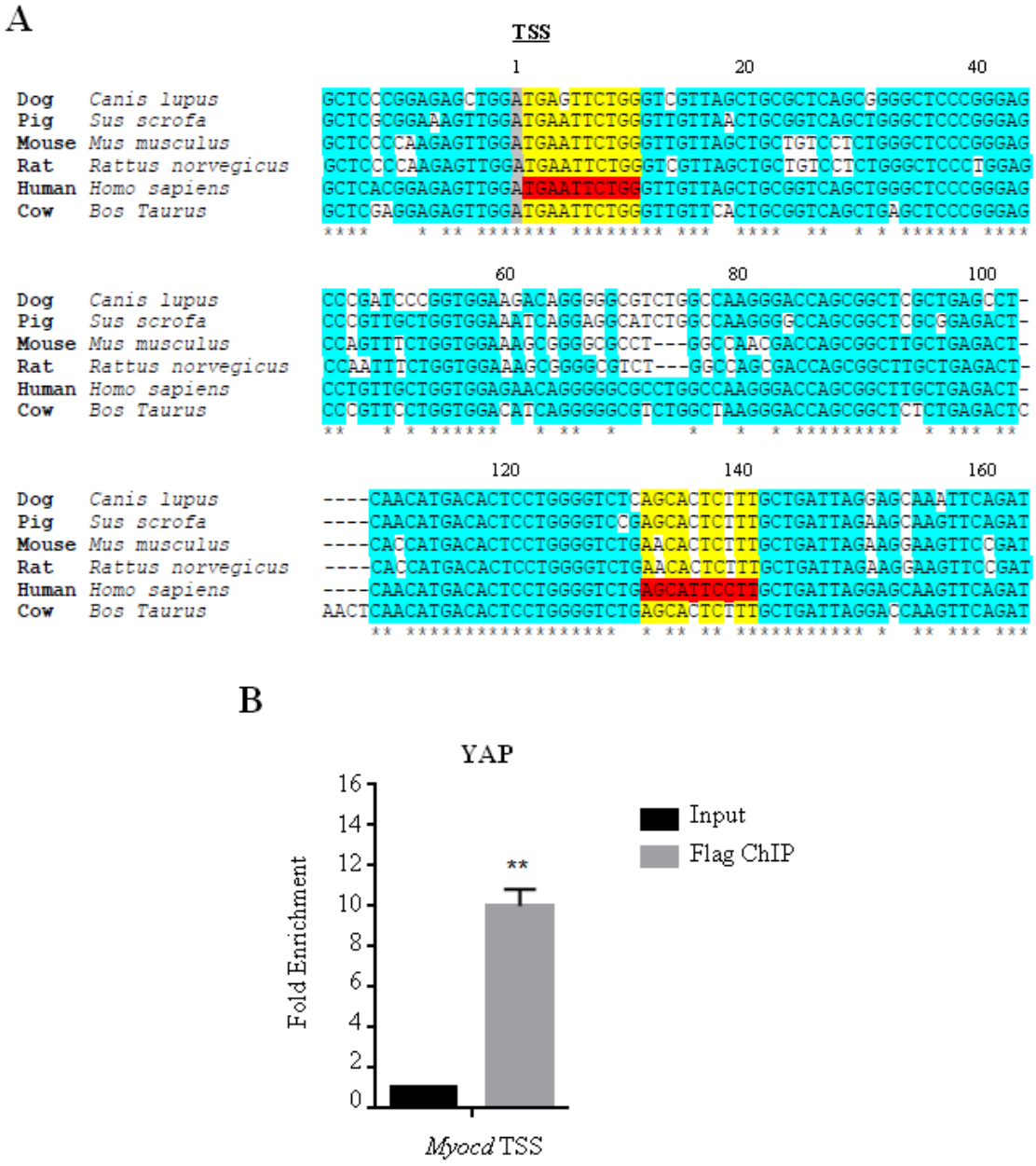


Figure 3.23. YAP binds directly to an evolutionarily conserved TEAD4 binding site in the *Myocd* TSS.
(A) The TEAD4 binding site *(yellow)* in the *Myocd* TSS, as well as the *Myocd* sequence *(blue)* is highly conserved across multiple vertebrate species, including Human *(red)*, Dog, Pig, Mouse, Rat, and Cow. *(B)* YAP binds directly to the TEAD4 binding site in the *Myocd* TSS. Data are mean ± S.D., n = 3, * = p value ≤0.05; ** = p value ≤0.01. Student's *t*-test.

and YAP/TAZ are excluded from the nucleus. Once YAP/TAZ leave the nucleus, the transcriptional repression on *Myocd* is relieved, and activated Hedgehog signaling is able to drive *Myocd* transcription. Myocardin then associates with the SRF protein to form the Myocardin-SRF master regulatory complex, and drive the smooth muscle differentiation target genes such as α-SMA, SMMHC, and SM22α.

Materials and Methods

Mouse Genetics

Myh11^{CreER} (Wirth et al., 2008), *R26*$^{mT/mG}$ (Muzumdar et al., 2007), *Lats1* (Heallen et al., 2011b) and *Lats2* (Heallen et al., 2013) mice were obtained from the Jackson laboratory. *R26*^{SmoM2} (Mao et al., 2006) mice were described previously. *Nkx3.2*^{Cre} (Verzi et al., 2009), *Yap*^{flox} (Xin et al., 2011b) and *Taz*^{flox} (Xin et al., 2013) mice were kindly provided by Drs. RA Shivdasani and EN Olson. To generate the *R26*^{YAP5SA} allele, the cDNA fragment encoding YAP5SA (a gift from Kunliang Guan, Addgene plasmid #27371) with the N-terminal Flag and NLS sequences was inserted into the pCN vector with a C-terminal IRES-nuclear lacZ before being cloned into the pROSA targeting vector. The University of Massachusetts Medical School Transgenic Animal Core performed ES cell targeting and blastocyst injection to generate chimeric animals. To target gastrointestinal mesenchyme and smooth muscle cells, *Nkx3.2*^{Cre} and *Myh11*^{CreER} mice were bred with *Yap*^{flox}, *Taz*^{flox}, *R26*^{YAP5SA}, *R26*^{SmoM2}, *R26*$^{mT/mG}$ mice. Cre-mediated recombination in *Myh11*^{CreER} *R26*^{YAP5SA} embryos was induced with intraperitoneal 200 mg/kg tamoxifen injection in pregnant females at 12.5 dpc and embryos harvested at 15.5 dpc. All mouse experiments were conducted according to the University of Massachusetts Medical School IACUC guidelines.

Tissue Collection and Histology

Following euthanasia, tissue was dissected from adult or embryonic mice and fixed in 10% Neutral Buffered Formalin (NBF) at 4°C overnight. For paraffin sections, tissue was dehydrated, embedded in paraffin, and sectioned at 6 μm. Paraffin sections were stained using standard hematoxylin & eosin reagents. Mesenchymal width was measured in triplicate and normalized to control samples to quantify relative mesenchyme width in mutant samples.

For frozen sections, tissue was dehydrated in 30% sucrose overnight at 4°C, embedded in OCT, and sectioned at 12 μm. Frozen sections were stained for lacZ using standard X-GAL staining reagents and incubated overnight at 37°C, followed by eosin counterstain.

Immunohistochemistry and Immunofluorescence

For immunohistochemistry (IHC), sections were deparaffinized and rehydrated before undergoing heat-induced antigen retrieval in 10mM sodium citrate buffer (pH 6.0) for 30 minutes. Slides were blocked for endogenous peroxidase for 20 minutes, then blocked for 1 hour in 5% BSA, 1% goat serum, 0.1% Tween-20 buffer in PBS, and incubated overnight at 4°C in primary antibody diluted in blocking buffer or SignalStain® Antibody Diluent (Cell Signaling). Slides were incubated in biotinylated secondary antibodies for 1 hour at room temperature and signal was detected using the Vectastain Elite ABC kit (Vector Laboratories).

For immunofluorescence (IF), sections were blocked for 1 hour and incubated overnight at 4°C in primary antibody diluted in blocking buffer. Slides were then incubated for 1 hour at room temperature in Alexa Fluor-conjugated secondary antibodies (Invitrogen) at a 1:500 dilution in blocking buffer and mounted using mounting media with DAPI (EMS). Primary antibodies used for IHC/IF are listed in Table 3.1. Image quantification of Ki67 immunofluorescence staining in the gastrointestinal mesenchyme were performed using Fiji software (Schindelin et al., 2012) and the Renyi Entropy algorithm.

C3H10T1/2 cell culture and smooth muscle differentiation

C3H10T1/2, clone 8 cells were acquired from ATCC (CCL-226™) and maintained in Eagle's Basal medium with 2 mM L-glutatmine, 1.5 g/L sodium bicarbonate, Earle's BSS, Pen/Strep, and 10% FBS at 37°C in 5% CO2. C3H10T1/2 cells were maintained at 30-50% confluency to prevent density-induced differentiation. For Hedgehog-induced smooth muscle differentiation, cells were transitioned to induction media (Eagle's Basal media with 0.5% FBS) for 12 hrs, following by addition of 0.1 μM Smoothened Agonist (SAG) dissolved in DMSO for 24-48 hours.

RNAseq and Affymetrix gene chip analysis

Embryonic stomachs were dissected from E13.5 control and *Nkx3.2*^{Cre}*R26*^{YAP5SA} embryos, and RNA was extracted for subsequent RNAseq analysis, using independent biological quadruplicates. RNAseq reads (paired end 75 bp) were aligned to the mouse

genome (mm10) using TopHat, followed by running samtools to filter the reads with MAPQ lower than 20. Differential expressed genes were determined by cufflinks. Transcriptional profiling of E13.5 *Nkx3.2*^{Cre}*R26*^{SmoM2} gastrointestinal using Affymetrix Mouse Gene 1.0ST chips was described previously (Huang & Cotton et al., 2013). Briefly, gastrointestinal tissue from E13.5 *Nkx3.2*^{Cre}*R26*^{SmoM2} and littermate control animals was dissected and RNA was isolated, labeled, and hybridized to mouse GeneST1.0 chips (Affymetrix) according to manufacturer's protocols. Three independent biological samples were used for chip analysis and statistical analysis was performed using R, a system for statistical computation and graphics. Genes with p values <0.05 and fold change ≥1.5 were investigated further. The heatmap was generated using heatmap (v 1.0.7) package.

Western Blotting

Tissue from perinatal and adult intestine was dissected. Protein lysates were probed with primary antibodies. Primary antibodies are listed in Table 3.2. HRP-conjugated Secondary antibodies were obtained from Jackson Laboratories.

Chromatin immunoprecipitation-qPCR analysis

Chromatin immunoprecipitation (ChIP) assays were performed according to manufacturer's instructions (Active Motif, High Sensitivity Kit). Briefly, 6×10^6 cells per sample were fixed using 1% formaldehyde, washed with PBS, and lysed in lysis buffer. Fixed cells were sonicated and sheared DNA was incubated with 4 μg of primary

antibody on a rotator at 4℃ overnight. Primary antibodies used were Flag, H3K27ac, and CHD4. Antibody concentrations and company are listed in Table 3.3. After washing with buffer, Protein G agarose beads were added to ChIP reactions and incubated on a rotator for 3 hours at 4℃ before elution. Cross-links were reversed and DNA was purified for qPCR analysis. Primers used for ChIP-qPCR of YAP, H3K27ac, and CHD4 cis-regulatory elements are listed in Table 3.4.

Quantitative Real-Time PCR

RNA of animal tissues or cultured cells was isolated using Trizol reagent (Invitrogen), followed by reverse-transcription using SuperscriptII Reverse Transcriptase (Invitrogen). Quantitative real-time PCR was performed using Sybr Mastermix (Kapa Bioscience). The primers used for real-time PCR were described in Table 3.5. All qPCR experiments were conducted in biological triplicates, error bars represent mean ± standard deviation, and Student's t-test was used to generate p-values (* = p value ≤0.05; ** = p value ≤0.01; *** = p value ≤0.001; **** = p value ≤0.0001).

Myocd Promoter Sequence Analysis

The upstream promoter region and transcriptional start site (TSS) for the *Myocd* gene were analyzed for putative TEAD4 binding sites. Sequences from a number of vertebrates (human, dog, rat, cow, mouse, and pig) were analyzed and overlaid to reveal homology.

Table 3.1: IHC/IF Antibodies

Antibody	Dilution	Company
YAP	1:200	Cell Signaling
YAP/TAZ	1:200	Cell Signaling
Ki67	1:10000	Abcam
H/K-ATPase	1:500	MBL
β-catenin	1:500	BD Biosciences
CD44	1:400	eBioscience
Desmin	1:400	ThermoFisher
α-SMA	1:5000	Abcam
β-tubulin III	1:800	Covance
CD31	1:200	BD Pharmingen
cleaved caspase 3	1:400	Cell Signaling
β-galactosidase	1:2000	Abcam
PDGFRα	1:400	BD Biosciences

Table 3.2: Western Blot Antibodies

Antibody	**Dilution**	**Company**
YAP	1:1000	Cell Signaling
TAZ	1:1000	BD Pharmingen
β-actin	1:1000	Genescript

Table 3.3: Antibodies for ChIP

Antibody	**Concentration**	**Company**
Flag	4 ug	Sigma
H3K27Ac	4ug	Active Motif
CHD4	4ug	Abcam

Table 3.4: ChIP-qPCR Primers

ChIP-qPCR Primers	**Sequence**
mouse Cyr61 forward	CTCTGATGGATCTGAGAAGAGG
mouse Cyr61 reverse	GCCCTTTATAATGCCTGCCTA
mouse Ctgf forward	CAATCCGGTGTGAGTTGATG
mouse Ctgf reverse	GGCGCTGGCTTTTATACG
mouse Ankrd1 forward	CCAAGAGGGAGATGACAAGC
mouse Ankrd1 reverse	GTGGTCACTGCCAAAGGAAT
mouse Myocd forward	ATTCTCTGGGTTGCACCAAT
mouse Myocd reverse	AGTTGAGTAGCAGGGCTCCA
mouse Myocd TSS forward	ACTGTGCGTCCTCCTACCC
mouse Myocd TSS reverse	CCCAGAGGACAGCAGCTAAC
mouse α-SMA forward	AGCAGAACAGAGGAATGCAGTGGAAGAGAC
mouse α-SMA reverse	CCTCCCACTCGCCTCCCAAACAAGGAGC
mouse GADPH forward	GCCTCTGCGCCCTTGAGCTA
mouse GADPH reverse	GATGCGGCCGTCTCTGGAAC
mouse intergenic region forward	GCTCCGGGTCCTATTCTTGT
mouse intergenic region reverse	TCTTGGTTTCCAGGAGATGC

Table 3.5: qPCR Primers

qPCR Primers	Sequence
mouse CTGF forward	TGTGCACTGCCAAAGATGGTGCAC
mouse CTGF reverse	TGGGCAGGCGCACGTCCATG
mouse ANKRD1 forward	GGAACAACGGAAAAGCGAGAA
mouse ANKRD1 reverse	GAAACCTCGGCACATCCACA
mouse α-SMA forward	ATTGTGCTGGACTCTGGAGATGGT
mouse α-SMA reverse	TGATGTCACGGACAATCTCACGCT
mouse NG2 forward	ACCATGCTACTCCGCAACAG
mouse NG2 reverse	CCGGTGAACATCTATGTGTACG
mouse Vimentin forward	CGGCTGCGAGAGAAATTGC
mouse Vimentin reverse	CCACTTTCCGTTCAAGGTCAAG
mouse PDGRA forward	CCTGTGCCCATCCGCAGGAAGAGA
mouse PDGRA reverse	TTGGCCACCTTGACGCTGCGGTG
mouse PDGRB forward	ATCGCCGAGTGCAAGACGCG
mouse PDGRB reverse	AAGCACCATTGGCCGTCCGA
mouse PDGFRα forward	CGACTCCAGATGGGAGTTCCC
mouse PDGFRα reverse	TGCCATCCACTTCACAGGCA
mouse PDGFRβ forward	AGCTACATGGCCCCTTATGA
mouse PDGFRβ reverse	GGATCCCAAAAGACCAGACA
mouse Gli1 forward	CGCCAAGCACCAGAATCGG
mouse Gli1 reverse	CCGAGACACAAGGTCCTTCATCC
mouse Ptch1 forward	AACAAAAATTCAACCAAACCTC
mouse Ptch1 reverse	TGTCTTCATTCCAGTTGATGTG

mouse Smo forward	CGCCAAGGCCTTCTCTAAGCG
mouse Smo reverse	CCTCTGCCTGGGCTCAGCAT
mouse Shh forward	CAAAGCTCACATCCACTGTTCTG
mouse Shh reverse	GAAA CAGC CGCC GGATTT
mouse Ihh forward	CACGTGCATTGCTCTGTCAA
mouse Ihh reverse	AGGAAAGCAGCCACCTGTCTT
mouse Myocd forward	AAGGTCCATTCCAACTGCTC
mouse Myocd reverse	CCATCTCTACTGCTGTCATCC
mouse SMMHC forward	GAGAAAGGAAACACCAAGGTCAAGC
mouse SMMHC reverse	AACAAATGAAGCCTCCTGGTGGCTC
mouse SM22α forward	CTCTAATGGCTTTGGGCAGTTTGG
mouse SM22α reverse	GCTCCTGGGCTTTCTTCATAAACC
mouse GAPDH forward	GTGAAGGTCGGTGTGAACG
mouse GAPDH reverse	ATTTGATGTTAGTGGGGTCTCG

CHAPTER IV

Discussion

Functional requirement of YAP/TAZ in the mammalian GI

The fundamental question I sought to answer in this dissertation was: What is the function of YAP/TAZ in the mammalian gastrointestinal tract? In order to adequately discuss how my data address this question, it is first necessary to briefly review what was known in the Hippo Pathway/Gastrointestinal field and remember the main open-ended questions when this work was initiated five years ago. Following this concise review, I will discuss how my current findings in the gastrointestinal epithelia fit into the somewhat contentious field of YAP/TAZ in the gastrointestinal tract, and what additional open-ended questions remain. Finally, I will discuss the newly identified requirement for YAP/TAZ in the gastrointestinal mesenchyme, including the implications for mesenchymal progenitor cell maintenance and important future directions for this work.

When I began this work in 2011, the Mammalian Hippo Pathway field was arguably still in its infancy. Just four years earlier, in 2007, it was discovered that the Hippo organ size control pathway identified in *Drosophila melanogaster* was also conserved in mammals (Dong et al., 2007). Another group had just announced that YAP subcellular nuclear localization could be regulated by cell density and that mutation of 5 canonical LATS1/LATS2 phosphorylation sites in YAP5SA mutants allowed cells to overcome contact inhibition (Zhao et al., 2007). Finally, Camargo et al., were the first group to link the Hippo Pathway to the mammalian gastrointestinal tract; they showed that endogenous YAP is restricted to the intestinal epithelial stem cell compartment and that YAP overexpression rapidly expanded an intestinal progenitor cell population

(Camargo et al., 2007). Three years later in 2010, another group added to the intestinal epithelia commentary when they showed that genetic ablation of YAP has no effect on normal intestinal homeostasis but causes severe regeneration defects following DSS treatment (Cai et al., 2010). The authors also showed that genetic knockout of Salvador (SAV1), a scaffold protein that interacts with MST1/MST2, generates a serrated polyp phenotype, presumably due to accumulation of nuclear YAP protein (Cai et al., 2010). Together, these findings indicated that YAP is an oncogene in colorectal cancer and that further work was needed to fully elucidate how YAP contributed to tumorigenesis.

Given the understanding of the YAP/intestinal epithelia field at the time when I began my work in 2011, I decided to focus on the main unanswered questions relevant to the Mao Lab's previous expertise: (1) Does TAZ functionally compensate for YAP in the epithelia during development and homeostasis? (2) What is the relationship between YAP/TAZ and Wnt Pathway Signaling in the intestinal epithelia?

Does TAZ functionally compensate for YAP in the epithelia during development and homeostasis?

YAP knockout in the intestinal epithelia during development has no effect on homeostasis or tumorigenesis, leading to the conclusion that YAP is dispensable in these cells (Barry et al., 2013; Cai et al., 2010). This finding is somewhat surprising because YAP/TAZ are essential in many tissues during development and YAP/TAZ activity in GI tumors confers a poor prognosis for colorectal cancer patients (Morin-Kensicki et al.,

2006; Steinhardt et al., 2008). However, YAP and TAZ are functionally redundant *in vivo* under some conditions (Nishioka et al., 2009).

Knowing that YAP/TAZ can sometimes compensate for the other's loss *in vivo*, I hypothesized that endogenous TAZ protein was functionally compensating for YAP genetic knockout in the intestinal epithelia. I decided to investigate this by genetically knocking out both YAP and TAZ using floxed alleles and two different Cre alleles; Shh^{Cre} and $Villin^{Cre}$. I decided to use two Cre alleles because they are constitutively expressed at different developmental time points and in different tissue compartments. By using two different Cre driver alleles, I was able to investigate both the spatial and temporal requirement for YAP/TAZ during development. Shh^{Cre} is expressed starting in E8.5 in the developing endoderm, which eventually gives rise to the epithelial layer of both the lung and gastrointestinal tract. $Villin^{Cre}$ is expressed later in development, at E12.5, and expression is restricted to the intestinal epithelia.

I found that there is a compartmental requirement for YAP/TAZ in the developing endoderm. In $Shh^{Cre}Yap^{flox/flox}Taz^{flox/flox}$ embryos, YAP/TAZ is required in the developing lung epithelia but is dispensable in the intestinal epithelia. Knockout of YAP/TAZ in the developing endoderm caused a severe lung development defect but had no phenotype in the intestine *(Fig. 2.1)*. While our work was ongoing, a paper was published showing that YAP is required for epithelial tube formation and branching in the developing lung (Mahoney et al., 2014). The authors showed that in $Shh^{Cre}Yap^{flox/flox}$ animals, boundaries in the lung are demarcated by the subcellular localization of YAP protein. When YAP is knocked out, branching is inhibited because epithelial progenitor cells are unable to

properly distribute Sox2 protein (Mahoney et al., 2014). However, in their mutant animals, endogenous TAZ protein was still present. In our own $Shh^{Cre}Yap^{flox/flox}Taz^{flox/flox}$ mutant embryos, we knocked out both YAP and TAZ to investigate whether TAZ was compensating for YAP loss *in vivo*. However, I observed an identical phenotype in the $Shh^{Cre}Yap^{flox/flox}Taz^{flox/flox}$ as compared to the $Shh^{Cre}Yap^{flox/flox}$; both exhibited a defect in epithelial cell branching in the developing lung *(Fig. 2.1)*. Therefore, my work corroborates the epithelia branching defect findings published by Mahoney et al, and expands to confirm that TAZ does not functionally compensate for YAP in the developing lung endoderm.

When the YAP/TAZ knockout is limited to the intestinal epithelia, I found that $Villin^{Cre}Yap^{flox/flox}Taz^{flox/flox}$ animals appeared phenotypically normal and did not exhibit hyperplasia or polyposis even after aging to 12 months. Additionally, I observed genetic knockout of YAP/TAZ does not impact epithelial cell differentiation or proliferation *(Fig. 2.3, Fig. 2.4)*. Again, while this work was ongoing a number of papers were published with conclusions identical to my own (Azzolin et al., 2014; Cai et al., 2015; Gregorieff et al., 2015; Zanconato et al., 2015). In contrast to my findings, another group concluded that TAZ actually can compensate for YAP in the intestinal epithelia (Imajo et al., 2015). The authors in this study observed that YAP/TAZ knockdown inhibited progenitor cell growth and differentiation into goblet cells. However, it should be noted that in this particular study, the authors used a shRNA approach to knockdown YAP/TAZ, instead of the genetic knockout approach used by our lab and others, which may explain the differing results they observed. Overall, my data showing that TAZ does

not functionally compensate for YAP in the intestinal epithelia *in vivo* corroborates the current view in the Hippo field that neither YAP nor TAZ is required in the intestinal epithelia during both development and homeostasis.

Taken together, my data introduce a number of additional questions regarding the compartmental requirement for endodermal YAP/TAZ. Firstly, why is YAP/TAZ required for lung epithelial development but not gastrointestinal epithelial development? I showed evidence via immunohistochemistry that YAP/TAZ localization is predominantly nuclear in lung epithelial cells, whereas YAP/TAZ are cytoplasmic in gastrointestinal epithelial cells during normal development *(Fig. 3.1)*. Given this observation, a logical hypothesis would be that the difference in subcellular localization explains the tissue compartment requirement for activity. Another directly related question is: Does the YAP/TAZ spatial requirement continue through postnatal and adult stages? One experiment to investigate this would be to use inducible Cre alleles, such as the $Sox2^{CreER}$ allele, to delete YAP/TAZ postnatally in the lung epithelia (Arnold et al., 2011).

Finally, what other organs have a similar tissue compartment-specific requirement for YAP/TAZ? In solid tumors, YAP and TAZ represent attractive targets for novel therapies because they have been shown to drive growth and proliferation. As such, treating patients with small molecule YAP/TAZ inhibitors may be a way to reduce tumor growth. However, one caveat to drug treatments is that in order to inhibit YAP/TAZ in a tumor, the drug will also be disseminated systemically and will inhibit YAP/TAZ in a wide range of cells in the body. Therefore, if there are tissues where YAP/TAZ activity is absolutely required for normal homeostasis, this would negatively impact the feasibility

of using a YAP/TAZ inhibitor as a cancer therapy. Ultimately, a greater understanding of the spatial and temporal requirement for YAP/TAZ is needed.

What is the relationship between YAP/TAZ and Wnt Pathway signaling in the intestinal epithelia?

When I first began this work, part of the experimental rationale was to dissect the exact relationship between YAP/TAZ and Wnt pathway signaling in the intestine. Wnt/β-catenin signaling is required to maintain the intestinal epithelial stem cell population; inhibiting Wnt signaling or knocking out β-catenin in the intestinal epithelia causes rapid loss of intestinal crypts and differentiation of stem cells into terminally differentiated lineages (Fevr et al., 2007). In *Drosophila melanogaster,* Yorkie activation causes the upregulation of Wingless target gene expression, suggesting that YAP activates Wnt signaling (Cho et al., 2006). Conversely, other reports showed that elevated YAP nuclear protein is detected in *Apc*-mutant tumor cells, suggesting that in certain contexts Wnt signaling could activate YAP (Camargo et al., 2007). Finally, one publication showed that endogenous YAP knockout had no effect on the intestinal stem cell compartment until challenged with DSS, when it exhibited a severe regeneration defect (Cai et al., 2010).

Based on these findings, I hypothesized that the $Villin^{Cre}Yap^{flox/flox}$ mutants had no apparent phenotype due to functional compensation by endogenous TAZ. I additionally hypothesized that if both YAP and TAZ were genetically ablated from the intestinal crypt

stem cell, I would observe an effect on Wnt pathway signaling during normal homeostatic conditions. However, I was surprised to find that in both *Shh*^{Cre}*Yap*$^{flox/flox}$*Taz*$^{flox/flox}$ and *Villin*^{Cre}*Yap*$^{flox/flox}$*Taz*$^{flox/flox}$ intestine, β-catenin localization remains indistinguishable from controls *(Fig. 2.2* and *Fig. 2.4)*. These observations indicated that YAP/TAZ are likely not involved in the regulation of β-catenin localization during either embryogenesis or postnatal stages. Additionally, I also observed no change in either the expression of stem cell marker Lgr5 or of bona fide Wnt target genes CD44 and Sox9. Taken together, these data told us that YAP/TAZ are dispensable for Wnt pathway signaling in the intestinal crypt stem cell *in vivo* during both development and homeostasis.

Given the lack of consensus regarding YAP/TAZ and Wnt at the time this work began, I was particularly interested in investigating the relationship between YAP/TAZ and Wnt during tumorigenesis. I found that both YAP and TAZ are required when Wnt pathway signaling is hyperactivated both *in vitro* and *in vivo (Fig. 2.6* and *Fig. 2.7)*. When Wnt pathway signaling was inhibited by expressing a dominant-negative TCF4 construct in either HCT116 (*β-catenin* mutation) or DLD1 (*APC* mutation) human colorectal cancer cell lines, I observed that expression of YAP and TAZ mRNA is decreased. Importantly, I also observed that YAP/TAZ is required for tumorigenesis when Wnt pathway signaling is hyperactivated, such as in *in vitro* organoid cultures as well as in a APC-mutant mouse polyps. Finally, I found that Wnt pathway signaling activates YAP/TAZ directly at the transcriptional level *(Fig. 2.5)*.

While this work was being finished, a number of groups published observations relating to YAP/TAZ/Wnt in the intestinal epithelia. My data showing that YAP/TAZ are dispensable for Wnt pathway signaling in the intestinal crypt stem cell *in vivo* under normal conditions were corroborated when a number of groups published nearly identical findings (Azzolin et al., 2014; Cai et al., 2015; Gregorieff et al., 2015; Zanconato et al., 2015). However, my results represent the first record of YAP/TAZ double knockout in the early endodermal lineage, as the $Shh^{Cre}Yap^{flox/flox}Taz^{flox/flox}$ mutant data has not yet been published. Overall, my observations that YAP/TAZ are not involved with Wnt pathway signaling in the intestinal stem cell under normal conditions corroborates the recently published findings. My data showing that YAP/TAZ activation is required in Wnt-driven tumorigenesis also serves to corroborate the recent findings published by other labs. In the recent months, a number of groups, in addition to our own lab, have reported that genetic ablation of YAP/TAZ in an APC-mutant background is sufficient to inhibit tumorigenesis *in vivo* and proposed that YAP/TAZ are attractive therapeutic targets in tumors with Wnt hyperactivation (Azzolin et al., 2014; Cai et al., 2015; Gregorieff et al., 2015).

In the Hippo Pathway/Gut field, the controversy relating to YAP/TAZ/Wnt signaling in the intestinal epithelial stem cell can be distilled down to the following question: Is YAP/TAZ a member of the APC-Axin-GSK3β destruction complex? In response to this question, two core fields of thought have emerged. One hypothesis suggests that YAP/TAZ are not members of the APC-Axin-GSK3β destruction complex, but rather inhibit Wnt pathway signaling through inhibition of the Wnt effector

Dishevelled (Barry et al., 2013; Varelas et al., 2010). The second hypothesis suggests that YAP/TAZ are critical components of the APC-Axin-GSK3β destruction complex (Azzolin et al., 2014; Azzolin et al., 2012). In this alternative hypothesis, it has been suggested that YAP/TAZ are inhibited during normal Wnt pathway conditions, and can inhibit β-catenin through association with Axin. However, when Wnt pathway signaling is hyperactivated, such as in tumorigenesis, the APC-Axin-GSK3β destruction complex dissociates, and nuclear YAP/TAZ accumulate.

In this study, my data does not directly address the relationship between the destruction complex and YAP/TAZ, but rather introduces an additional layer of YAP/TAZ transcriptional regulation mediated by TCF4 during Wnt pathway hyperactivation. I show that during tumorigenesis, Wnt pathway directly activates *Yap* and *Taz* transcription through TCF4 binding in HCT116 cells *(Fig. 2.5)*. A previously published study linked Wnt pathway and YAP activity via transcriptional regulation in HCT116 colorectal cancer cells (Konsavage et al., 2012). However, my work differs somewhat from this report. Konsavage et al. observed β-catenin/TCF4 bind a DNA enhancer within the first intron of the *Yap* gene in HCT116 cells, whereas I observed TCF4 occupancy in the *Yap* promoter region. My research also uncovers an unknown connection between Wnt pathway regulation of TAZ via TCF4 binding to an enhancer region upstream of *Taz*. Together, these findings help to highlight the complex relationship between Wnt and YAP/TAZ in the intestinal epithelia.

However, there are experimental limitations to the conclusions presented here regarding the Wnt/YAP/TAZ relationship. One caveat to my conclusions about the direct

TCF4 transcriptional regulation of YAP/TAZ is that these experiments were performed in colorectal cancer cells. Taken together, my *in vitro* and *in vivo* data suggests that YAP/TAZ are Wnt targets in tumorigenesis because of direct transcriptional regulation by TCF4. To robustly show that the TCF4 transcriptional regulation occurs *in vivo*, a future experiment would be to perform chromatin immunoprecipitation in both APC-mutant polyps as well as control epithelium tissue. However, the data from the *in vitro* ChIP experiments I performed represent evidence that this transcriptional regulation is important for tumorigenesis in colorectal cancer.

This work also raises new and unanswered questions. Primarily, why is YAP/TAZ a downstream Wnt pathway target only during pathway hyperactivation? Wnt activity is required for maintenance of the ISC population, but data from our lab and others conclusively shows that YAP/TAZ are not required for ISCs under normal homeostasis. To begin to answer this question, further work is needed to understand the fundamental differences that exist between ISCs in homeostasis versus ISCs in regeneration or tumorigenesis. One hypothesis is that YAP/TAZ regulate a set of genes that are required in Wnt-driven tumorigenesis but are dispensable in development. Therefore, a future experiment to investigate this would be to induce transformation in ISCs and identify a transcriptional signature specific to tumorigenesis.

Another question raised by these data is whether YAP/TAZ are transcriptional targets of Wnt pathway signaling in a range of different cancer types. Our lab has previously shown that Wnt signaling regulates YAP activity at the transcriptional level in hepatocellular carcinoma cells (Wang et al., 2013). This suggests that transcriptional

regulation of YAP/TAZ by Wnt/TCF4 may be a conserved mechanism, but further work is needed to confirm this. One future experiment to investigate this would be to perform chromatin immunoprecipitation for TCF4 in cancer cell lines as well as non-transformed cell lines. Data from this experiment would reveal if the molecular mechanism we uncovered in APC-mutant colorectal cancer is important in other cancer types.

Answering these questions will help to understand how YAP/TAZ transcriptional regulation by the Wnt pathway contributes to tumorigenesis. Overall, my work uncovers an additional level of YAP/TAZ regulation mediated by Wnt/TCF4 in addition to protein-protein interactions in the cytoplasm, and highlights the complexity that exists with Wnt/YAP/TAZ signaling in the intestinal epithelia *(Fig. 4.1)*.

Are endodermal YAP/TAZ required for mesodermal differentiation?

In addition to the complex relationship that exists between YAP/TAZ and Wnt signaling within the epithelia, I also investigated how YAP/TAZ contributes to signaling between the epithelia and mesenchyme. We and others have shown that signaling between the GI endoderm and mesoderm in development is important for patterning and differentiation (Huang & Cotton et al., 2013; Walton et al., 2012; Zacharias et al., 2011). In our own lab, we previously showed that endodermal knockout of Hedgehog ligands Shh and Ihh inhibits smooth muscle differentiation and mesenchymal growth in $Shh^{Cre/flox}Ihh^{flox/flox}$ embryos (Mao et al., 2010). These data led us to conclude that Hedgehog ligands are required to transmit signals from endoderm to mesoderm signaling

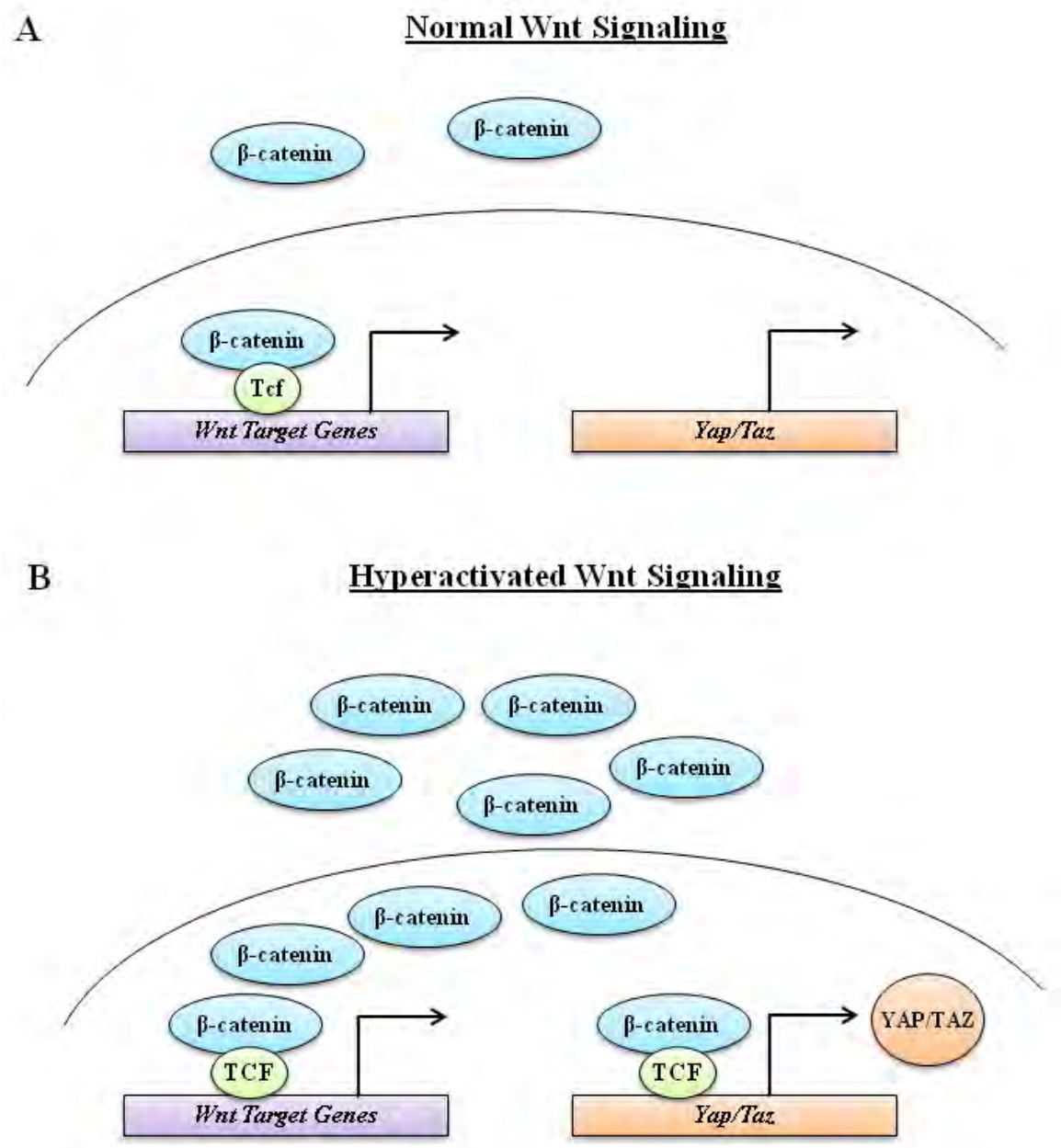


Figure 4.1. Proposed model for YAP/TAZ as Wnt targets during in intestinal epithelial transformation.
(A) Under conditions of normal Wnt signaling, such as intestinal development and stem cell maintenance, YAP/TAZ is dispensable. *(B)* When Wnt signaling is hyperactivated, such as during regeneration and tumorigenesis, β-catenin/TCF bind to cis-regulatory elements of both *Yap* and *Taz* to activate transcription. YAP/TAZ protein is required during conditions of Wnt hyperactivation.

during differentiation. If endodermal YAP/TAZ were similarly involved with this crosstalk between tissue compartments, I would have observed a similar defect in gastrointestinal mesenchymal differentiation and growth in $Shh^{Cre}Yap^{flox/flox}Taz^{flox/flox}$ embryos. However I did not observe this. GI mesoderm in $Shh^{Cre}Yap^{flox/flox}Taz^{flox/flox}$ embryos successfully differentiated into mesenchyme and muscle cells, as assayed by expression of markers for differentiated tissue *(Fig. 2.2)*. Taken together, these observations led us to conclude that endodermal YAP/TAZ is not required for mesenchymal differentiation induced by paracrine Hedgehog Pathway signaling.

However, there are caveats to this conclusion, due in part to experimental limitations. I reached this conclusion on expression pattern of differentiation markers α-smooth muscle actin and desmin, as assayed by IHC. By relying on the expression of these two differentiation markers as my experimental strategy, it is possible that I missed detecting minor defects in differentiation. Comparing the gene expression signature in $Shh^{Cre}Yap^{flox/flox}Taz^{flox/flox}$ mesenchyme to control tissue would provide a deeper experimental analysis.

Additionally, IHC analysis does not provide information regarding functionality of the differentiated mesenchyme and muscle tissue. It is possible that the $Shh^{Cre}Yap^{flox/flox}Taz^{flox/flox}$ mesenchyme has functional defects that are not readily observed due to perinatal lethality. I showed that $Villin^{Cre}Yap^{flox/flox}Taz^{flox/flox}$ animals are viable and do not exhibit any functional defects in the GI mesenchyme. These data support my conclusion that endodermal YAP/TAZ are dispensable for mesenchymal differentiation and functionality. However, a caveat of this conclusion exists due to experimental

limitations. *Shh*^{Cre} is expressed beginning from E8.5, whereas *Villin*^{Cre} expression does not begin until E12.5. Paracine Hedgehog signaling initiates mesenchymal differentiation, which begins at approximately E12.0. Therefore, YAP/TAZ knockout in *Villin*^{Cre}*Yap*$^{flox/flox}$*Taz*$^{flox/flox}$ animals occurs just after this mesenchymal differentiation begins. One way to investigate the developmental window between E8.5 and E12.5 would be to use the tamoxifen inducible *Shh*^{CreER} allele (Harfe et al., 2004). The inducible *Shh*^{CreER} allele allows for greater temporal control over YAP/TAZ knockout. Overall, this experiment would yield insight into whether YAP/TAZ are involved in mesenchymal differentiation between E8.5 and E12.5.

Functional requirement of YAP/TAZ in the GI mesenchyme

We and others have shown that YAP/TAZ are required in the developing lung endoderm but are dispensable in the intestinal endoderm during development and homeostasis (Barry et al., 2013; Cai et al., 2015; Mahoney et al., 2014; Zanconato et al., 2015). Our data also shows that YAP/TAZ act as downstream Wnt pathway effectors only when Wnt pathway is hyperactivated, such as in tumorigenesis.

However, to date there has been nothing reported regarding the role of YAP/TAZ in non-epithelial cells in the gastrointestinal tract. We have previously shown that the relationship between the intestinal epithelia and underlying mesenchyme is both complex and required for normal development and patterning (Huang & Cotton et al., 2013; Mao et al., 2010). Given our lab's extensive experience with gastrointestinal mesenchymal

development, I believed that the next logical scientific leap was to investigate whether the Hippo Pathway played a role in this process.

Tissue compartment-specific YAP/TAZ localization and expression

I first set out to determine the normal expression pattern for YAP/TAZ protein in the developing gastrointestinal tract at midgestation, as this has not been investigated previously. I showed via immunohistochemistry that YAP/TAZ subcellular localization in the developing gastrointestinal tract is predominantly nuclear in the mesenchyme but is predominantly cytoplasmic in the developing epithelia *(Fig. 3.1)*. This is in contrast to the subcellular YAP/TAZ protein localization in the developing lung, where YAP/TAZ is localized in the nucleus in both the epithelia and mesenchyme. These data led me to ask why YAP/TAZ protein localization was predominantly nuclear in the mesenchyme but was cytoplasmic in the epithelia. I speculated whether the differential YAP/TAZ subcellular localization was related to functionality, because I showed previously that the endoderm requires YAP/TAZ in the developing lung, but not in the developing gastrointestinal tract.

Similar to the developing gastrointestinal tract, the developing lung also requires tightly regulated reciprocal signaling between the lung epithelia and lung mesenchyme. The lung mesenchyme secretes factors such as Wnt and FGF to adjacent epithelial cells to regulate critical developmental and differentiation processes, such as branching morphogenesis, epithelial differentiation, and lineage distinctions (Chow et al., 2013;

McCulley et al., 2015). Recently, it was shown that nuclear YAP in the lung epithelia regulates branching and establishes the proximal-distal axis in the developing lung bud (Lange et al., 2015; Mahoney et al., 2014); however, to date no studies have investigated the role of YAP/TAZ in the developing lung mesenchyme.

An unanswered question that remains is whether YAP/TAZ are required in the lung mesenchyme during development. Given the importance of the lung mesenchyme in lung development and the robust nuclear YAP/TAZ subcellular localization observed in normal lung tissue, it is logical to hypothesize that, like the gastrointestinal mesenchyme, the lung mesenchyme also requires YAP/TAZ protein for appropriate epithelial patterning. If this is true, mutant animals would likely exhibit a profound lung branching defect if YAP/TAZ were genetically ablated in the developing lung mesenchyme. To test this hypothesis in a future experiment, I would use the recently generated *Tbx4-rtTA/TetO-Cre* to investigate YAP/TAZ in the lung mesenchyme during embryogenesis (Zhang et al., 2013). These experiments would help to reveal the contribution of mesenchymal YAP/TAZ activity in lung development and branching.

If YAP/TAZ are shown in the future to be required for lung mesenchymal development, this then opens up the possibility that YAP/TAZ could be required in the mesenchyme of other organs, such as the pancreas or breast. If so, what does this indicate about YAP/TAZ in the maintenance of mesenchymal cells more broadly, and is there a conserved YAP/TAZ mechanism in mesenchymal tissue development? The overall importance of the mesenchyme during normal development, as well as during maintenance of the tumor microenvironment, is starting to attract significant attention by

the scientific community. If an immunohistochemical staining for YAP/TAZ protein can reveal other tissue compartments with robust YAP/TAZ nuclear localization during development, we may uncover additional mesenchymal compartments that are critical for organogenesis. Ultimately, furthering the understanding of Hippo/YAP signaling, and its contribution to normal development, will reveal how YAP/TAZ maintain a pro-survival tumor microenvironment in a multitude of solid tumors.

YAP/TAZ in normal GI mesenchymal development

Once I established that there was a dichotomy between subcellular YAP/TAZ localization in the mesenchyme and subcellular localization in the epithelia, the next logical question I sought to answer was whether YAP/TAZ are required for normal gastrointestinal development. To answer this question, I used the mesodermal Cre allele $Nkx3.2^{Cre}$ to knock out both copies of *Yap* and *Taz* in the developing gastrointestinal mesenchyme *(Fig. 3.2)*. I also investigated mesenchymal YAP gain of function using both a novel transgenic gain of function allele, $R26^{YAP5SA}$, as well as genetic ablation of the upstream Hippo Pathway kinases *Lats1* and *Lats2 (Fig. 3.7* and *Fig. 3.19)*.

I found that mesodermal deletion of both *Yap* and *Taz* is perinatal lethal and results in a reduced mesenchymal compartment when both are knocked out during development. This is the first study to implicate YAP/TAZ as having a required role in gastrointestinal development; previous studies, including those in our own laboratory described in **Chapter II**, focused on the intestinal epithelia and showed that YAP/TAZ

are dispensable during both intestinal epithelial development and homeostasis (Barry et al., 2013; Cai et al., 2015; Zanconato et al., 2015).

Here, I showed that deletion of YAP/TAZ in the developing gastrointestinal mesenchyme caused a significantly smaller mesenchymal compartment *(Fig. 3.2* and *Fig. 3.4)*. This was due to a reduction in mesenchymal proliferation, as opposed to an increase in apoptosis. This was not overly surprising; I had hypothesized that a decrease in proliferation was the explanation for this phenotype because many other labs have reported a significant decrease in proliferation when YAP/TAZ proteins are either knocked down via RNAi or knocked out using Cre-recombination both *in vitro* and *in vivo* (Meng et al., 2016; Zanconato et al., 2016; Zhou et al., 2015a). Non-mesodermal cell lineages, such as enteric neurons and endothelial cells, are present in the YAP/TAZ deficient mesenchyme. This data suggests that mesenchymal YAP/TAZ are dispensable for either enteric neuron intravasation or angiogenesis in the developing gastrointestinal tract.

Additionally, I observed that mesenchymal cells in $Nkx3.2^{Cre}Yap^{flox/flox}Taz^{flox/flox}$ animals still were able to undergo differentiation, as assayed by detection of α-smooth muscle actin and desmin expression via IHC/IF *(Fig. 3.3* and *Fig. 3.4)*. These data suggest that YAP/TAZ are not required for differentiation of mesenchymal progenitor cells into differentiated mesenchyme and smooth muscle cells. This is in agreement with an observation from another group, which observed that in myoblasts, YAP is required for myoblast proliferation, but that YAP knockdown does not impact differentiation (Fischer et al., 2016; Judson et al., 2012). However, future experiments are needed to

determine if YAP/TAZ are truly dispensable in differentiated mesenchymal cells. One way to investigate this would be to investigate postnatal knockout of YAP/TAZ in the GI mesenchyme using either the *Gli1*^{CreER} allele or the *Myh11*^{CreER} allele.

While my data supports my hypothesis that YAP/TAZ are dispensable for gastrointestinal mesenchymal progenitor cell differentiation, it would be interesting to further investigate if the ratio of the resulting YAP/TAZ deficient differentiated mesenchymal cells is skewed from the wildtype expected ratios. Specifically, does loss of YAP/TAZ cause differentiation of more smooth muscle cells versus fibroblasts versus myofibroblast cells? The best way to investigate this would be with a cell lineage experiment, where it would be possible to track the percentages of differentiated subpopulations of mesenchymal cells within both control and mutant gut. I can also use fluorescence activated cell-sorting (FACS) to identify individual mesenchymal cell populations based on expression of distinct surface markers. Overall, uncovering the relationship between YAP/TAZ and mesenchymal differentiation could also provide insight into how aberrant YAP/TAZ activity in stromal cells may be contributing to the tumor microenvironment.

After observing the growth defect in the GI mesenchyme of *Nkx3.2*^{Cre}*Yap*$^{flox/flox}$*Taz*$^{flox/flox}$ mutant animals, I hypothesized that a mesenchymal YAP gain of function would result in the opposite phenotype. The mesenchyme in *Nkx3.2*^{Cre}*R26*$^{YAP5SA/+}$ mutant animals did in fact exhibit mesenchymal overgrowth due to elevated mesenchymal cell proliferation *(Fig. 3.7)*. This observation is in agreement with the majority of the previously published work investigating transgenic YAP gain of

function *in vivo*— other labs previously showed that YAP gain of function in the mouse liver generates a profound overgrowth phenotype due to increased proliferation (Camargo et al., 2007; Dong et al., 2007).

However, it is always possible that the overgrowth phenotype was due to an artifact of the transgenic allele design. The $R26^{YAP5SA}$ transgene is the human cDNA copy of YAP5SA, rather than the mouse cDNA, and is knocked into the *Rosa26* locus instead of the endogenous YAP locus. Therefore, to confirm that I was not observing an artifact of the transgene, I repeated the experiment using *Lats1* and *Lats2* floxed alleles, to more faithfully interrogate YAP activation *in vivo*. I observed that $Nkx3.2^{Cre}Lats1^{flox/flox}Lats2^{flox/flox}$ animals exhibited identical phenotypes to the $Nkx3.2^{Cre}R26^{YAP5SA/+}$ animals, including the mid-gestational embryonic lethality, accumulation of nuclear YAP, and downregulation of α-smooth muscle actin expression in the mesenchymal compartment *(Fig. 3.20)*. Based on these data, I concluded that the $Nkx3.2^{Cre}R26^{YAP5SA/+}$ phenotype is physiologically relevant and mesenchymal $R26^{YAP5SA}$ transgene expression faithfully recapitulates upstream Hippo Pathway kinase inactivation and subsequent YAP activation. Additionally, these data also confirm that in the developing gastrointestinal mesenchyme, YAP/TAZ protein localization are regulated by canonical Hippo Pathway kinases LATS1/LATS2, instead of through a non-canonical mechanism.

Why are the YAP/TAZ mutant phenotypes most severe in the developing stomach?

Interestingly, I observed that the mutant phenotypes in both the YAP/TAZ knockouts mutants (*Nkx3.2*^{Cre}*Yap*$^{flox/flox}$*Taz* $^{flox/flox}$) and the YAP gain of function mutants (*Nkx3.2*^{Cre}*R26*$^{YAP5SA/+}$ and *Nkx3.2*^{Cre}*Lats1*$^{flox/flox}$*Lats2*$^{flox/flox}$) was organized along a caudal-rostral gradient, with the most severe phenotype observed in the stomach and least severe in the intestine and colon. This phenotype is likely explained by the endogenous expression pattern of *Nkx3.2* (also known as *Bapx1*). Nkx3.2 protein expression is first detected in the hindstomach at E9.5, and then expression continues on a caudal-rostral gradient during development (Faure et al., 2013; Hecksher-Sorensen et al., 2004; Tribioli and Lufkin, 1999; Verzi et al., 2009). When we used the *Nkx3.2*^{Cre} allele to specifically restrict genetic ablation of *Yap* and *Taz* to the developing mesoderm, we were directing the YAP/TAZ knockout to follow the same caudal-rostral expression pattern as *Nkx3.2*.

Therefore, I hypothesize that the stomach tissue exhibits the strongest phenotype because these cells underwent Cre-mediated recombination earliest in development. To test this, an inducible gastrointestinal mesenchymal Cre allele, such as the *Gli1*^{CreER} or the *Myh11*^{CreER} allele, could be used to delay *Yap/Taz* knockout until tamoxifen administration. This would direct the genetic ablation to occur simultaneously along the length of the gastrointestinal tract. I hypothesize that if I induce Cre-recombination in the developing mesenchyme simultaneously instead of along an expression gradient, the phenotype severity would be the same across the caudal-rostral axis in the GI tract. However, it is still possible that the requirement for YAP/TAZ in the developing

mesoderm is organized along this caudal-rostral gradient and is higher in the stomach mesenchyme than in the colon. If the results from an inducible Cre still yield a phenotype that presents in a gradient, this would indicate that the requirement for YAP/TAZ must be different along the length of the gastrointestinal tract.

A Goldilocks Effect for YAP/TAZ activity in the developing GI mesoderm

Paradoxically, I observed that in the developing mesoderm, both YAP/TAZ knockout (*Nkx3.2*^{Cre}*Yap*$^{flox/flox}$*Taz*$^{flox/flox}$) as well as constitutive YAP activation (*Nkx3.2*^{Cre}*R26*$^{Yap5SA/+}$ and *Nkx3.2*^{Cre}*Lats1*$^{flox/flox}$*Lats2*$^{flox/flox}$) is embryonic lethal. These data suggest that the Hippo Pathway maintains tight regulation of YAP/TAZ activity to support progenitor cell proliferation while also inhibiting uncontrolled growth and tissue expansion. Another tissue where YAP/TAZ levels must be tightly regulated to allow for proper growth and differentiation is in the developing heart; both YAP knockout as well as YAP constitutive activation are embryonic lethal in this compartment (von Gisea et al., 2012; Xin et al., 2013; Xin et al., 2011c; Zhou et al., 2015a). Therefore, I propose that, as in the developing heart, a Goldilocks Principle exists within the gastrointestinal mesenchymal. YAP/TAZ activity is confined to a narrow window for homeostasis, and either too little or too much YAP/TAZ activity is detrimental.

What remains unanswered, however, is why the deviation from the optimal window of YAP/TAZ activity is so detrimental during mesodermal development. Rather, why are both YAP/TAZ loss of function, as well as YAP/TAZ gain of function,

embryonic lethal? Does YAP/TAZ loss cause lethality in the same manner that YAP/TAZ gain of function does? Or are two critical developmental events being perturbed, one that requires YAP/TAZ activation and another that requires YAP/TAZ inactivation?

To begin to address these questions, I considered the previously published work surrounding YAP/TAZ and mammalian development. To explore this further, one must consider that the mesodermal Cre allele I used, *Nkx3.2*^{Cre}, is constitutively expressed during development and expression is not restricted to the gastrointestinal mesenchyme. In addition to the developing gastrointestinal mesenchyme, *Nkx3.2* is also expressed predominantly in the developing skeletal system (Akazawa et al., 2000; Sivakamasundari et al., 2012; Tribioli et al., 1997; Tribioli and Lufkin, 1999). In fact, one group showed that a whole body knockout of Nkx3.2 protein in *Nkx3.2*$^{-/-}$ mutants resulted in a severe skeletal dysplasia phenotype and perinatal lethality (Akazawa et al., 2000).

In my study, *Nkx3.2*^{Cre}*Yap*$^{flox/flox}$*Taz*$^{flox/flox}$ mutant animals also exhibited a severe perinatal lethal phenotype, with 100% of mutant animals dying within hours of birth. Therefore I hypothesize that this observed lethality could be due to YAP/TAZ knockout in the developing skeletal system, especially considering a number of groups have identified links between YAP/TAZ and maintenance of the skeletal stem cells (SSCs) population. One group determined that the transcription factors Slug and Snail associate with YAP/TAZ to regulate skeletal stem/stromal cell (SSC) proliferation and differentiation (Tang et al., 2016). Another group showed that RASSF2, which is known

to regulate YAP/TAZ through direct binding to upstream Hippo Pathway kinases MST1/MST2, regulates osteoblast and osteoclast differentiation (Song et al., 2012).

This could be experimentally investigated by staining with Alizarin Red/Alcian Blue to determine if *Nkx3.2*^{Cre}*Yap*$^{flox/flox}$*Taz*$^{flox/flox}$ mutants exhibit skeletal defects as compared to control animals. Additionally, another experiment to test this hypothesis would be to use a bone cell specific Cre allele, such as the *OC*^{Cre} transgene (expressed in osteoblasts), to restrict *Yap* and *Taz* knockout to the developing bone and see if a similar phenotype occurred (Zhang et al., 2002).

The embryonic lethality I observed in the genotypes with mesodermal YAP gain of function, *Nkx3.2*^{Cre}*R26*$^{Yap5SA/+}$ and *Nkx3.2*^{Cre}*Lats1*$^{flox/flox}$*Lats2*$^{flox/flox}$, occurred during mid-gestation around approximately E14.5. Interestingly, another group observed embryonic lethality at a similar developmental timepoint when YAP was overexpressed in the developing mouse heart (von Gisea et al., 2012). In this study, the authors used a Tet-On system to express a doxycycline-induced *Yap-S127A* overexpression construct starting from E8.5, and observed lethality by E15.5 (von Gisea et al., 2012). To suggest that this may be a reason for my observed phenotype, the GenePaint Atlas shows that *Nkx3.2* expression has been detected in the developing heart via RNA in situ hybridization (Visel et al., 2004).

It is possible that the mid gestatation lethality in YAP gain of function mutants could be due to cardiac developmental defects. A closer examination of the developing heart could confirm this; for example, lacZ staining would reveal whether the *R26*^{YAP5SA} transgene had been expressed in the heart of *Nkx3.2*^{Cre}*R26*$^{Yap5SA/+}$ animals. To better

investigate this, inducible Cre-alleles, such as the ubiquitiously expressed, tamoxifen-inducible Cre allele Ubc^{CreER} can be used to bypass critical developmental points and induce genetic knockout of YAP/TAZ at a later developmental time point.

Epithelial Wnt signaling and mesenchymal YAP/TAZ

It is well acknowledged that paracrine signaling from the mesenchyme to the epithelia is important for both development and stem cell maintenance in the gastrointestinal tract (Kabiri et al., 2014; Li et al., 2007; Mao et al., 2010). Interesting, in my study, I observed that epithelial cells adjacent to YAP/TAZ deficient mesenchyme in $Nkx3.2^{Cre}Yap^{flox/flox}Taz^{flox/flox}$ animals displayed seemingly normal Wnt pathway signaling and parietal cell differentiation *(Fig. 3.3)*. Given the great interest (and controversy) that exists regarding the YAP/TAZ/Wnt relationship in the gastrointestinal tract (Hong et al., 2016; Imajo et al., 2015; Yu et al., 2015; Zanconato et al., 2016), we were particular surprised to observe apparently normal canonical Wnt pathway signaling in the epithelia adjacent to YAP/TAZ deficient mesenchymal tissue.

The relationship between YAP/TAZ deficient mesenchyme and overlying epithelia needs additional attention to fully characterize how loss of YAP/TAZ impacts the reciprocal signaling relationship between the two tissue compartments. To investigate this, an experiment to characterize the expression signature of the both the mesenchyme and epithelia compartments, such as RNAseq or proteomics, will be helpful in identifying differentially expressed transcripts in YAP/TAZ deficient tissue as compared to control.

Additionally, the overall architecture of the overlying epithelia appears disrupted in *Nkx3.2^{Cre}Yap$^{flox/flox}$Taz$^{flox/flox}$* mutant animals as compared to controls *(Fig. 3.3)*. Therefore, it is necessary to further investigate this epithelial cell compartment to elucidate whether this is due to defects in proliferation or differentiation, and how mesenchymal YAP/TAZ contributes to this phenotype.

Finally, my data brings us to an unanswered question regarding whether YAP/TAZ mesenchymal knockout affects Wnt signaling in the epithelia during regeneration or tumorigenesis. This question is somewhat tricky to answer because the *Nkx3.2^{Cre}Yap$^{flox/flox}$Taz$^{flox/flox}$* animals die at birth. In order to thoroughly probe this question *in vivo*, an inducible mesenchymal Cre allele such as *Gli1^{CreER}* should be used to genetically ablate YAP/TAZ protein from the gastrointestinal mesenchyme after the critical developmental timepoint before injury is induced via DSS treatment. Understanding the mesenchymal function of YAP/TAZ during regeneration could reveal important information about how mesenchymal YAP/TAZ contributes to the tumor microenvironment. Ultimately, this type of experiment would provide important insight as to whether targeting stromal YAP/TAZ may be a viable treatment strategy in cancer patients.

Is Hedgehog signaling impacted by YAP/TAZ mesenchymal knockout?

Hedgehog pathway signaling is critical for normal GI development. Our lab previously published a Hedgehog mutant phenotype with a mesenchymal growth defect

that was reminiscent of the phenotype I observed in *Nkx3.2^{Cre}Yap$^{flox/flox}$Taz$^{flox/flox}$* animals (Mao et al., 2010). In that study, our group showed that gastrointestinal epithelial knockout of Hedgehog ligands Shh and Ihh in *Shh$^{Cre/flox}$Ihh$^{-/flox}$* animals yielded a severe gastrointestinal mesenchymal defect and embryonic lethality by E18.5 (Mao et al., 2010). In a critical follow-up study published a few years later, we further found that mesenchymal knockout of the Hedgehog pathway component Smoothened in *Nkx3.2^{Cre}Smo$^{flox/flox}$* animals yielded a mesenchymal growth defect (Huang & Cotton et al., 2013) quite similar to the YAP/TAZ mesenchymal knockout.

It is important to note that in the *Nkx3.2^{Cre}Smo$^{flox/flox}$* mutants, we observed a severe intestinal villi developmental defect in addition to Wnt pathway signaling being virtually abolished in the adjacent epithelia (Huang & Cotton et al., 2013). However, in the intestinal epithelia of *Nkx3.2^{Cre}Yap$^{flox/flox}$Taz$^{flox/flox}$* mutants, I observed normal crypt-villi architecture and normal Wnt pathway signaling *(Fig. 3.2)*. I also detected a reduction in expression of Hedgehog pathway ligands Ihh and Shh in mutant gastrointestinal tract with mesenchymal YAP gain of function when analyzed by RNAseq. Together, these observations led me to the hypothesis that mesenchymal YAP/TAZ signaling is not required for establishing intestinal villi structures but could be involved with reciprocal signaling to the gastrointestinal epithelia to regulate the Hedgehog pathway. A critical follow-up study would be to perform transcriptional analysis of *Nkx3.2^{Cre}Yap$^{flox/flox}$Taz$^{flox/flox}$* gastrointestinal tissue. I expect to see an increase in both Ihh and Shh expression following mesenchymal YAP/TAZ loss of function if my hypothesis

is true. This experiment would help expand our understanding of YAP/TAZ in the mesenchyme.

Nevertheless, it is clear that loss of either YAP/TAZ or Smoothened in the developing gastrointestinal mesenchyme generates a severe growth defect. Therefore both YAP/TAZ and Hedgehog pathway signaling are required during gastrointestinal mesenchymal development. However, further studies are needed to fully elucidate how mesenchymal YAP/TAZ could be regulating epithelial Hedgehog signaling.

The YAP/TAZ mesenchymal knockout (*Nkx3.2*^{Cre}*Yap*$^{flox/flox}$*Taz*$^{flox/flox}$) described in this study displayed a similar mesenchymal growth defect as compared to the Smoothened mesenchymal knockout (*Nkx3.2*^{Cre}*Smo*$^{flox/flox}$) we published previously (Huang & Cotton et al., 2013). Additionally, the YAP gain of function mutant (*Nkx3.2*^{Cre}*R26*$^{YAP5SA/+}$) exhibited a mesenchymal overgrowth and tissue compartment expansion phenotype akin to the Smoothened gain of function mutant (*Nkx3.2*^{Cre}*R26*$^{SmoM2/+}$). We and others have shown that activated Hedgehog signaling is sufficient to induce differentiation of mesenchymal progenitor cells into smooth muscle progenitor cells both *in vitro* and *in vivo* (Huang & Cotton et al., 2013; Mao et al., 2010; Zacharias et al., 2011). Transgenic overexpression of a mutant Smoothened allele, *R26*$^{SmoM2/+}$, in the developing mesoderm drives the expansion of a differentiated gastrointestinal mesenchymal cell population in *Nkx3.2*^{Cre}*R26*$^{SmoM2/+}$ animals (Mao et al., 2010; Zacharias et al., 2011). Based on these previously published findings, I initially hypothesized that in the *Nkx3.2*^{Cre}*R26*$^{YAP5SA/+}$ animals, YAP5SA was activating Hedgehog-mediated differentiation to drive mesenchymal cell overproliferation.

However, my data convincingly showed that YAP inhibits the Hedgehog-mediated differentiation program both *in vitro* and *in vivo (Fig. 3.16* and *Fig. 3.17)*. In C3H10T1/2 cells, expression of the mutant YAP5SA is sufficient to inhibit Hedgehog-induced differentiation following SAG treatment. *In vivo* nuclear YAP localization is sufficient to halt and perhaps even reverse the smooth muscle differentiation program. In a differentiated $SMMHC^{+}$ population of cells, YAP5SA expression is sufficient to inhibit α-SMA expression. Finally, it is important to note that constitutive Hedgehog activity is not sufficient to override YAP constitutive activity *in vivo.* My data showed that endogenous nuclear YAP/TAZ inhibits the smooth muscle differentiation program, even when SmoM2 is overexpressed. Additionally, mesenchymal differentiation is still inhibited in $SMMHC^{+}$ cells when both YAP5SA and SmoM2 overexpression transgenic alleles are co-expressed in the $Myh11^{CreER}R26^{SmoM2/YAP5SA}$ animals. Based on my data showing that the SmoM2 mutant phenotype is not sufficient to override the YAP5SA mutant phenotype, I hypothesized that in the gastrointestinal tract, YAP and Hedgehog are not acting in a direct linear pathway to regulate differentiation, but rather the interaction between the two must be more complex.

One question that remains is whether YAP/TAZ directly inhibit Hedgehog-mediated mesenchymal differentiation. Results from the RNA transcriptional analysis showed Hedgehog Pathway targets such as *Gli1, Ptch1, Ihh,* and *Shh* were downregulated in the gastrointestinal tract of $Nkx3.2^{Cre}R26^{YAP5SA/+}$ embryos. However, in C3H10T1/2 cells that are overexpressing YAP5SA, I did not observe a significant difference in expression of *Gli1* or *Ptch1*. This discrepancy is likely due to experimental conditions.

For the RNA-seq experiment, I isolated RNA from the entire *Nkx3.2*^{Cre}*R26*$^{YAP5SA/+}$ gastrointestinal tract— a heterogenous cell population that included epithelial and non-mesenchymal cells. However, the C3H10T1/2 cells were a homogenous culture of mouse mesenchymal progenitor cells. The downregulated Hedgehog Pathway target expression in the microarray could therefore be due to the epithelial cell contribution.

Another possibility to explain why I observed downregulation of Hedgehog target gene expression in the *Nkx3.2*^{Cre}*R26*$^{YAP5SA/+}$ it that mesenchymal YAP/TAZ may not inhibit mesenchymal Hedgehog signaling (i.e. Gli-mediated transcription) directly. Rather, mesenchymal YAP/TAZ could inhibit Hedgehog pathway signaling indirectly, through paracrine inhibition of epithelial secretion of the Hedgehog Pathway ligands. To examine this further, a similar experiment could be performed where the GI epithelium and mesenchyme are physically separated via EDTA treatment. Then, RNA would be isolated from separated populations of epithelial tissue and mesenchymal tissue. In this way, the gene expression profiles specific to the epithelium and mesenchyme can be individually analyzed using RNA isolated from homogenous cell populations.

Overall, future experiments will help to elucidate whether YAP/TAZ directly regulate Hedgehog pathway activity intrinsically, or whether mesenchymal YAP/TAZ are involved with paracine regulation of Hedgehog pathway in the epithelia.

The molecular mechanism underlying YAP/TAZ inhibition of mesenchymal differentiation

The link between Hedgehog pathway and the differentiation program in the gastrointestinal mesenchymal tract has been studied by a number of labs (Huang & Cotton et al., 2013; Kolterud et al., 2009; Madison et al., 2005; Mao et al., 2010; van den Brink, 2007; Walton et al., 2012; Zacharias et al., 2011). It is suggested that Hedgehog pathway directly induces smooth muscle differentiation through direct Gli-binding to the *Myocd* gene to drive transcription and formation of the Myocardin-SRF master regulatory complex (Zacharias et al., 2011). What is less understood, however, is how the progenitor cell population is maintained in the gastrointestinal mesenchyme. Additionally, to date there have been no studies investigating YAP/TAZ in the developing gastrointestinal mesenchyme.

I have shown that YAP/TAZ activity must be tightly regulated during gastrointestinal development: both the loss of YAP/TAZ in $Nkx3.2^{Cre}Yap^{flox/flox}Taz^{flox/flox}$ mutants as well as YAP gain of function in $Nkx3.2^{Cre}R26^{YAP5SA/+}$ mutants yields severe gastrointestinal mesenchymal growth defects. When I further explored the relationship between YAP/TAZ protein subcellular localization and differentiation in the gastrointestinal mesenchyme, I observed that there was a robust relationship between α-SMA^{+} and subcellular YAP/TAZ localization; α-SMA^{-} cells showed robust nuclear YAP/TAZ localization whereas in α-SMA^{+} cells, YAP/TAZ was excluded from the nucleus *(Fig. 3.14)*.

Based on this information, I hypothesized that in the gastrointestinal mesenchyme, YAP/TAZ protein must be excluded from the nucleus and either sequestered in the cytoplasm or degraded, before the Hedgehog-mediated smooth muscle differentiation program can begin. While there has been no link between YAP/TAZ and gastrointestinal mesenchymal progenitor cells, YAP has been linked to smooth muscle differentiation in vascular smooth muscle cells (Xie et al., 2012a). Similar to my findings, another group found that YAP is upregulated in vascular smooth muscle progenitor cells following vascular injury to expand the progenitor cell population, and that YAP knockdown induces differentiation into vascular smooth muscle contractile cells (Xie et al., 2012a).

I showed that in both C3H10T1/2 mouse mesenchymal progenitor cells *in vitro* and in the mouse gastrointestinal tract *in vivo*, YAP inhibits Hedgehog-mediated smooth muscle differentiation by acting as a transcriptional co-repressor by directly binding to the *Myocd* promoter region at an evolutionarily conserved TEAD binding site to inhibit *Myocd* transcription, while also simultaneously acting as a transcriptional co-activator for its canonical targets *Cyr61* and *Ctgf (Fig. 3.21* and *Fig. 3.23)*. It is important to note that in mesenchymal progenitor cells, my data showed that YAP/TAZ do not bind directly to the α-SMA promoter to directly inhibit α-SMA transcription. This provides further evidence that downregulation of α-SMA is a read-out of Myocardin-SRF differentiation, rather than YAP/TAZ directly inhibiting α-SMA expression. My work agrees with a recent study by Dae-Sik Lim's group, which was one of the first studies to identify an oncogenic role for YAP/TAZ by acting as transcriptional co-repressors in MCF10A cells

in vitro by repressing transcription of tumor suppressors *Ddit4* and *Trail* (Kim et al., 2015b). My data support the observations made by Kim et al., and expand them by providing the first *in vivo* example of YAP/TAZ acting as transcriptional co-repressors.

Based on my data, I proposed a model wherein YAP/TAZ function as molecular gatekeepers in the gastrointestinal mesenchyme to regulate progenitor cell differentiation *(Fig. 4.2)*. In mesenchymal progenitor cells, YAP/TAZ migrate to the nucleus where they associate with TEAD proteins to bind to the *Myocd* TSS at an evolutionarily conserved TEAD binding site. YAP/TAZ function as transcriptional co-repressors, along with TEAD, to inhibit *Myocd* transcription and prevent the formation of the Myocardin-SRF master regulatory complex. When differentiation begins, canonical Hippo Pathway inhibits YAP/TAZ through LATS1/LATS2-mediated inhibitory phosphorylation. Once this occurs, activated Hedgehog signaling is able to initiate *Myocd* transcription, allowing for the Myocardin-SRF master regulatory complex to form. Myocardin-SRF in turn drives the smooth muscle differentiation program by upregulating target genes such as α-SMA, SMMHC, and SM22α.

One potential caveat to this model is that it is possible that YAP/TAZ potentiate part of their inhibitory role through protein-protein interactions, rather than just through transcriptional co-repression via TEAD binding. Other groups have shown that YAP inhibits differentiation in vascular smooth muscle cells through inhibiting the formation of the Myocardin-SRF master regulatory complex. One group found that YAP binds directly to and sequesters the Myocardin protein away from associating with SRF, thereby inhibiting the formation of the Myocardin-SRF master regulatory complex and

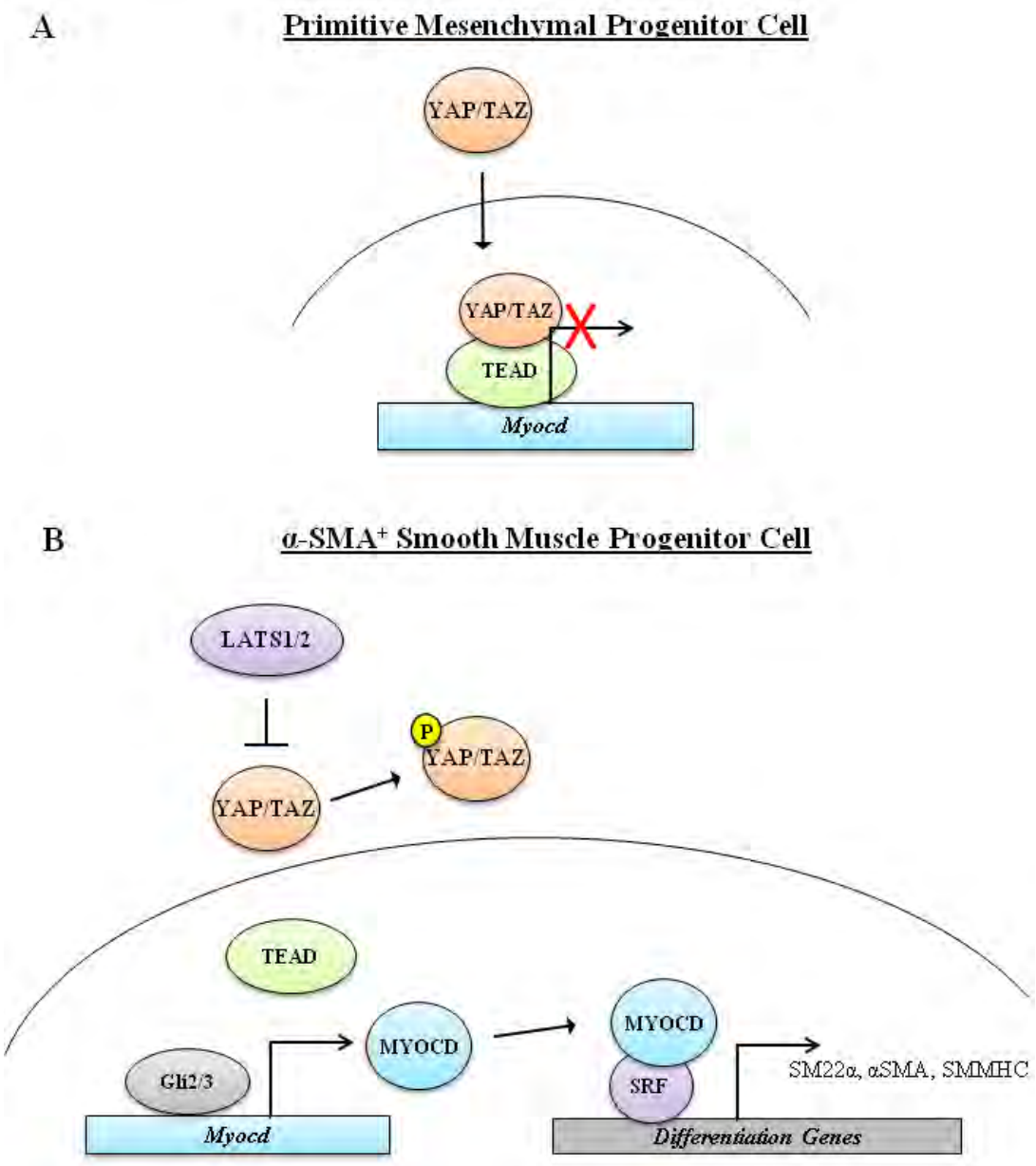


Figure 4.2. Model for YAP/TAZ in gastrointestinal mesenchymal differentiation. *(A)* In primitive mesenchymal progenitor cells, YAP/TAZ/TEAD bind to the *Myocd* promoter to repress transcription. *(B)* During differentiation, YAP/TAZ are inhibited by LATS1/2, and excluded from the nucleus. Activated Hedgehog activates *Myocd* transcription through direct Gli binding. MYOCD associates with SRF to form the Master Regulatory Complex and upregulate expression of differentiation target genes such as SM22α, α-SMA, and SMMHC.

preventing differentiation (Xie et al., 2012a). Additionally, TEAD proteins have also been linked to vascular smooth muscle differentiation; another group has observed that TEAD binds directly to SRF proteins to inhibit the formation of the Myocardin-SRF complex and inhibit differentiation in vascular smooth muscle progenitor cells (Gupta et al., 2001; Liu et al., 2014). Therefore, it is possible that a secondary level of regulatory inhibition exists in mesenchymal progenitor cells, similar to vascular smooth muscle cells, via protein-protein regulation and sequestration. Future work is needed to investigate if YAP/TAZ protein binds to members of the Myocardin-SRF master regulatory complex as a secondary level of differentiation in mesenchymal progenitor cells. If so, this would add an additional level of complexity to the YAP/TAZ regulatory mechanism for differentiation in the gastrointestinal mesenchyme.

Future Directions

The data in this study represents the first evidence implicating YAP/TAZ as having a critical role in the developing gastrointestinal mesenchyme and in progenitor cell differentiation. It has also generated a number of additional questions that will be important to consider for future studies. The following speculations represent what I believe to be the most logical next questions to ask and the exciting future directions for mesenchymal YAP/TAZ in tumorigenesis.

Does YAP/TAZ act as transcriptional co-repressors in other tissues?

I identified a TEAD binding site motif that is present in the *Myocd* transcriptional start site (TSS) that is evolutionarily conserved amongst human, mouse, rat, dog, cow, and pig *(Fig. 3.23)*. Given that the TEAD binding site is highly conserved in the *Myocd* TSS, it is possible that I have uncovered an evolutionarily critical mechanism through which YAP/TAZ control gastrointestinal mesenchymal progenitor cell differentiation in vertebrates. If so, this raises a number of questions about YAP/TAZ functioning as transcriptional co-repressors: Do YAP/TAZ function as transcriptional co-repressors in other progenitor cell populations during postnatal homeostasis or is this function specific to development? Given that Xie et al. previously reported that YAP knockdown induces differentiation in vascular smooth muscle progenitor cells (Xie et al., 2012a), it is logical to investigate whether the YAP/TAZ transcriptional co-repression mechanism I uncovered in the gastrointestinal mesenchyme is also conserved in vascular smooth muscle progenitor cells.

Another question brought to light by my data is whether the YAP/TAZ mechanism is specific to the gastrointestinal mesenchyme or if YAP/TAZ function as transcriptional co-repressors in other organs? For example, similar to the gastrointestinal mesenchyme, mammalian heart development requires tight regulation of YAP/TAZ activity in cardiomyocytes; both embryonic YAP/TAZ knockout as well as YAP activation in cardiomyocytes results in lethality (von Gisea et al., 2012). Therefore, it is possible that YAP/TAZ also function as transcriptional co-repressors in cardiomyocyte

progenitor cells to control appropriate differentiation. Cardiac stem cells are quiescent at adult stages; it is impossible to regenerate cardiac tissue following myocardial infarction (Madonna et al., 2014). However, if YAP/TAZ function as transcriptional co-repressors in cardiac progenitor cells at adult stages, then inhibiting YAP/TAZ in this population might allow for cardiac regeneration. Conversely, another group has shown that constitutive YAP activation following myocardial infarction actually drives cardiac regeneration (Xin et al., 2013). Clearly, more work is needed to understand how YAP/TAZ regulate progenitor cell populations in the heart and whether YAP/TAZ function as transcriptional co-repressors in the cardiomyocyte progenitor cell population. Beginning to address these questions in future work will allow for a greater understanding of normal mammalian development and how YAP/TAZ, as critical members of the Hippo organ size control pathway, regulate growth and proliferation.

How is the YAP/TAZ differentiation program regulated?

I have shown that the canonical Hippo Pathway is responsible for inhibiting YAP/TAZ prior to Hedgehog-mediated differentiation in the gastrointestinal mesenchyme, but what signals upstream of LATS1/LATS2 to initiate this regulation? There are several possibilities for this mechanism, including a morphogen gradient or mechanical regulation.

Morphogen gradients, established when signaling molecules are secreted and form a concentration gradient, have been shown to be integral to many developmental

processes such as establishing polarity and determining cell fate identity (Ashe and Briscoe, 2006). For example, secreted Shh ligand organizes into a concentration gradient to pattern the ventral neural tube in mammalian development (Ericson et al., 1997; Pierani et al., 1999). Additionally, the Hippo Pathway has previously shown to be responsive to a morphogen gradient; one group showed that in the developing *Drosophila melanogaster* wing, the morphogen Decapentaplegic (Dpp) regulates the Hippo pathway through the membrane receptor Fat (Rogulja et al., 2008). Therefore, it is possible that a similar morphogen gradient activates canonical Hippo Pathway signaling to inhibit nuclear YAP/TAZ through LATS1/LATS2-mediated phosphorylation and allow for upregulation of mesenchymal progenitor cell differentiation.

Another possible mechanism for the upstream differentiation mechanism could be mechanical forces. In recent years, the importance of physical and mechanical forces present in the tissue microenvironment has been appreciated. A number of groups have demonstrated that specific lineage of mesenchymal stem cell differentiation can be controlled by changing the rigidity of the extracellular matrix (ECM) because of the different forces acting on the progenitor cells (Engler et al., 2006; McBeath et al., 2004). For example, mesenchymal stem cells plated on a soft ECM will differentiate into neural cells but will differentiate into either muscle cells or bone cells when plated on firmer ECM (Engler et al., 2006). Additionally, it has been shown by a number of groups that YAP/TAZ nuclear localization can be regulated by mechanical forces, such as stretching (Driscoll et al., 2015; Dupont et al., 2011; Yu et al., 2012; Zhao et al., 2012). As the gastrointestinal tract grows and expands in size, the stretching forces and resulting

tension on each individual cell is presumably also changing. It is possible that these stretching forces in the developing GI tract might contribute to Hippo Pathway activation. To investigate this further, C3H10T1/2 cells can be cultured on different ECM substrates to begin to investigate whether mechanical forces can induce smooth muscle differentiation. Overall, future experiments to identify how Hippo Pathway maintains this mesenchymal progenitor cell population may also provide greater insight into how the Hippo Pathway contributes to tumorigenesis.

What does this developmental study reveal about mesenchymal YAP/TAZ in tumorigenesis?

My work has revealed that accumulation of nuclear YAP/TAZ in mesenchymal cells is sufficient to drive the hyperproliferation and expansion of a progenitor cell population during gastrointestinal development. However, several unknown questions remain regarding the requirement for YAP/TAZ in the mesenchyme during normal postnatal homeostasis as well as tumorigenesis. Is mesenchymal YAP/TAZ required for normal homeostasis in the adult gastrointestinal tract? Is postnatal mesenchymal YAP/TAZ activation sufficient to initiate an overgrowth phenotype in the gastrointestinal tract? Does mesenchymal YAP/TAZ contribute to the tumor cell niche in colorectal cancer and/or in other solid tumors?

One future experiment to investigate the role for YAP/TAZ in postnatal mesenchymal cell populations would be to use a conditional Cre allele, such as the

Gli1^{CreER} or the *Myh11*^{CreER} allele, to target the GI mesenchyme during postnatal stages. I would use these Cre drivers to investigate both YAP/TAZ loss of function as well as YAP5SA activation in adult mice. This type of experiment would reveal whether mesenchymal YAP/TAZ activity is required in normal homeostasis. This experiment would also reveal whether YAP activation in a GI mesenchymal cell population contributes to tumorigenesis.

Preliminary work in our lab has revealed that YAP/TAZ appear to be dispensable in a gastrointestinal myofibroblast population during postnatal stages, but that YAP activation is sufficient to drive expansion of a mesenchymal cell population. Additionally, another project revealed that YAP/TAZ are required for polyp initiation in an LKB1-deficient background, indicating that the gastrointestinal hamartomatous polyposis disorder, Peutz Jegher's Syndrome, has a molecular requirement for Hippo Pathway signaling (See **Appendix B**). From these data combined, we are confident that investigating the function of YAP/TAZ in the gastrointestinal mesenchyme is certain to enhance our understanding of the Hippo Pathway in the tumor microenvironment.

My work presented here also revealed a critical mechanism whereby YAP/TAZ function as transcriptional co-repressors to maintain a mesenchymal progenitor cell population. Based on these data, the next logical question is whether YAP/TAZ function similarly in cancer associated fibroblasts (CAFs). CAFs are mesenchymal cells that exist in the tumor microenvironment and play a tumor-promoting role through secretion of growth factors in a number of solid tumors, such as breast, prostate, and pancreas (Luo et al., 2015; Olumi et al., 1999; Pan et al., 2015; Spaeth et al., 2009). As a result of their

tumor promoting role through paracrine signaling to tumor cells, CAFs represent attractive drug targets for targeted therapies in patients with solid tumors (Calon et al., 2015; Conti and Thomas, 2011; Isella et al., 2015). However, further work is needed to understand exactly how CAFs maintain the pro-growth niche environment, and whether this mechanism involves YAP/TAZ. One such experiment could involve genetic mesenchymal *Yap/Taz* knockout in the Apc^{Min} model for colorectal cancer. If mesenchymal YAP/TAZ activity contributes to the tumor microenvironment in APC-mutant polyps, I would expect to observe a reduction in tumor burden when YAP/TAZ are genetically removed from the mesenchyme.

Furthering our understanding of YAP/TAZ during normal gastrointestinal development will ultimately help us explain the role YAP/TAZ is playing during tumorigenesis. However, my work has also revealed a multitude of new and exciting questions. Answering these questions will allow us to better understand how YAP/TAZ contribute to tumorigenesis and if YAP/TAZ are attractive drug targets for targeted therapies in the clinic.

Final Thoughts

Cumulatively, the data presented here highlight the critical importance of tissue compartment specificity during both development and tumorigenesis. YAP/TAZ clearly are dispensable in the epithelia under normal conditions and are only required during Wnt hyperactivation. In the developing gastrointestinal mesenchyme however, YAP/TAZ are required. Uncovering the temporal and spatial requirements for developmental signaling pathways, such as the Hippo Pathway, helps to understand whether it is efficacious to develop YAP/TAZ inhibitors for clinical use.

This study is also the first to link Hippo Pathway signaling to the maintenance of gastrointestinal mesenchymal progenitor cells and to discover a relationship between Hippo Pathway and Hedgehog-Pathway mediated differentiation in the mesenchyme. Given the tumor-promoting role that the stroma has been shown to play through paracrine signaling to associated tumor cells in the tumor microenvironment, the identification of the Hippo Pathway as a critical mediator of the mesenchymal progenitor cell population expands understanding of both Hippo Pathway signaling, as well as the importance of the mesenchyme in gastrointestinal development and tumorigenesis.

APPENDICES

Preface

Of the most commonly used model organisms— bacteria, yeast, worms, flies, and mice— the laboratory mouse is the most similar to humans in both genomic homology as well as in anatomical similarity (Eppig et al., 2012). Using mice to develop genetically engineered mouse models (GEMMs) allows for precise spatial and temporal control of gene expression in an *in vivo* system. Despite advances in genetic engineering, these mouse models are not always a perfect recapitulation of human disease; mouse models of colorectal cancer in particular highlight the dichotomy that exists between polyp initiation in mice as compared to humans. Similar to humans with an *APC* mutation, mice also develop polyps driven by elevated Wnt pathway signaling in the GI epithelia (Su et al., 1992). However, humans generally develop APC-mutant polyps in their large bowel, whereas mice predominantly develop APC-mutant polyps in the small intestine (Jackstadt and Sansom, 2016). Additionally, humans born with Peutz Jeghers' Syndrome caused by an inherited autosomal dominant mutation in *LKB1* develop multiple hamartomatous polyps throughout the GI tract, whereas mice carrying a heterozygous knockout of *Lkb1* develop hamartomatous polyps only at the pylorus (Bardeesy et al., 2002; Jishage et al., 2002; Miyoshi et al., 2002). The small intestine and large bowel are both functionally and structurally different; the small intestine is involved with nutrient absorption and is organized in crypt-villi architecture, whereas the large bowel is primarily involved with water absorption and villi are absent (Ménard, 2004). Therefore, comparing a small intestine polyp in mouse to a large bowel polyp in a human can be challenging due to the

vast differences that exist along the GI tract. Finally, the majority of mouse models of CRC do not undergo metastasis and are primarily experimental examples of adenomas and carcinoma in situ; these models do not recapitulate the metastatic CRC which ultimately leads to death in human patients (Deming et al., 2013). Overall, there still remain many experimental questions which the mouse models currently available are incapable of addressing.

While GEMMs are not perfect facsimiles of human disease, mouse models are still considered to be a powerful experimental tool for cancer research. The time to adulthood for a laboratory mouse is approximately 4-6 weeks, whereas this process takes 18 years in humans (Vandamme, 2014). While the chimpanzee is the most similar animal to humans according to genome sequence homology, the costs and space required for primate research are prohibitive for many studies, whereas mice take significantly less space and cost per animal (Conlee and Rowan, 2012). Finally, the ideal experiment to study polyp progression and tumorigenesis would be conducted within humans; however, strict ethical regulations and morality prevent scientists from conducting these type of experiments with human subjects (Festing and Wilkinson, 2007). Therefore, mouse models represent an ideal *in vivo* model organism to conduct tumor studies due to the abbreviated mouse lifespan, biological similarities to humans, and the strict ethical regulations involved with human research. The CRC mouse models available represent the best technology to investigate polyp initiation and progression *in vivo*, however, development of new mouse models that more closely recapitulate human disorders will continue to bridge the gaps that exist between mouse and human. Ultimately, developing

and studying these new mouse models should lead to new drugs and treatments for human patients.

APPENDIX A

SMAD7 promotes serrated polyposis in the GI epithelia

Preface

Appendix A represents work I performed in Dr. Junhao Mao's lab at the University of Massachusetts. The data presented herein is my own, with the following exceptions listed below:

Fig. A.7.A: Dr. He Huang isolated RNA from *Villin*^{Cre}*R26*$^{Smad7/+}$ polyps. Dr. Jianhong Ou performed data set comparison and analysis.

These data are unpublished. Further experiments are needed to complete this manuscript. Therefore the story presented in Appendix A represents our current understanding of the science based upon our interpretation of the data. Additional data and conclusions may be added in the published version.

Abstract

Mutations in SMAD7 have been shown to be a prognostic marker in colorectal cancer (CRC) patients, but the exact relationship between SMAD7 and tumorigenesis remains elusive. To determine the effect of SMAD7 on transformation, I stably expressed a SMAD7 expression construct in FET cells, a noninvasive colorectal cancer cell line, as well as IEC6 cells, an immortalized, non-transformed intestinal epithelial cell line. I observed that SMAD7 expression allows cells to overcome contact-mediated inhibition. To investigate this finding further, we generated a novel mouse allele for SMAD7 expression, the $R26^{Smad7}$ allele. SMAD7 expression in the intestinal epithelia is sufficient to initiate serrated polyposis, independently of WNT-pathway mutations. Furthermore, SMAD7-mutant polyps express the serrated polyp markers *Vsig1* and *Anxa10*. Together, SMAD7 may initiate serrated polyposis through the upregulation of canonical serrated polyp markers. Thus, this study describes a novel mouse model that expresses the serrated polyp molecular signature and links SMAD7 to the serrated polyp initiation cascade.

Introduction

Colorectal cancer (CRC) is the third leading lethal cancer subtype in the United States and is responsible for almost 50,000 deaths per year in the United States (Siegel et al., 2016). CRC has long believed to arise according to the Vogelstein model for tumorigenesis with an initiating mutation in the APC/Wnt signaling pathway initiating hyperplasia, followed by a stepwise accumulation of genetic events as polyps progress from adenomas to carcinoma in situ and eventually malignant carcinoma (Fearon and Vogelstein, 1990).

The serrated polyp subtype, including both traditional serrated polyps and sessile serrated polyps, are histologically distinct from conventional adenomatous polyps (Torlakovic et al., 2008). Adenomatous polyps, such as pedunculated adenomas, are characterized by a smooth or tube-like crypt structure (Bariol et al., 2003; Fodde, 2002; Shibata et al., 1997). Serrated polyps are characterized by a "saw-tooth" pattern in the crypts, and can also be organized into a "tennis-racket" type structure in early lesions (Bariol et al., 2003; Bordacahar et al., 2015; Salaria et al., 2012; Torlakovic et al., 2008).

For many years it was believed that the serrated polyp group were benign and did not progress to carcinoma (Longacre and Fenoglio-Preiser, 1990). However, it has recently been appreciated that histologically serrated polyps represent as many as 30% of all malignant colorectal cancer cases (Makkar et al., 2012; Salaria et al., 2012). Additionally, it has been shown that serrated polyps arise through a genetically distinct molecular pathway, known as the Serrated Polyp Pathway (Laiho et al., 2007). The

Serrated Polyp Pathway is independent of the canonical APC/Wnt signaling pathway, which is responsible for conventional adenoma initiation and progression (Laiho et al., 2007). Activating mutations in KRAS and BRAF have been proposed to be involved in the Serrated Polyp Pathway, however many questions remain about whether other genes are instrumental in serrated polyp pathogenesis (Davies et al., 2014; Feng et al., 2011; Laiho et al., 2007; Patai et al., 2013)

SMAD7 is the inhibitory R-SMAD for both the TGFβ and BMP pathways and mechanistically prevents the phosphorylation of either SMAD2/3 or SMAD1/5/8 (Zhu et al., 2011). Recently, SMAD7 has been identified as a prognostic marker for colorectal cancer (Slattery et al., 2010; Stolfi et al., 2013; Tenesa et al., 2008; Thompson et al., 2009). Additionally, recent Genome-wide Association Studies (GWAS) identified single-nucleotide polymorphisms (SNPs) within the *Smad7* gene that confers an elevated risk for developing colorectal cancer (Broderick et al., 2007; Jiang et al., 2013; Pittman et al., 2009; Slattery et al., 2010; Tenesa et al., 2008).

There are conflicting reports regarding whether SMAD7 gain of function or SMAD7 loss of function is tumorigenic. The *Smad7* gene is located within the chromosomal region 18q21, which has been frequently observed to be deleted in colorectal cancer (CRC) (Boulay et al., 2003). One report suggests that SMAD7 inhibits tumorigenicity through inhibiting TGFβ growth factor secretion (Javelaud et al., 2005). However, there is also evidence that SMAD7 gain of function can also be tumorigenic by blocking TGFβ-mediated apoptosis (Halder et al., 2005; Halder et al., 2008).

We report here that SMAD7 gain of function is tumorigenic in the intestinal epithelia, and is sufficient to initiate polyposis independently of WNT pathway activation. Furthermore, we show that SMAD7 overexpression drives growth of serrated polyps, through a Hippo Pathway-independent mechanism. Additionally, we report that SMAD7 expression is sufficient to drive the expression of the molecular signature for serrated polyposis. Overall we have uncovered a link between SMAD7 and serrated polyposis *in vivo*, and have developed a novel mouse model that recapitulates the histological and molecular signature of human serrated polyposis.

Results and Discussion

SMAD7 increases anchorage-independent cell growth and induces foci formation

Recent GWAS studies have identified SNPs in SMAD7 that correlate with a greater risk for colorectal cancer as well as a poorer prognosis. To investigate this further, I first investigated SMAD7 expression in FET cells, a non-invasive colorectal cancer cell line (Brattain et al., 1980). I first cloned the mouse *Smad7* cDNA containing a flag-tag into the *pGIPZ2a* plasmid. I then transfected FET cells with either the *pGIPZ2a* or *pGIPZ2a-Smad7* plasmid and assayed for anchorage-independent cell growth using a soft agar assay. I confirmed that overexpression of the SMAD7 protein is sufficient to increase anchorage-independent growth of FET colorectal carcinoma cells *in vitro (Fig. A.1.A),* corroborating previously published findings (Halder et al., 2005; Halder et al., 2008).

I next wanted to investigate the role of SMAD7 in the process of transformation. I used lentiviral infection to express the *pGIPZ2a* or *pGIPZ2a-Smad7* plasmid in IEC6 cells, an immortalized non-transformed cell line (Quaroni et al., 1979). I then assayed whether overexpression of SMAD7 was sufficient to allow IEC6 cells to overcome contact-mediated growth inhibition in a foci formation assay. I found that after three weeks of growth in culture, *IEC6-pGIPZ2a-Smad7* cells formed significantly more foci colonies than control cells *(Fig. A.1.B,C)*. Together, my analysis suggested a role for SMAD7 in anchorage-independent cell growth and in foci formation.

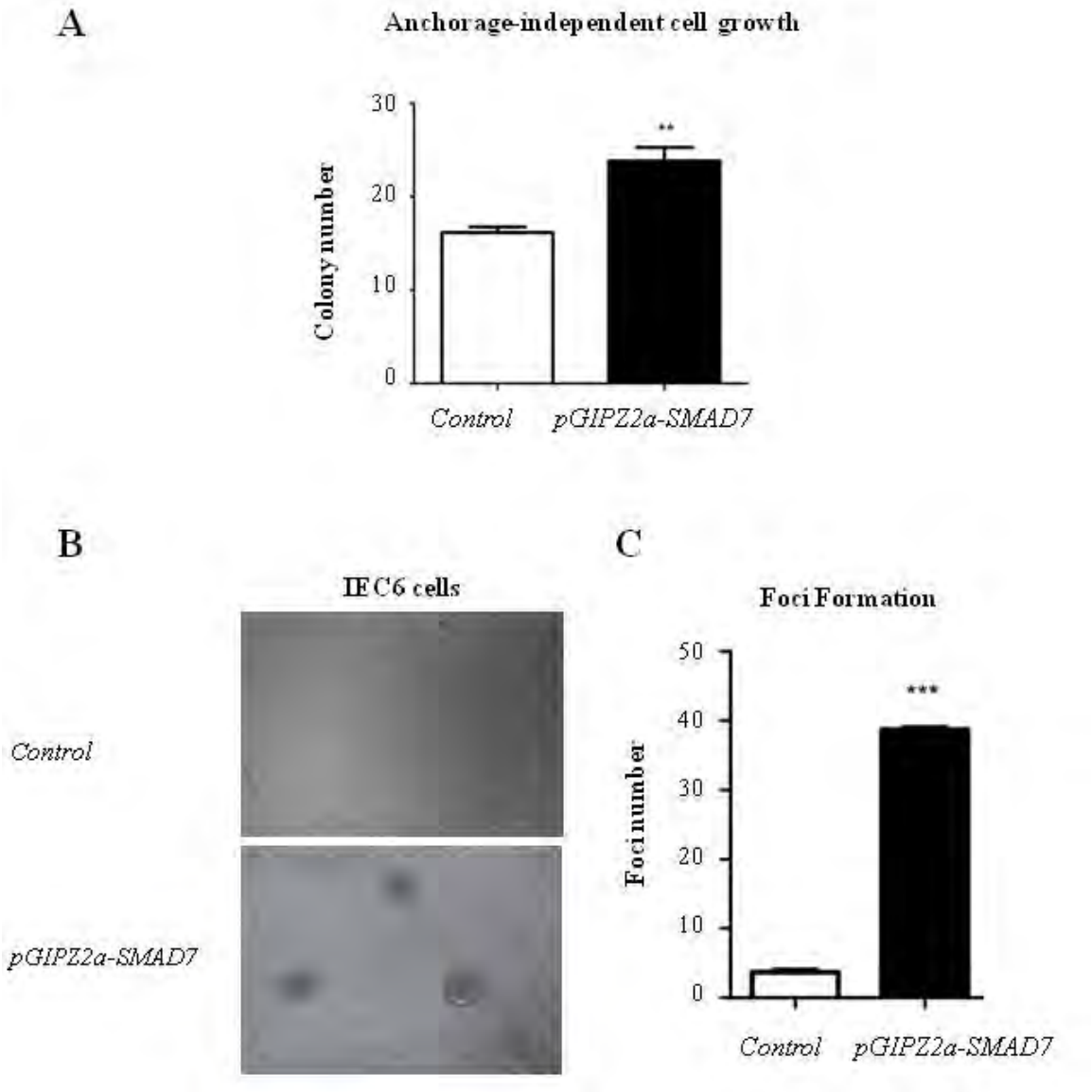


Figure A.1. SMAD7 drives colony and foci formation.
(A) In vitro SMAD7 expression increases anchorage-independent cell growth and soft agar colony formation in FET cells and *(B,C)* induces foci formation in the non-transformed rat intestinal epithelia cell line IEC6.

SMAD7 does not affect WNT pathway signaling *in vitro*

I next investigated whether SMAD7 overexpression was activating Wnt pathway signaling *in vitro*. Activation of Wnt pathway signaling in IEC6 cells is sufficient to induce foci formation *in vitro* (Ouko et al., 2004). Given that SMAD7 has been shown to interact with and stabilize β-catenin, I hypothesized that SMAD7 was initiating tumorigenesis by stabilizing β-catenin, thereby activating Wnt pathway target gene expression (Edlund et al., 2005; Tang et al., 2008).

Interestingly, I observed that SMAD7 expression in IEC6 cells does not result in elevated β-catenin protein levels *(Fig. A.2.A)*. Additionally, protein expression of canonical Wnt pathway targets axin2 and c-myc were not increased following expression of the *pGIPZ2a-Smad7* construct *(Fig. A.2.B,C)*. Finally, I found that SMAD7 expression does not affect TCF/LEF luciferase reporter activity in IEC6 cells *(Fig. A.2.D)*. From these data, I concluded that SMAD7 does not affect Wnt pathway signaling *in vitro*.

SMAD7 overexpression induces polyposis in the intestinal epithelia

I next wanted to investigate whether SMAD7 expression in the intestinal epithelia *in vivo* was sufficient to initiate polyposis. To do this, our lab generated a conditional allele targeted into the *Rosa26* locus, $R26^{Smad7}$, which expresses *Smad7* and a C-terminal IRES-nuclear LacZ under control of a hybrid *CMV* enhancer/β-actin CAGGS promoter following traditional Cre-Lox mediated recombination *(Fig. A.3.A)*. To restrict the expression of the $R26^{Smad7}$ allele specifically to the intestinal epithelia, I crossed the $R26^{Smad7}$ mice to $Villin^{Cre}$ mice to generate mice with the $Villin^{Cre}R26^{Smad7/+}$ genotype. I

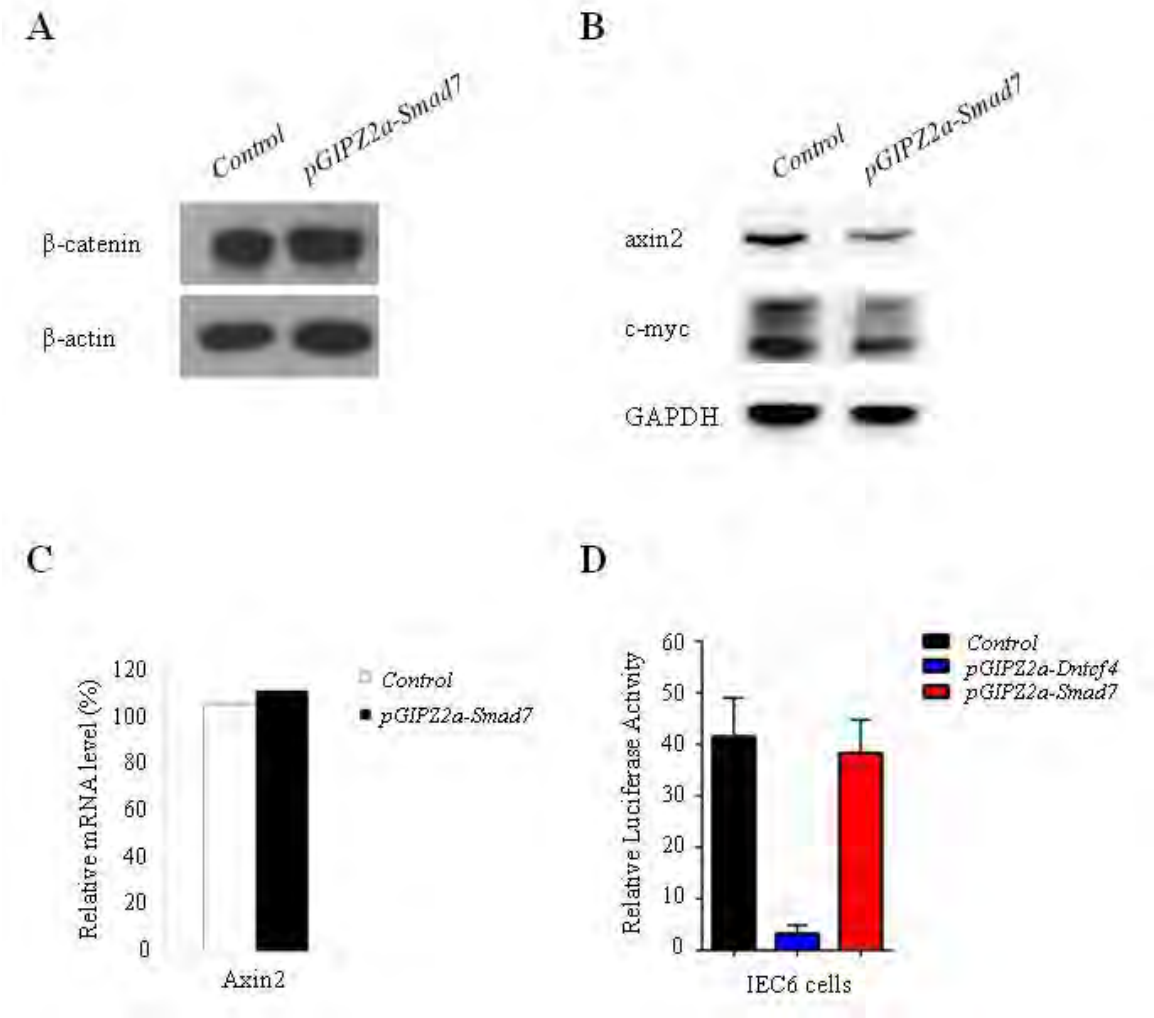


Figure A.2. SMAD7 does not affect Wnt pathway signaling *in vitro*.
(A) β-catenin protein levels are unchanged in IEC6 cells following SMAD7 expression. *(B,C)* Canonical Wnt pathway targets axin2 and c-myc affect TCF/LEF reporter activity *in vitro*.

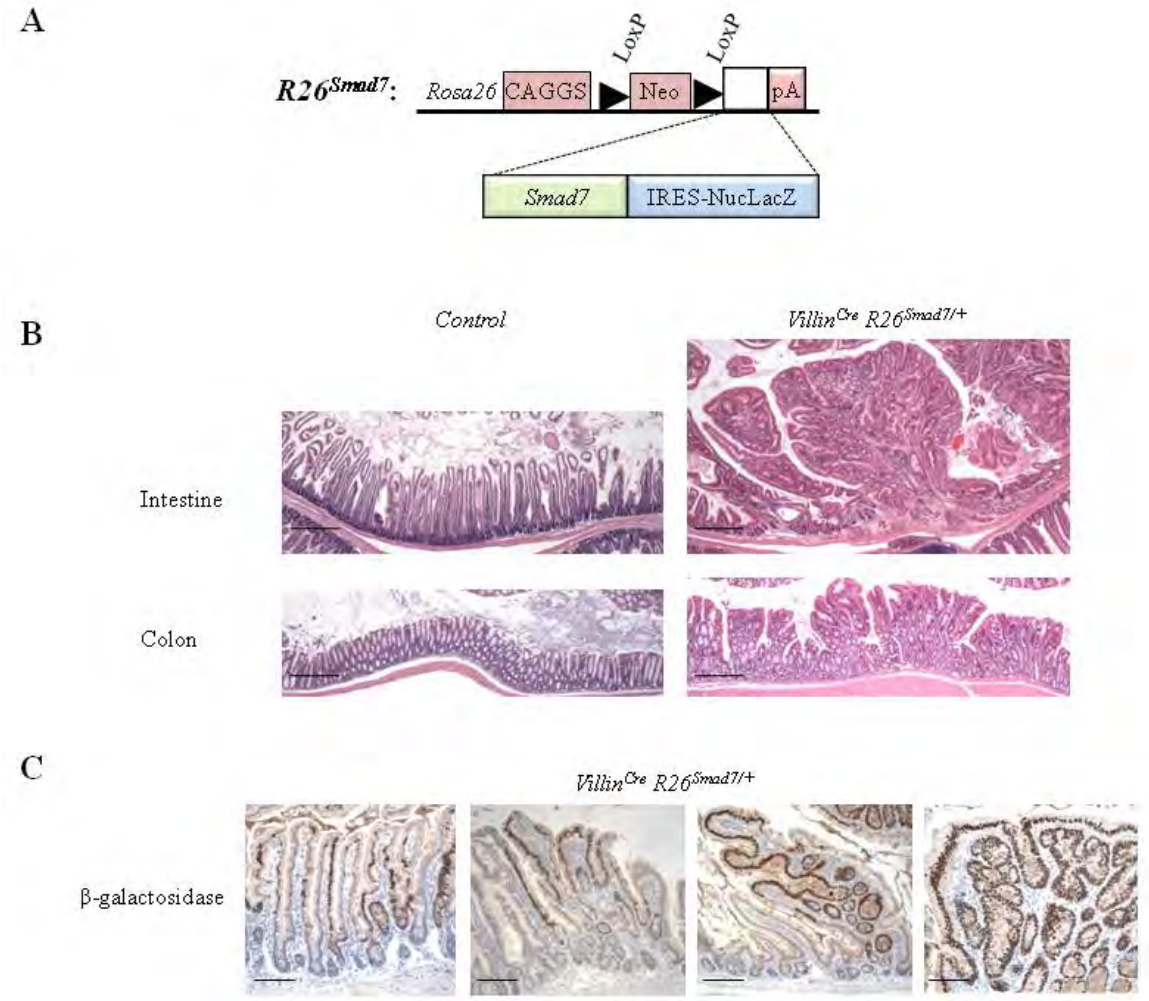


Figure A.3. *Villin^{Cre}R26$^{Smad7/+}$* animals develop intestinal and colon polyps.
(A) Smad7 gain of function transgenic allele. *(B) Villin^{Cre}R26$^{Smad7/+}$* animals develop polyps in both the intestine and colon. Scale bar = 100 µM. *(C)* Mosaic expression of the *R26^{Smad7}* transgene in the intestinal epithelia demonstrates the oncogenic capability of SMAD7; polyps are comprised of βgal^{+} cells whereas adjacent wildtype appearing tissue is negative for SMAD7 transgene expression. Scale bar = 5 µM.

allowed *Villin*^{Cre}*R26*$^{Smad7/+}$ animals to age and observed that animals developed polyps in both intestine and colon *(Fig. A.3.B)*, and animals survived to approximately 14 months of age *(Fig. A.8.A)*. Expression of the *Villin*^{Cre}*R26*$^{Smad7/+}$ transgene appeared to be mosaic, yet I observed that both hyperplastic villi and polyps were comprised exclusively of β-galactosidase^{+} cells *(Fig. A.3.C)*, indicating that expression of the *R26*^{Smad7} allele in intestinal epithelia cells is sufficient to initiate hyperplasia and polyposis.

Villin^{Cre}*R26*$^{Smad7/+}$ polyps exhibit serrated morphology

I next decided to compare the morphology of the *Villin*^{Cre}*R26*$^{Smad7/+}$ polyps to the classic adenomatous polyp morphology. According to the widely accepted Vogelstein model of tumorigenesis, colorectal cancer progresses through a sequential series of accumulating genetic aberrations (Fearon and Vogelstein, 1990). One of the earliest genetic mutations is the loss of *APC*, which results in the stabilization of nuclear β-catenin and upregulation of Wnt signaling. Elevated Wnt pathway activity has been correlated with tumorigenesis in colorectal cancer in patients.

To test this, I first generated *Villin*^{Cre}*Apc*$^{flox/+}$ animals; genetic ablation of *Apc* specifically in the intestinal epithelia is sufficient to drive polyposis through activation of the Wnt pathway and stabilization of β-catenin (Fearon and Vogelstein, 1990; Powell et al., 1992; Siu et al., 1997; Su et al., 1992; Yuan et al., 2001). Polyps in *Villin*^{Cre}*Apc*$^{flox/+}$ animals exhibited classic adenomatous histology with rounded polyp morphology.

I consulted with the UMass Pathology Department to review the pathology of the APC-mutant polyps to SMAD7-mutant polyps. Dr. Zhong Jiang confirmed that the

Villin^{Cre}*R26*$^{Smad7/+}$ polyps exhibited the characteristics of serrated polyps, included the "saw-tooth" like crypt structures, rather than classic adenomatous polyps *(Fig. A.4.A,B)* (Bariol et al., 2003). Additionally, early *Villin*^{Cre}*R26*$^{Smad7/+}$ polyp lesions displayed the "tennis racket" morphology characteristic of serrated polyps, rather than the rounded adenomatous morphology characteristic of APC-mutant polyps (Patai et al., 2013; Torlakovic et al., 2008). A previous publication showed that whole-body *Smad4* heterozygous knockout mice can generate serrated adenomas *in vivo* (Hohenstein et al., 2003). My findings corroborate this finding that aberrant TGFβ/BMP signaling in the intestinal epithelia is sufficient to initiate serrated polyposis, and also links SMAD7 to this process for the first time.

Finally, immunohistochemical analysis revealed that in contrast to robust nuclear accumulation of β-catenin in APC-mutant polyps, β-catenin localization in SMAD7-mutant polyps is primarily membranous *(Fig. A.5.A)*. Additionally, canonical Wnt pathway targets CD44 and Sox9 are highly elevated in APC-mutant polyps, but not in SMAD7-mutant polyps *(Fig. A.5.B,C)*. The molecular mechanisms and mutations involved with serrated polyp tumorigenesis are believed to be independent of the Vogelstein/WNT pathway model for adenoma tumorigenesis (Carragher et al., 2010; Davies et al., 2014; Hawkins et al., 2002; Makinen, 2007; Patai et al., 2013). Therefore, I conclude that SMAD7 is driving polyposis independently of Wnt pathway signaling, and is initiating a serrated polyp pathway to drive tumorigenesis.

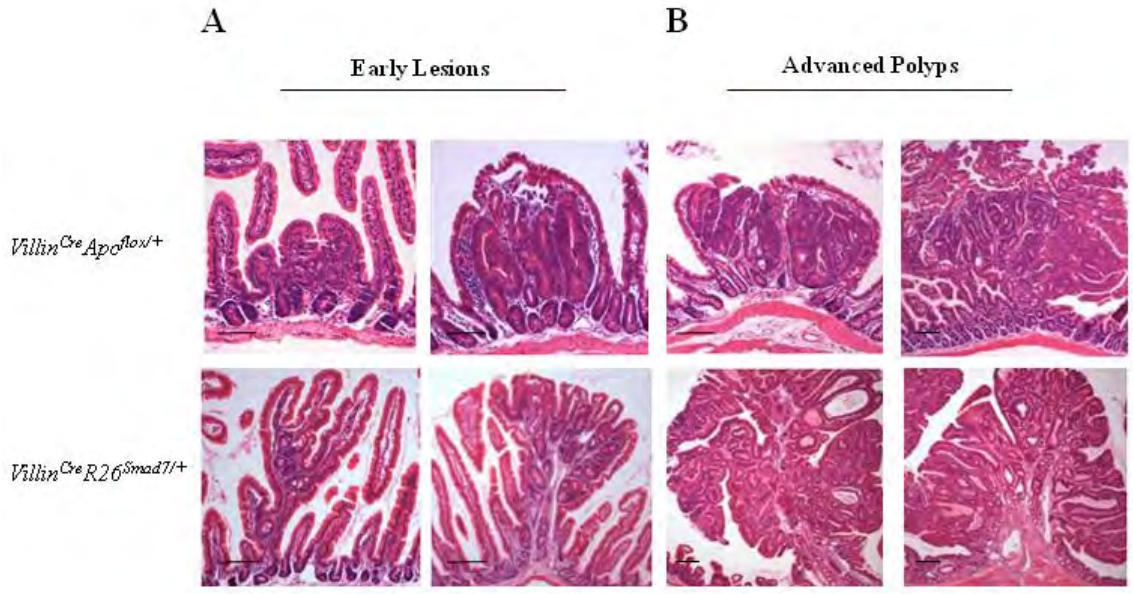


Figure A.4. *Villin*^{Cre}*R26*$^{Smad7/+}$ polyps exhibit serrated morphology.
(A-B) Villin^{Cre}*Apc*$^{flox/+}$ polyp histology is representative of classical adenomatous polyps, whereas *VillinCre R26*$^{Smad7/+}$ polyp histology is representative of serrated polyposis. *Villin*^{Cre}*R26*$^{Smad7/+}$ polyps display "tennis-racket" morphology rather than the rounded adenomatous morphology in *Villin*^{Cre}*Apc*$^{flox/+}$. Scale bar = 50 µM.

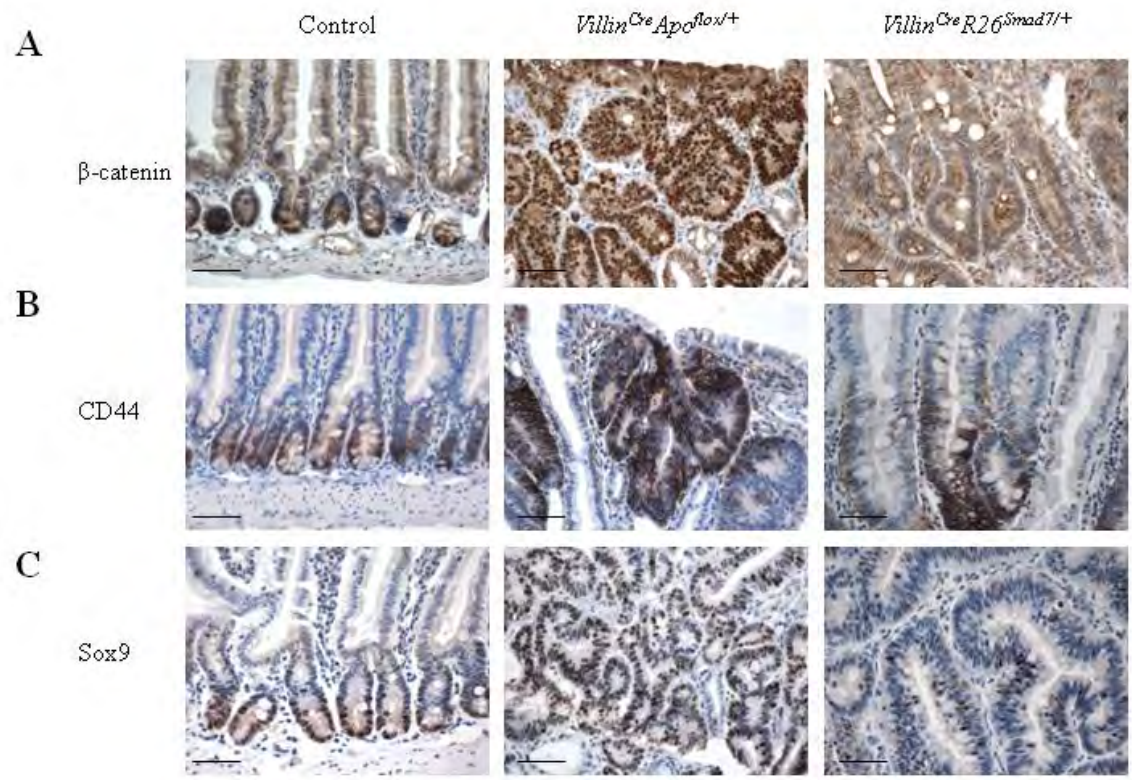


Figure A.5. Wnt pathway signaling is not affected in *Villin^{Cre}R26$^{Smad7/+}$* polyps *in vivo*.
(A) Subcellular localization of β-catenin is nuclear in *Villin^{Cre}Apc$^{flox/+}$* polyps but is membranous in *Villin^{Cre}R26$^{Smad7/+}$* polyps. *(B, C)* Canonical Wnt pathway targets CD44 *(B)* and Sox9 *(C)* are not elevated in *Villin^{Cre}R26$^{Smad7/+}$* polyps. Scale bar = 5 μM.

Villin^{Cre}*R26*$^{Smad7/+}$ polyps express markers specific to serrated polyps

It has recently been reported that human serrated polyps express a core set of signature markers, Vsig1 and ANXA10, specific to serrated polyps (Bae et al., 2015; Kim et al., 2015a). I obtained patient samples from the UMass Pathology Department for both traditional adenomas as well as serrated adenomas. I observed that the *Villin*^{Cre}*R26*$^{Smad7/+}$ polyps exhibit the characteristic "sawtooth" epithelial morphology, consistent with the human serrated polyps (Fleming et al., 2012; Makinen, 2007).

As expected, I found that the human serrated adenomas samples exhibited high expression for serrated polyp markers Vsig1 and ANXA10, whereas the traditional adenomas were negative for these markers *(Fig. A.6.B,C)*. Next, I wanted to investigate whether the *Villin*^{Cre}*R26*$^{Smad7/+}$ polyps exhibit the serrated polyp expression signature. I found that the APC-mutant adenomatous polyps in the *Villin*^{Cre}*Apc*$^{flox/+}$ animals were negative for Vsig1 and ANXA10 expression, but that the *Villin*^{Cre}*R26*$^{Smad7/+}$ polyps expressed Vsig1 and ANXA10 in the intestinal epithelia.

I then decided to investigate the transcriptional profile in the *Villin*^{Cre}*R26*$^{Smad7/+}$ polyps via microarray. I found 1683 differentially expressed transcripts in the *Villin*^{Cre}*R26*$^{Smad7/+}$ polyps as compared to control tissue *(Fig. A.7.A)*. Interestingly, I found that *Vsig1* was the highest expressed transcript, and *Anxa10* was one of the highest expressed transcripts *(Fig. A.7.B)*. Finally, I compared the *Villin*^{Cre}*R26*$^{Smad7/+}$ microarray data to two publically available datasets: GSE46513, a human serrated polyp RNAseq data set, and GSE43841, a human serrated polyp microarray data set *(Fig. A.7.A)*. I found a shared set of 46 genes that were common amongst the three data sets, including both

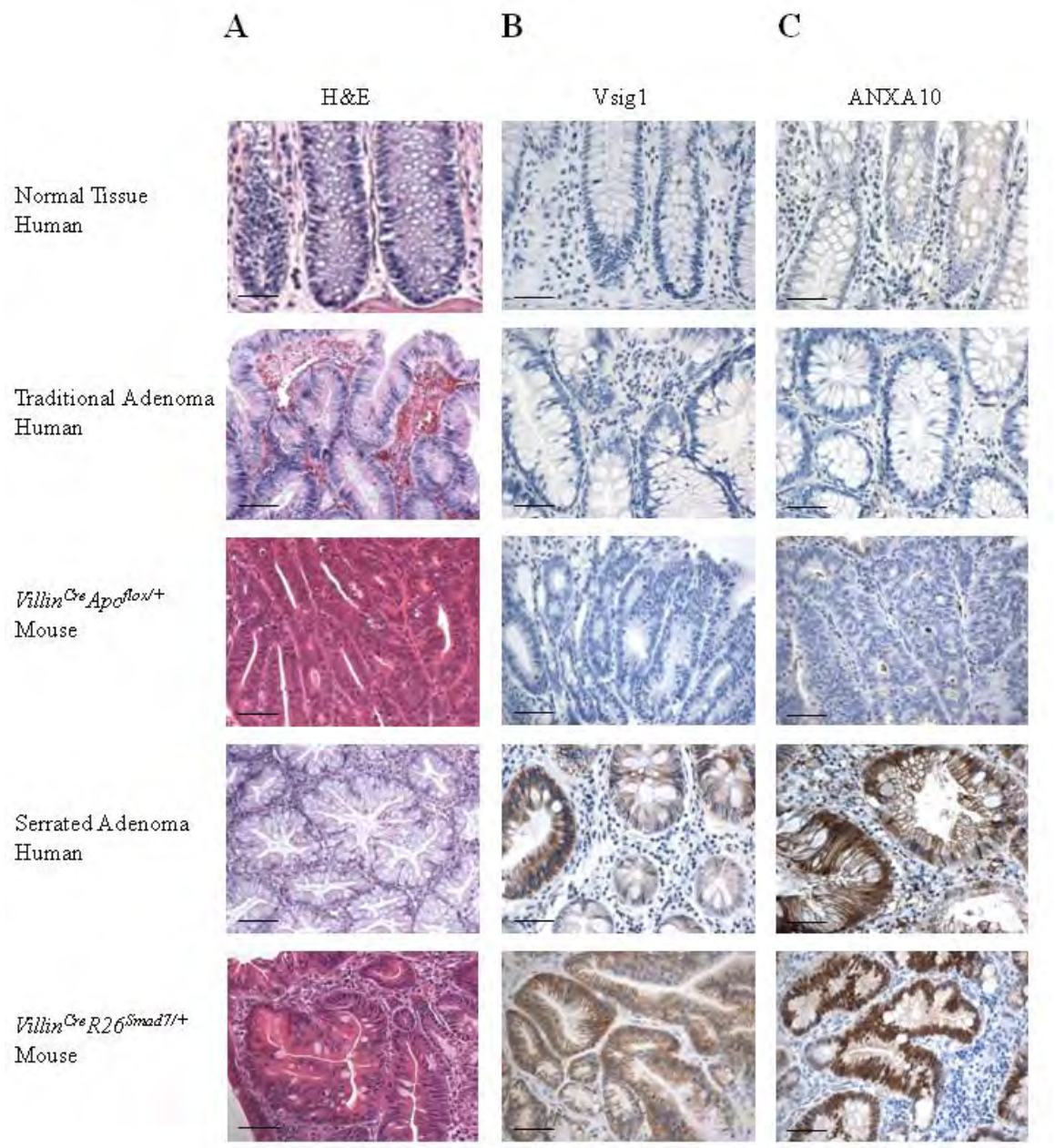
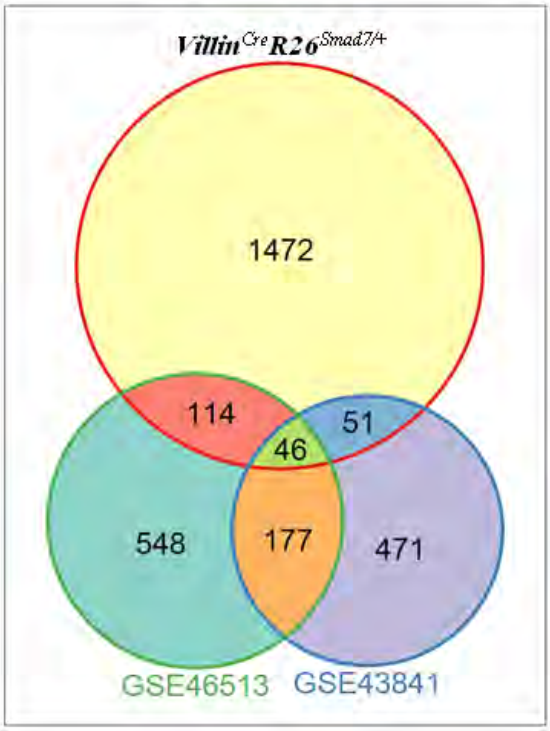


Figure A.6. SMAD7-driven polyps in mouse phenocopy human serrated polyps. *(A) Villin^{Cre}R26$^{Smad7/+}$* polyps exhibit histological characteristics of serrated polyps, not adenomatous polyps. *(B-C) Villin^{Cre}R26$^{Smad7/+}$* polyps exhibit upregulation of bona fide serrated polyposis markers Vsig1 and ANXA10. Scale bar = 10 μM.

A



B

Upregulated Genes	Fold Change
VSIG1	4.325
ANXA1	2.977
ANXA10	2.773
S100A6	2.398
IL1RN	2.291
CLDN2	1.763
SPINK4	1.528

Figure A.7. Gene expression of *Villin^{Cre}R26$^{Smad7/+}$* polyps overlaps with canonical serrated polyp signature markers.
(A) Comparison of gene expression. Affymetrix microarray data from the *Villin^{Cre}R26$^{Smad7/+}$* polyps compared to GSE46513, a human serrated polyp RNAseq data set, and GSE43841, a human serrated polyp microarray data set. *(B)* Canonical serrated polyp markers *Vsig1* and *AnxA10* are amongst the highest expressed genes in *Villin^{Cre}R26$^{Smad7/+}$* polyps.

Vsig1 and *AnxA10*. Therefore I concluded that the *Villin*^{Cre}*R26*$^{Smad7/+}$ polyps are both histologically consistent with human serrated adenomas and are robustly recapitulating the molecular serrated polyp expression *in vivo*.

Villin^{Cre}*R26*$^{Smad7/+}$ polyps are not regulating Hippo Pathway components YAP/TAZ

It has been reported that intestinal epithelia knockout of the Hippo Pathway component *Sav1* is sufficient to drive a serrated polyp histological phenotype in an *in vivo* mouse model (Cai et al., 2010). Additionally, we and others have shown that YAP/TAZ are critical during conditions of WNT pathway overactivation and that genetic knockout of both YAP/TAZ in an APC-mutant background is sufficient to prevent polyp formation (Azzolin et al., 2014; Cai et al., 2015; Gregorieff et al., 2015; Zanconato et al., 2015). Finally, SMAD7 has been shown to interact with YAP through direct binding (Ferrigno et al., 2002). Therefore, I decided to investigate whether YAP/TAZ are also required for SMAD7-driven serrated polyp formation.

I first investigated overall YAP protein expression in the *Villin*^{Cre}*R26*$^{Smad7/+}$ polyps. I observed that the *Villin*^{Cre}*Apc*$^{flox/+}$ polyps exhibited elevated nuclear YAP levels, whereas *Villin*^{Cre}*R26*$^{Smad7/+}$ polyps did not exhibit elevated nuclear YAP localization. I next decided to use mouse genetics to investigate the requirement for YAP/TAZ in *Villin*^{Cre}*R26*$^{Smad7/+}$ mutant polyps. I generated *Villin*^{Cre}*R26*$^{Smad7/+}$*Yap*$^{flox/flox}$*Taz*$^{flox/flox}$ animals and aged them to 14 months. In an *Apc*-mutant background, I observed that genetic ablation of both *Yap* and *Taz* was sufficient to inhibit polyposis. However, I found no change in overall lifespan of animals *(Fig. A.8.A)* as compared to

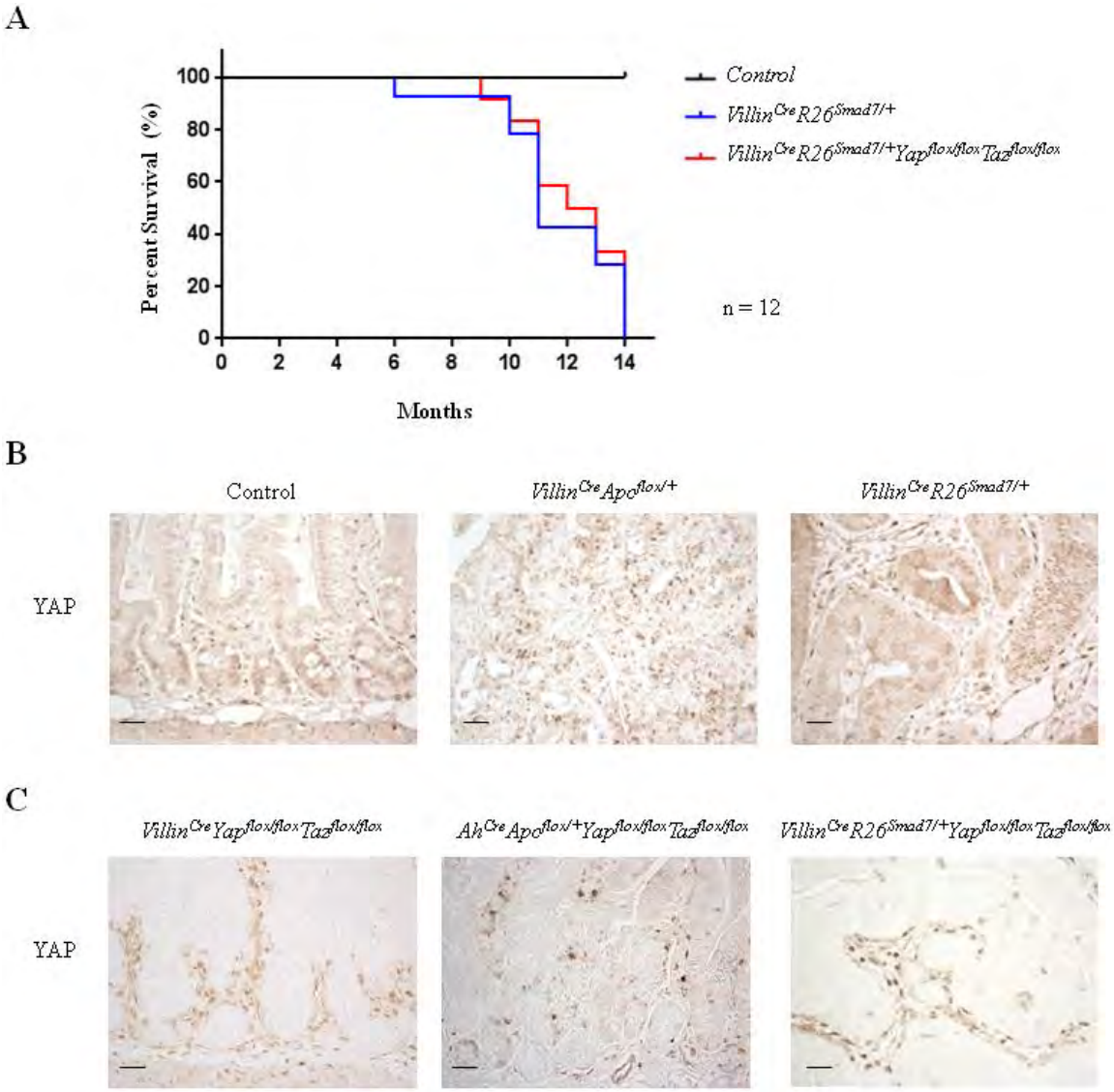


Figure A.8. YAP/TAZ are not required for SMAD7-driven serrated polyposis. *(A)* Survival curve of *Villin*^{Cre}*R26*$^{Smad7/+}$ and *Villin*^{Cre}*R26*$^{Smad7/+}$*Yap*$^{flox/flox}$ *Taz*$^{flox/flox}$ animals as compared to controls. *(B)* YAP protein is predominantly nuclear in *Villin*^{Cre}*Apc*$^{flox/+}$ polyps, whereas YAP is predominantly cytoplasmic in *VillinCre R26*$^{Smad7/+}$ polyps *in vivo*. *(C)* Genetic knockout of both *Yap* and *Taz* is insufficient to inhibit SMAD7-driven serrated polyposis. Scale bar = 5.0 μM.

Villin^{Cre}*R26*$^{Smad7/+}$ animals, despite robust knockout of YAP/TAZ *(Fig. A.8.C)*. This leads me to conclude that YAP/TAZ are dispensable for SMAD7-driven serrated polyposis in the intestinal epithelia.

Conclusions

This is the first study to link SMAD7 to serrated polyposis. Furthermore, this work also represents the first mouse model that robustly recapitulates human serrated polyposis both histologically and molecularly through expression of a bona fide serrated polyposis molecular signature. This work also provides evidence that SMAD7 expression drives the growth of serrated polyps independently of either Wnt pathway activation or YAP/TAZ signaling, uncovering a novel genetic mechanism for serrated polyposis through SMAD7 activation. The mouse model presented in this work will be hugely beneficial for future studies investigating the molecular basis for serrated polyposis, and will hopefully provide clinical benefit from the knowledge gained.

Materials and Methods

Mouse Genetics

The $Villin^{Cre}$, Ah^{Cre}, and Apc^{flox} alleles were obtained from the Jackson Laboratory. The Yap^{flox} and Taz^{flox} alleles were a gift from Drs. WE Zimmer, OJ Sansom, DJ Winton, and EN Olson. To generate the $R26^{Smad7}$ allele, the *Smad7* cDNA was cloned into the pROSA targeting vector. The construct was electroporated into mouse ES cells for blastocyst injection and chimeric animals were generated. To target the intestinal epithelia, $Villin^{Cre}$ or Ah^{Cre} was crossed to $R26^{Smad7}$, Apc^{flox}, Yap^{flox}, and Taz^{flox} alleles. Ah^{Cre} expression was induced at postnatal day 30 through intraperitoneal injection of 80 mg/kg β-napthoflavone.

Tissue Collection and Histology

Mice were dissected and tissue was fixed in 10% neutral buffered formalin overnight at 4°C. Tissues were dehydrated in ethanol, embedded in paraffin, and sectioned at 6 μm for paraffin sections. Standard hematoxylin & eosin reagents were used to stain paraffin sections. For frozen sections, tissues were dehydrated in sucrose, embedded in OCT media, and sectioned at 12 μm.

Human clinical samples

Clinical samples representing normal colon tissue, adenomatous polyps, and serrated polyps were provided by the UMass Medical School Department of Pathology.

Histological analyses of mouse tissues were conducted by Dr. Zhong Jiang, Department of Pathology, UMass Medical School.

Immunohistochemistry

For immunohistochemistry (IHC), paraffin sections were deparaffinized and antigen retrieval was performed using heat-induced epitope retrieval in 10mM sodium citrate buffer, pH 6.0. Slides were blocked for endogenous peroxidase in a 3% H_2O_2-MeOH solution before being blocked for 1 hour in a 5% BSA, 1% goat serum, 0.5% Tween-20 solution. Slides were incubated in primary antibody diluted in either blocking buffer or SignalStain® Antibody Diluent (Cell Signaling) overnight at 4°C. Slides were incubated in biotinylated secondary antibody for 1 hour and then signal was amplified and detected using the Vectastain Elite ABC kit and the DAB Peroxidase Substrate kit (Vector Laboratories). Primary antibodies used for IHC were: β-galactosidase (1:2,000, Abcam), β-catenin (1:500, BD Biosciences), CD44 (1:400, eBioscience), Sox9 (1:200, Abcam), Vsig1 (1:100, Novus Biologicals), ANXA10 (1:100, Novus Biologicals), and YAP (1:200, Cell Signaling).

Cell Culture

FET and IEC6 cells were obtained from ATCC. FET cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and IEC6 cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and 0.1 Unit/mL insulin.

To generate the *pGIPZ2a-Smad7* plasmid, *Smad7* cDNA was cloned and ligated into the *pGIPZ2a* plasmid. For anchorage-independent growth assay, HCT116 cells were grown in soft agar and colonies were counted. For foci formation assay, IEC6 cells were seeded and grown for 3 weeks, after which point foci colonies were counted. For luciferase assay, either *pGIPZ2a* empty vector, *pGIPZ2a-Smad7,* or *pGIPZ2a-dnTcf4* were transfected along with TCF/LEF reporter constructs, and luciferase activity monitored per Dual-Luciferase Reporter Assay System manufacturer's protocol (Promega).

Western Blotting

Cells were lysed and probed for the following primary antibodies: β-catenin (1:1,000, BD Biosciences), β-actin (1:1000, Genescript), axin2 (1:1000, Abcam), c-myc (1:1000, Abcam), GAPDH (1:1000, CST). Secondary antibodies (1:5000) were purchased from Jackson Laboratories.

Quantitative Real-Time PCR

RNA was isolated from cell lines using Trizol and was reverse-transcribed into DNA using the SuperscriptII kit (Invitrogen). Quantitative real-time PCR was performed using SYBR MasterMix (Kapa Biosystems). Primers for real-time PCR were:

Axin2 (rat): Forward: 5′-TGGTGCATACCTCTTCCGGACTTT-3′

Reverse- 5′-TTTCCTCCATCACCGCCTGAATCT-3′

GAPDH (rat): Forward: 5'-GGCAAGTTCAATGGCACAGT-3'

Reverse: 5'-TGGTGAAGACGCCAGTAGACTC-3'.

Affymetrix Gene Chip Transcriptional Profiling

Intestinal polyps from *Villin*^{Cre}*R26*$^{Smad7/+}$ animals were carefully dissected and RNA was isolated using Trizol reagent. For Affymetrix Gene Chip analysis, RNA was labeled and hybridized to Mouse Gene 1.0ST chips according to Affymetrix manufacturer protocols. Independent biological triplicates were used for chip analysis and data was analyzed using the statistical language R. Genes with a p value <0.05 and a fold change > 1.0 were identified for further analysis. The *Villin*^{Cre}*R26*$^{Smad7/+}$ transcriptional profile was then compared against publically available data sets for human serrated polyposis (Accession numbers GSE46513 and GSE43841) to identify a common shared gene expression signature.

APPENDIX B

Genetically distinct GI hamartomatous polyps arise from a common mesenchymal progenitor cell

Preface

The work presented in this Appendix, including all mouse models, is exclusively my own work at the University of Massachusetts in the lab of Dr. Junhao Mao.

These data are unpublished. Further experiments are needed to complete this manuscript. Therefore the results presented in Appendix B represent my current interpretation of the data based on the experiments I have performed. Additional data and conclusions will be performed and will be added in the published version.

Abstract

Gastrointestinal hamartomatous polyposis syndromes have been shown to confer an elevated risk for colorectal cancer in patients, but the exact mechanism of polyp initiation remains unclear. I report here the first evidence linking three genetically distinct gastrointestinal hamartomatous polyposis syndromes together through a shared mesenchymal cell of origin. I show that despite different initiating mutations and downstream signaling, Peutz Jeghers Syndrome, Juvenile Polyposis, and PTEN Hamartoma Syndrome/Cowden's Syndrome polyps all arise from a gastrointestinal myofibroblast cell. Furthermore, I report the $Gli1^{CreER}Pten^{flox/flox}$ mouse as the first mouse model for PTEN Hamartoma Syndrome with gastrointestinal polyposis and the $Gli1^{CreER}R26^{Smad7/+}$ mouse as the first mouse model using SMAD7 expression to model Juvenile Polyposis Syndrome. I also use mouse genetics to show that in Peutz Jeghers Syndrome polyps, mTOR pathway signaling is not required, but that YAP/TAZ are required. Overall this study describes a number of novel mouse models that recapitulate human gastrointestinal polyposis syndromes, identifies the gastrointestinal myofibroblast as a critical cell type in polyposis, and begins to elucidate the critical downstream signaling required for polyp growth.

Introduction

Gastrointestinal hamartomatous polyposis syndromes are rare, inherited genetic disorders that cause multiple hamartomatous polyps to grow in the gastrointestinal tract. Gastrointestinal hamartomatous polyps are generally comprised of fully differentiated cells native to the gastrointestinal tract, with a prominent mesenchymal contribution and a highly disorganized architecture. Patients with GI hamartomatous polyposis syndromes have an elevated risk of developing both colorectal cancer as well as other epithelial cancers.

Juvenile Polyposis Syndrome (JPS) is a gastrointestinal hamartomatous polyposis syndrome affecting approximately 1 in 160,000 people and linked to mutations in TGFβ/BMP pathway signaling (Chow and Macrae, 2005). JPS patients can have upwards of 50-200 polyps in their gastrointestinal tract, which are histologically characterized as being comprised of mucus-filled cystic lesions and a prominent stromal contribution. JPS patients have an approximately 50% chance of developing gastrointestinal cancer (Howe et al., 1998). Mechanistically, 40% of JPS patients carry an inherited mutation in either SMAD4 or BMPR1A (Merg and Howe, 2004). However, 60% of JPS patients have no identified genetic mutation, indicating that additional research is needed to uncover initiating mutations for this inherited syndrome.

PTEN Hamartoma Syndromes is a family of rare inherited genetic disorders caused by mutations in the tumor suppressor *Pten*. The most common of the PTEN Hamartoma Syndromes is Cowden's Syndrome, and affects approximately 1 in 250,000

people. Cowden's Syndrome patients have up to a 24% risk of developing colorectal cancer in their lifetime (Riegert-Johnson et al., 2010). Histologically, Cowden's Syndrome polyps usually arise in the colon, rarely have mucus-filled cystic lesions, and commonly have lymphoid follicles present (Shaco-Levy et al., 2016).

Peutz-Jeghers Syndrome (PJS) is a rare inherited autosomal dominant disorder affecting approximately 1 in 120,000 people and is characterized by the growth of multiple benign hamartomatous gastrointestinal polyps throughout the gastrointestinal tract, primarily in the small intestine (Jenne et al., 1998; McGarrity et al., 2000; Riegert-Johnson et al., 2009). Almost all Peutz Jeghers patients carry a mutation in the *Lkb1/Stk11* gene, a serine/threonine kinase known to phosphorylate and activate AMP-activated protein kinase (AMPK) (Hawley et al., 2003; Hemminki et al., 1998; Jenne et al., 1998; Shaw et al., 2004). Peutz Jeghers patients have a 93% chance of malignant transformation by the age of 65 and a 48% chance of dying from cancer by age 57 (Giardiello et al., 1987; Hearle et al., 2006; Spigelman et al., 1989).

PJS intestinal polyps are characterized by the hyperproliferation of disorganized yet fully differentiated cells, a prominent stromal contribution and a smooth muscle core (Jishage et al., 2002). Heterozygous *Lkb1* knockout in mice phenocopies human Peutz Jeghers Syndrome polyps (Bardeesy et al., 2002; Jishage et al., 2002; Miyoshi et al., 2002). Recently it was shown that homozygous loss of *Lkb1* in the intestinal epithelia does not initiate polyposis (Shorning et al., 2009), while heterozygous loss of *Lkb1* specifically in the smooth muscle compartment is sufficient to initiate PJS polyps (Katajisto et al., 2008) with a prominent contribution from the myofibroblast population.

These data suggest the myofibroblast population in the mesenchyme is of key importance in the initiation of PJS polyposis.

Even with the identification of *Lkb1* as the commonly mutated gene in Peutz Jeghers Syndrome, the exact downstream mechanism through which *Lkb1* loss leads to polyposis and tumorigenesis has been poorly characterized. It has been hypothesized that the LKB1-mediated mechanism disrupted in PJS patients is facilitated by the aberrant activation of mTORC1, which is downstream of AMPK (Shackelford et al., 2009). There are conflicting reports as to whether mTORC1 activity, as assayed through levels of pS6, is impacted in $Lkb1^{+/-}$ polyps and whether rapamycin, a partial mTORC1 inhibitor, can reduce the polyp burden in $Lkb1^{+/-}$ mice (Katajisto et al., 2008; Robinson et al., 2009; Wei et al., 2008). Additionally, there have been recent reports showing that LKB1 can interact with the Hippo Pathway transcriptional co-activator YAP *in vitro* and that YAP/TAZ are elevated in human Peutz Jeghers polyp samples (Mohseni et al., 2014). However, there is no genetic evidence to conclusively implicate either mTOR or YAP/TAZ as the downstream targets through which loss of *Lkb1* leads to polyp initiation in Peutz Jeghers Syndrome.

The role of the tumor stroma and disruption of homeostatic mesenchymal-epithelial signaling in tumorigenesis has only recently begun to be appreciated as a contributing factor to the initiation and progression of cancer (Ishiguro et al., 2006; Nakamura et al., 1997). The tumor stroma, which is composed of fibroblasts, myofibroblasts, inflammatory cells, along with extracellular matrix (ECM) proteins, is known to secrete various growth factors that stimulate cancer cell proliferation

(Shackelford et al., 2009; Ylikorkala et al., 2001). Given the prominent stromal contribution in GI hamartomatous polyps and patient predisposition to carcinomas, it can be hypothesized that the disruption of mesenchymal-epithelial signaling is a critical aspect of tumorigenesis in GI hamartomatous polyps. However, both the exact cell of origin as well as critical downstream signaling required for GI hamartomatous polyp initiation and growth remained poorly characterized.

Results and Discussion

Gli1^{+} mesenchymal cells are gastrointestinal myofibroblasts.

I first wanted to restrict Cre-Lox recombination specifically to the gastrointestinal mesenchymal compartment. To do this, I decided to use the *Gli1*^{CreER} allele. *Gli1* is expressed in a subpopulation of gastrointestinal mesenchymal cells during development and postnatal stages following induction via tamoxifen injection (Mao et al., 2010; Park et al., 2000). To induce expression, I first crossed *Gli1*^{CreER} to *R26*^{LacZ}, a nuclear-LacZ reporter construct. I then injected *Gli1*^{CreER}*R26*$^{LacZ/+}$ mice at postnatal day 30 (P30) with 120 mg/kg tamoxifen. Consistent with the previously published reports, I observed prominent nuclear LacZ staining in a subpopulation of mesenchymal cells in the gastrointestinal mesenchyme in both the intestine and colon when animals were dissected two weeks later *(Fig. B.1.A)* (Kolterud et al., 2009).

I then needed to determine the identity of the *Gli1*$^{+}$ mesenchymal cell. I decided to use immunofluorescence and double-staining to determine markers expressed by the Gli1^{+} cells. I found that in both the intestine and colon, the β-gal^{+} cells (marking the Gli1^{+} population) were also positive for α-Smooth Muscle Actin (αSMA) but negative for Desmin *(Fig. B.1.B,C)*. In the gastrointestinal mesenchyme, the myofibroblast population is characterized as being αSMA^{+}/Desmin^{-} (Mifflin et al., 2011; Pinchuk et al., 2010).

These data corroborate findings published by others. Members of the Gumucio lab showed that *Gli1*$^{+}$ cells are amongst the α-smooth muscle actin^{+}/desmin^{-} myofibroblast population in the intestine (Kolterud et al., 2009). More recently,

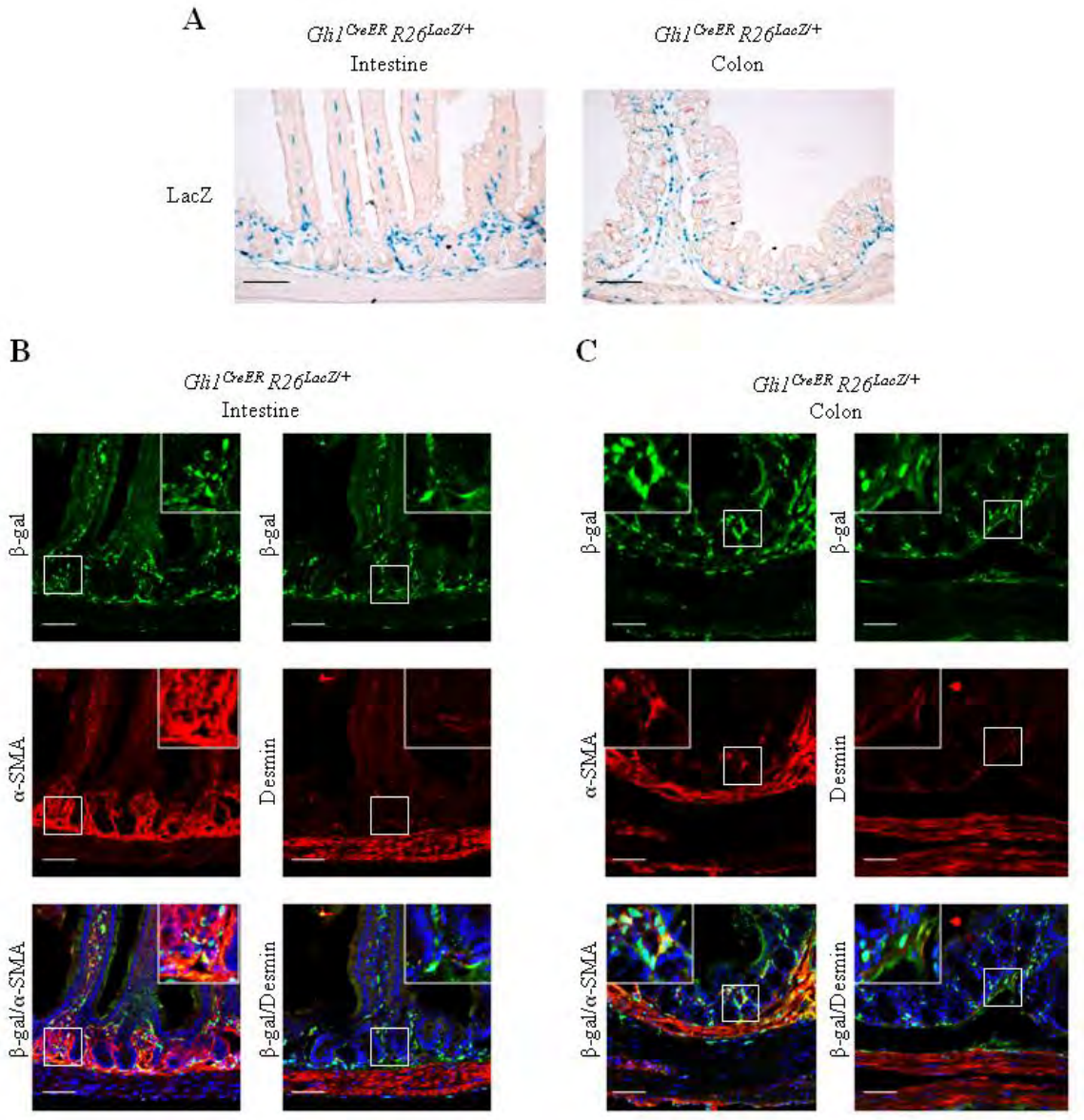


Figure B.1. Gli1^{+} mesenchymal cells are gastrointestinal myofibroblasts.
(A) LacZ staining in the intestine and colon of *Gli1*^{CreER}*R26*$^{lacZ/+}$ animals following P30 tamoxifen injection. *(B, C)* Intestine and colon tissue from *Gli1*^{CreER}*R26*$^{lacZ/+}$ animals probed for β-galactosidase, α-Smooth Muscle Actin (α-SMA), and Desmin. Scale bar = 50 μM.

Humphreys and colleagues showed that in a number of organs, Gli1 is a marker for a mesenchymal stem cell population that differentiates into myofibroblast cells following injury (Kramann et al., 2015). Therefore, I concluded that in my own system, the *Gli1*^{CreER} allele is restricting recombination to a gastrointestinal myofibroblast population.

SMAD7 expression in Gli1^{+} myofibroblasts promotes hamartomatous polyps that phenotype Juvenile Polyposis Syndrome.

I then decided to investigate if perturbing TGFβ/BMP pathway signaling in the gastrointestinal mesenchyme was sufficient to initiate polyp growth. To do this, I used the *R26*$^{Smad7/+}$ allele that our lab recently generated (see **Appendix A** for additional details) and again used the *Gli1*^{CreER} driver allele. I induced Cre-mediated recombination at postnatal day 10 (P10) by tamoxifen injection. I found that *Gli1*^{CreER}*R26*$^{Smad7/+}$ animals became moribund and died by 5 months of age, possibly due to obstruction caused by a large polyp at the pylorus *(Fig. B.2.A,B)*. *Gli1*^{CreER}*R26*$^{Smad7/+}$ mice also developed colon polyps with cystic lesions and a prominent stromal contribution, reminiscent of the colon polyps that develop in patients with Juvenile Polyposis Syndrome (JPS) *(Fig. B.2.C)*.

This observation provides further evidence that aberrant TGFβ/BMP pathway signaling is linked to Juvenile Polyposis. Previous labs have shown that whole body heterozygous *Smad4* knockout generates gastrointestinal polyps that phenocopy Juvenile Polyposis Syndrome, but this study definitively links JPS to a myofibroblast cell population (Sirard et al., 1998; Takaku et al., 1999; Xu et al., 2000). Additionally, it has been shown that 40% of all identified mutations in JPS are linked to mutations in

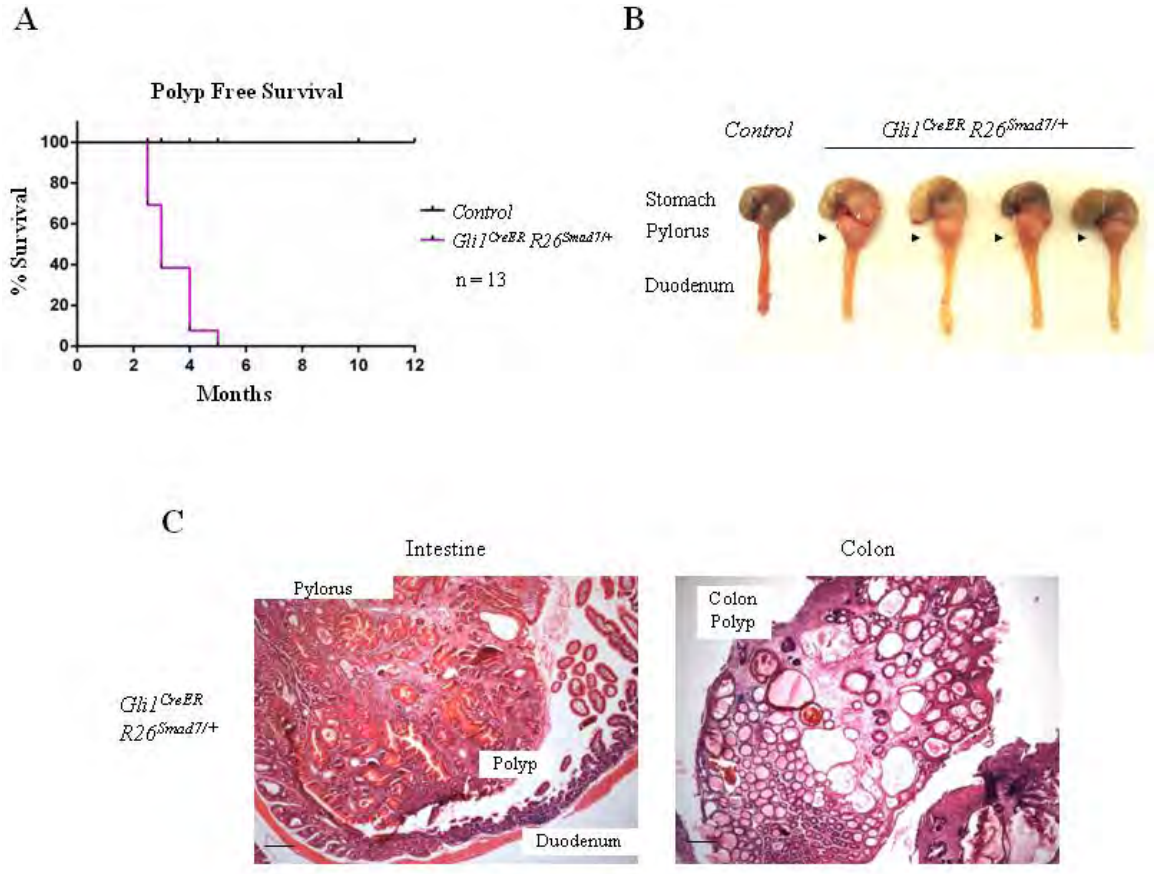


Figure B.2. SMAD7 expression in Gli1^{+} myofibroblasts promotes hamartomatous polyps that phenotype Juvenile Polyposis Syndrome.
(A) Survival of *Gli1^{CreER}R26$^{Smad7/+}$* animals injected with tamoxifen at postnatal day 10 (P10) as compared to littermate controls. *(B, C) Gli1^{CreER}R26$^{Smad7/+}$* animals injected with tamoxifen at P10 develop polyps at the pylorus between the stomach and duodenum, as well as in the colon. *Gli1^{CreER}R26$^{Smad7/+}$* colon polyps display cystic lesions and prominent mesenchymal contribution, characteristic of the gastrointestinal hamartomatous polyps in Juvenile Polyposis Syndrome (JPS). Scale Bar = 50 μM.

SMAD4 or BMPR1A; this data suggests that mutations in SMAD7 might also be involved in JPS as well.

PTEN knockout in Gli1^{+} myofibroblasts promotes hamartomatous polyps that phenotype PTEN Hamartoma Syndrome/Cowden's Syndrome.

I also wanted to investigate whether homozygous PTEN knockout in the gastrointestinal myofibroblast population is sufficient to initiate polyposis reminiscent to PTEN Hamartoma Syndrome/Cowden's Syndrome. I again used the *Gli1*^{CreER} allele and crossed it to *Pten*^{flox} mice to generate *Gli1*^{CreER}*Pten*$^{flox/flox}$ animals. I induced Cre recombination via tamoxifen injection at postnatal day 30 (P30). I observed that *Gli1*^{CreER}*Pten*$^{flox/flox}$ animals became moribund and died by 4 months of age *(Fig. B.3.A)*. Upon dissection, I observed numerous polyps in the colons of *Gli1*^{CreER}*Pten*$^{flox/flox}$ animals, with the greatest numbers present at the distal colon, although small polyps were present in the intestine as well *(Fig. B.3.B,C)*.

I observed lymphoid follicles were present in the majority of the *Gli1*^{CreER}*Pten*$^{flox/flox}$ colon polyps, but never observed them in the *Gli1*^{CreER}*Lkb1*$^{flox/+}$ or the *Gli1*^{CreER}*R26*$^{Smad7/+}$ polyps *(Fig. B.3.C,D)*. Lymphoid follicles are significantly more common in Cowden's Syndrome polyps than in other gastrointestinal hamartomatous polyps (Shaco-Levy et al., 2016). Even more confounding is that the lymphoid follicles appear to be αSMA^{-}/Desmin^{+}, providing evidence that the follicles are not densely populated regions of Gli1^{+} myofibroblasts. However, fibroblastic reticular cell networks in lymphoid follicles, which are comprised of both stromal and hematopoietic cells, have

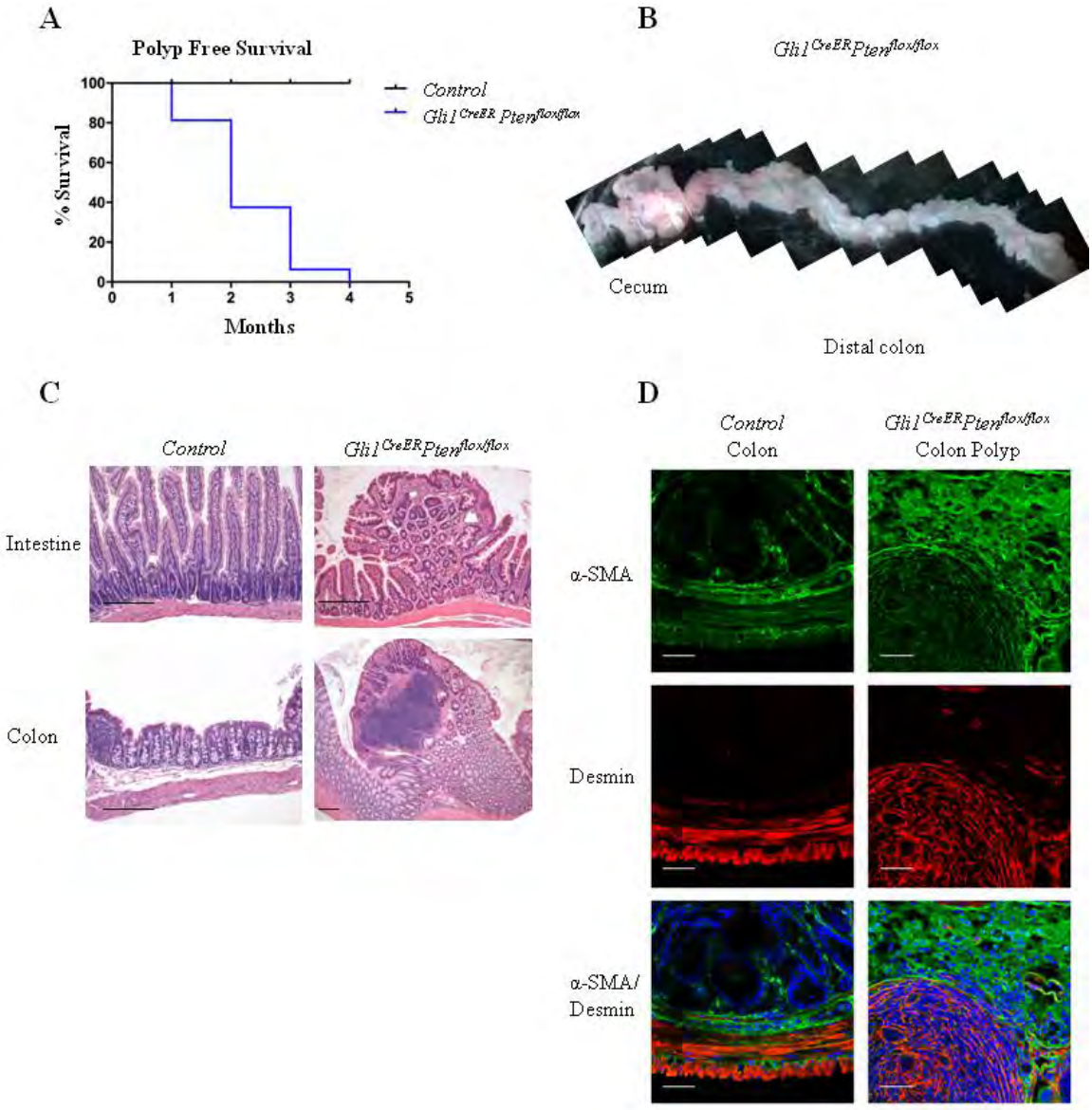


Figure B.3. PTEN knockout in Gli1^{+} myofibroblasts promotes hamartomatous polyps that phenotype PTEN Hamartoma Syndrome/Cowden's Syndrome.
(A) Survival of *Gli1^{CreER}Pten$^{flox/flox}$* animals injected with tamoxifen at postnatal day 30 (P30) as compared to controls. *(B)* Numerous polyps are observed in the colon of *Gli1^{CreER}Pten$^{flox/flox}$* animals. *(C,D)* *Gli1^{CreER}Pten$^{flox/flox}$* mice develop polyps in both the intestine and colon, with lympoid follicles characteristic of Cowden's Syndrome polyps. Scale Bar = 50 μM.

been shown to be αSMA^{-}/Desmin^{+} (Malhotra et al., 2013). From these data, I conclude that the *Gli1*^{CreER}*Pten*$^{flox/flox}$ mouse robustly recapitulates Cowden's Syndrome polyps, and represents the first mouse model for this gastrointestinal hamartomatous polyposis syndrome.

Heterozygous loss of *Lkb1* in Gli1^{+} myofibroblasts promotes hamartomatous polyps that phenotype Peutz Jeghers Syndrome.

I next used the *Gli1*^{CreER} to knock out a single copy of *Lkb1* in the gastrointestinal myofibroblast cell population. I induced Cre recombination through injection of 120 mg/kg tamoxifen at P30, and found that all *Gli1*^{CreER}*Lkb1*$^{flox/+}$ mice developed polyps by 12 months *(Fig. B.4.A).* Additionally, I observed that all LKB1-mutant polyps arose at the pylorus between the stomach and duodenum, and they were histologically consistent with human Peutz Jeghers Syndrome polyps *(Fig. B.4.B)*.

This work is consistent with previously published mouse models of Peutz Jeghers' Syndrome. Like the mouse models with whole body heterozygous knockout of *Lkb1*, the *Gli1*^{CreER}*Lkb1*$^{flox/+}$ mice developed polyps at the pylorus and had an overall survival of approximately 12 months (Bardeesy et al., 2002; Jishage et al., 2002; Miyoshi et al., 2002). Additionally, this work corroborates a previous study that showed that genetic ablation of *Lkb1* in a smooth muscle cell compartment, marked by expression of SM22α, was sufficient to initiate polyposis (Katajisto et al., 2008). Our work adds to this group's findings by showing that the myofibroblast population is sufficient to give rise to Peutz Jeghers polyps.

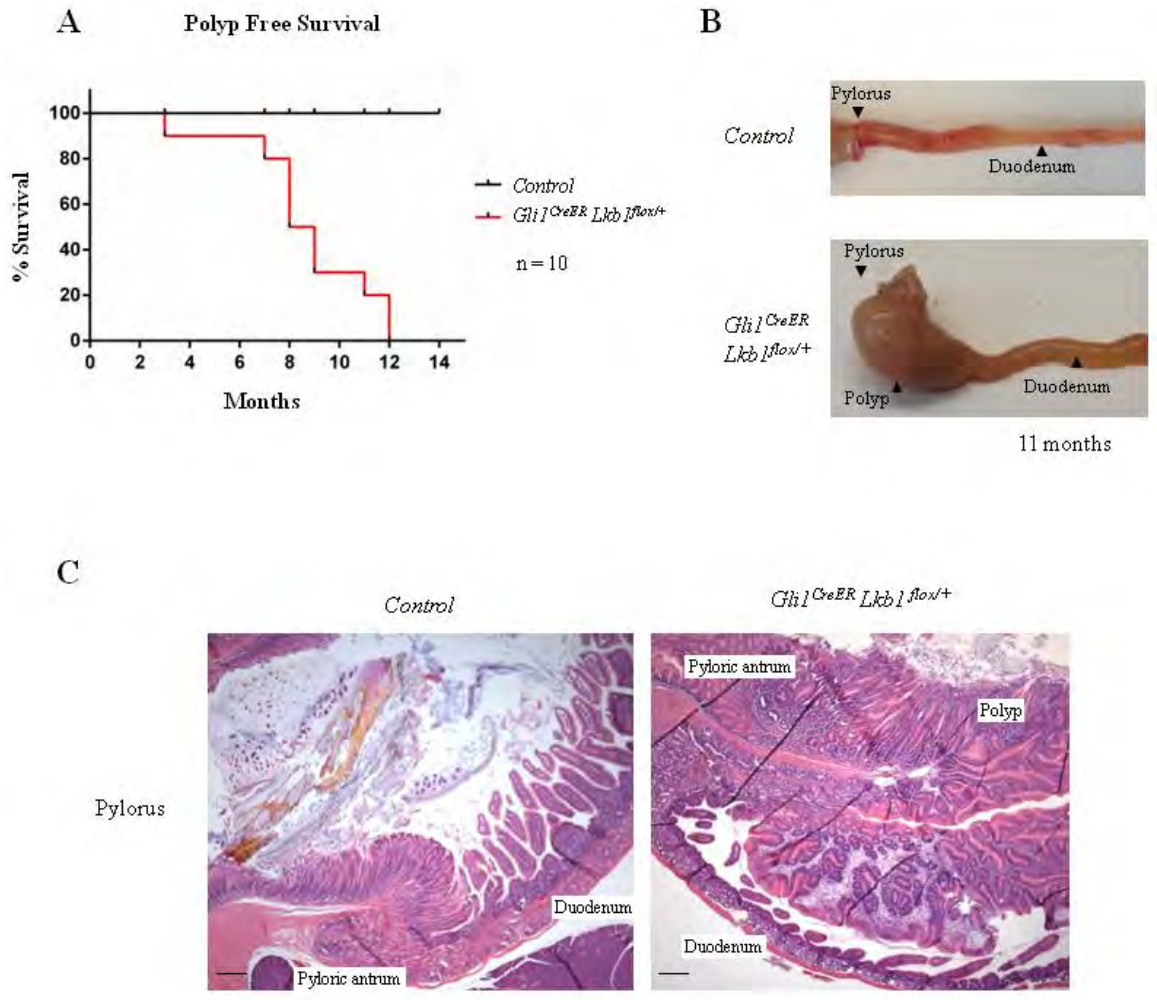


Figure B.4. Heterozygous loss of *Lkb1* in Gli1^{+} myofibroblasts promotes hamartomatous polyps that phenotype Peutz Jeghers' Syndrome.
(A) Polyp free survival in *Gli1*^{CreER}*Lkb1*$^{flox/+}$ animals as compared to controls following P30 tamoxifen injection. *(B)* *Gli1*^{CreER}*Lkb1*$^{flox/+}$ animals develop polyps at the pylorus between the stomach and duodenum. *(C)* Polyps in *Gli1*^{CreER}*Lkb1*$^{flox/+}$ animals exhibit a mesenchymal compartment and smooth muscle core, reminiscent of human Peutz Jeghers' Syndrome (PJS) polyps. Scale Bar = 50 μM.

PTEN mutant polyps but not LKB1-mutant or SMAD7-mutant polyps exhibit elevated mTOR pathway signaling.

I next decided to investigate mTOR pathway signaling in the three gastrointestinal hamartomatous polyposis mouse models. Via immunohistochemistry, I observed that the mesenchymal compartment as well as the epithelia of the *Gli1*^{CreER}*Pten*$^{flox/flox}$ polyps exhibited pS6 and pAktS473 staining. However, both the *Gli1*^{CreER}*Lkb1*$^{flox/+}$ and the *Gli1*^{CreER}*R26*$^{Smad7/+}$ polyps only displayed pS6 and pAktS473 staining in the epithelia, not the mesenchyme *(Fig. B.5.A,B)*. Further data supporting this initial observation came from the genetic ablation of mTOR in the PTEN-deficient background; *Gli1*^{CreER}*Pten*$^{flox/flox}$*mtor*$^{flox/flox}$ animals had a higher survival rate than *Gli1*^{CreER}*Pten*$^{flox/flox}$ animals *(Fig. B.5.C)*. This data indicated that in the gastrointestinal myofibroblast population, only PTEN knockout is sufficient to activate mTOR pathway signaling in hamartomatous polyps.

LKB1-mutant polyps do not require mTOR pathway signaling for polyp initiation and growth.

I shifted my focus back to the LKB1-mutant polyps to try to better understand the molecular mechanism through which *Lkb1* heterozygous knockout drives polyposis. At the time when this work began, the hypothesis in the field was that Peutz Jeghers Syndrome polyps arise through activation of mTOR due to loss of upstream LKB1/AMPK activity. Therefore, I hypothesized that if the polyposis mechanism in *Gli1*^{CreER}*Lkb1*$^{flox/+}$ polyps is through signaling from LKB1 to AMPK1/AMPK2 to inhibit

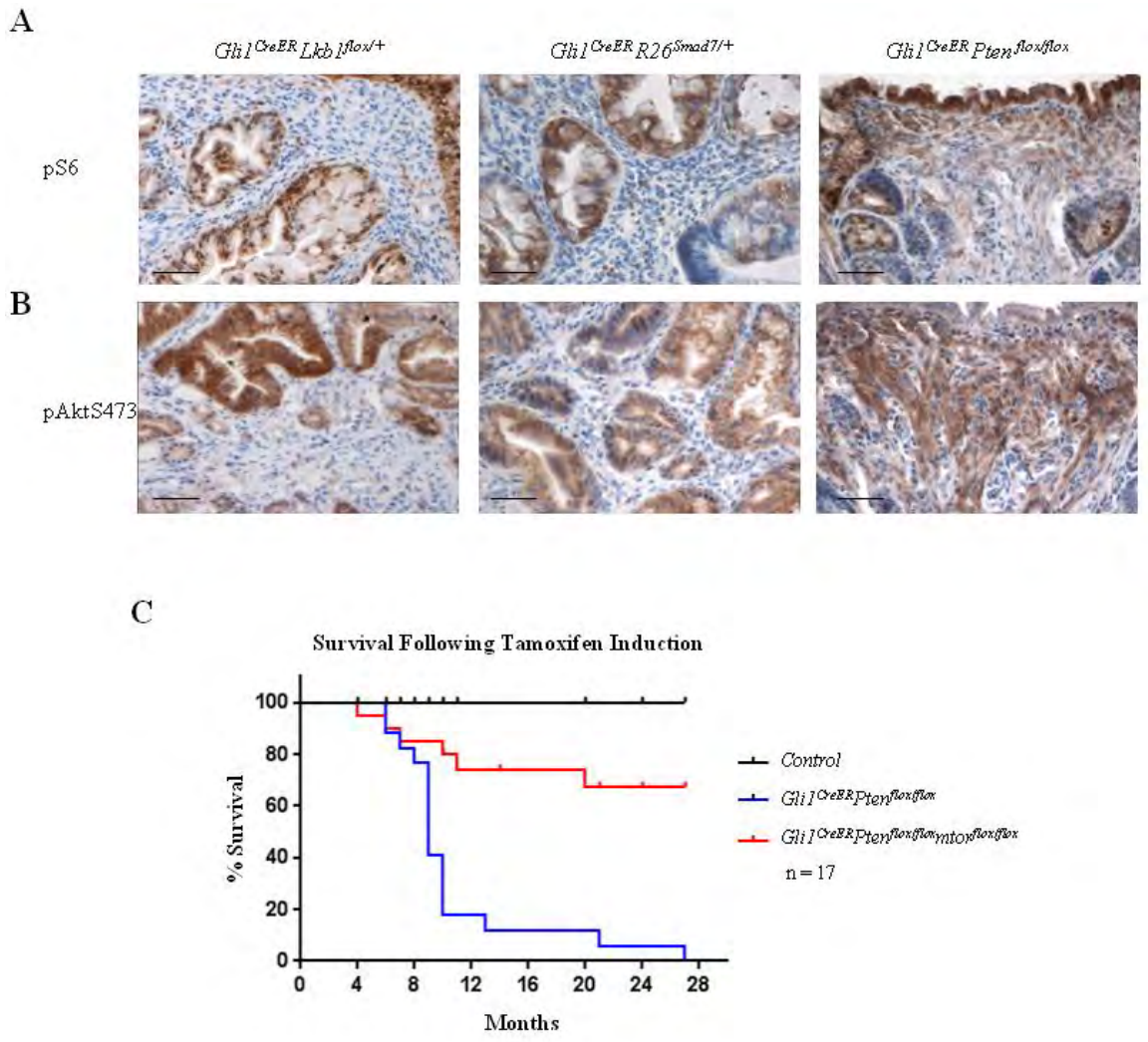


Figure B.5. PTEN mutant polyps but not LKB1-mutant or SMAD7-mutant polyps exhibit elevated mTOR pathway signaling.
(A,B) Immunohistochemical analysis of pS6 and pAkt-S473 expression in *Gli1*^{CreER}*Lkb1*$^{flox/+}$, *Gli1*^{CreER}*R26*$^{Smad7/+}$, and *Gli1*^{CreER}*Pten*$^{flox/flox}$ polyps. *(C)* Overall survival of *Gli1*^{CreER}*Pten*$^{flox/flox}$*mtor*$^{flox/flox}$ animals as compared to *Gli1*^{CreER}*Pten*$^{flox/flox}$ animals. Scale Bar = 10 μM.

mTOR, then the *Gli1*^{CreER}*Ampk1*$^{flox/flox}$*Ampk2*$^{flox/flox}$ phenotype should recapitulate the *Gli1*^{CreER}*Lkb1*$^{flox/+}$ polyposis phenotype.

I started by knocking out both *Ampk1* and *Ampk2* in the Gli1^{+} myofibroblast population. I found that after tamoxifen injection, *Gli1*^{CreER}*Ampk1*$^{flox/flox}$*Ampk2*$^{flox/flox}$ had no difference in overall survival as compared to control mice *(Fig. B.6.A)*. Furthermore, *Gli1*^{CreER}*Ampk1*$^{flox/flox}$*Ampk2*$^{flox/flox}$ mice never developed polyps in the gastrointestinal tract *(Fig. B.6.B).*

To further investigate the relationship between LKB1 and mTOR in the gastrointestinal mesenchyme, I next crossed the *Gli1*^{CreER}*Lkb1*$^{flox/+}$ to *mtor*^{flox} alleles to generate *Gli1*^{CreER}*Lkb1*$^{flox+}$*mtor*$^{flox/flox}$ animals. I hypothesized that if mTOR pathway is activated in LKB1-mutant tissue in Peutz Jeghers Syndrome, then genetic knockout of *mtor* in conjunction with *Lkb1* knockout should inhibit polyposis. However, I observed no difference in polyp-free survival in *Gli1*^{CreER}*Lkb1*$^{flox+}$*mtor*$^{flox/flox}$ animals as compared to *Gli1*^{CreER}*Lkb1*$^{flox+}$ animals *(Fig. B.6.C,D)*. I additionally observed expansion of a proliferative mesenchymal compartment comprised of predominantly αSMA^{+}/Desmin^{-} myofibroblast cells in both *Gli1*^{CreER}*Lkb1*$^{flox+}$*mtor*$^{flox/flox}$ polyps and in *Gli1*^{CreER}*Lkb1*$^{flox+}$ polyps *(Fig. B.6.E and Fig. B.7.A-C)*.

Overall, these data showed that in LKB1-deficient polyps, activation of the mTOR pathway is not the molecular mechanism for polyposis. This finding is contradictory to findings published reporting that administration of rapamycin was sufficient to suppress polyp formation in *Lkb1*$^{+/-}$ mice (Robinson et al., 2009; Wei et al., 2008). Therefore, it would be interesting to investigate whether rapamycin is somehow

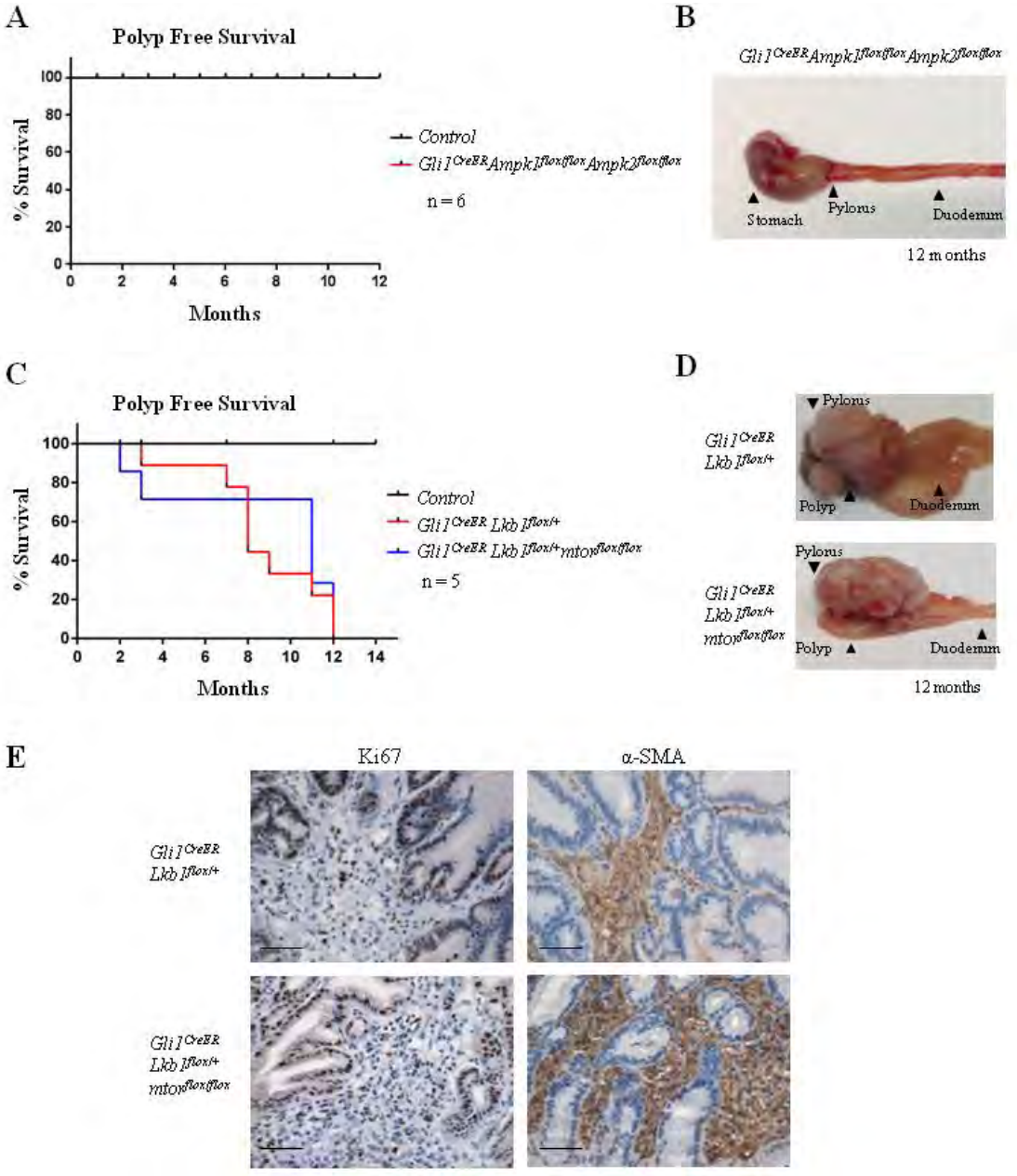


Figure B.6. LKB1-mutant polyps do not require mTOR pathway signaling for polyp initiation and growth.
(A,B) Genetic knockout of AMPK1 and AMPK2 does not affect overall survival and is not sufficient to drive polyp growth in $Gli1^{CreER}Ampk1^{flox/flox}Ampk2^{flox/flox}$ animals. *(C,D)* Genetic knockout of mTOR in conjunction with Lkb1 heterozygous knockout does not inhibit polyp initiation and growth in $Gli1^{CreER}Lkb1^{flox/+}mtor^{flox/flox}$ animals. *(E)* $Gli1^{CreER}Lkb1^{flox/+}mtor^{flox/flox}$ polyps are histologically identical to $Gli1^{CreER}Lkb1^{flox/+}$ polyps, as assayed through immunohistochemical Ki67 and α-SMA staining. Scale Bar = 50 μM.

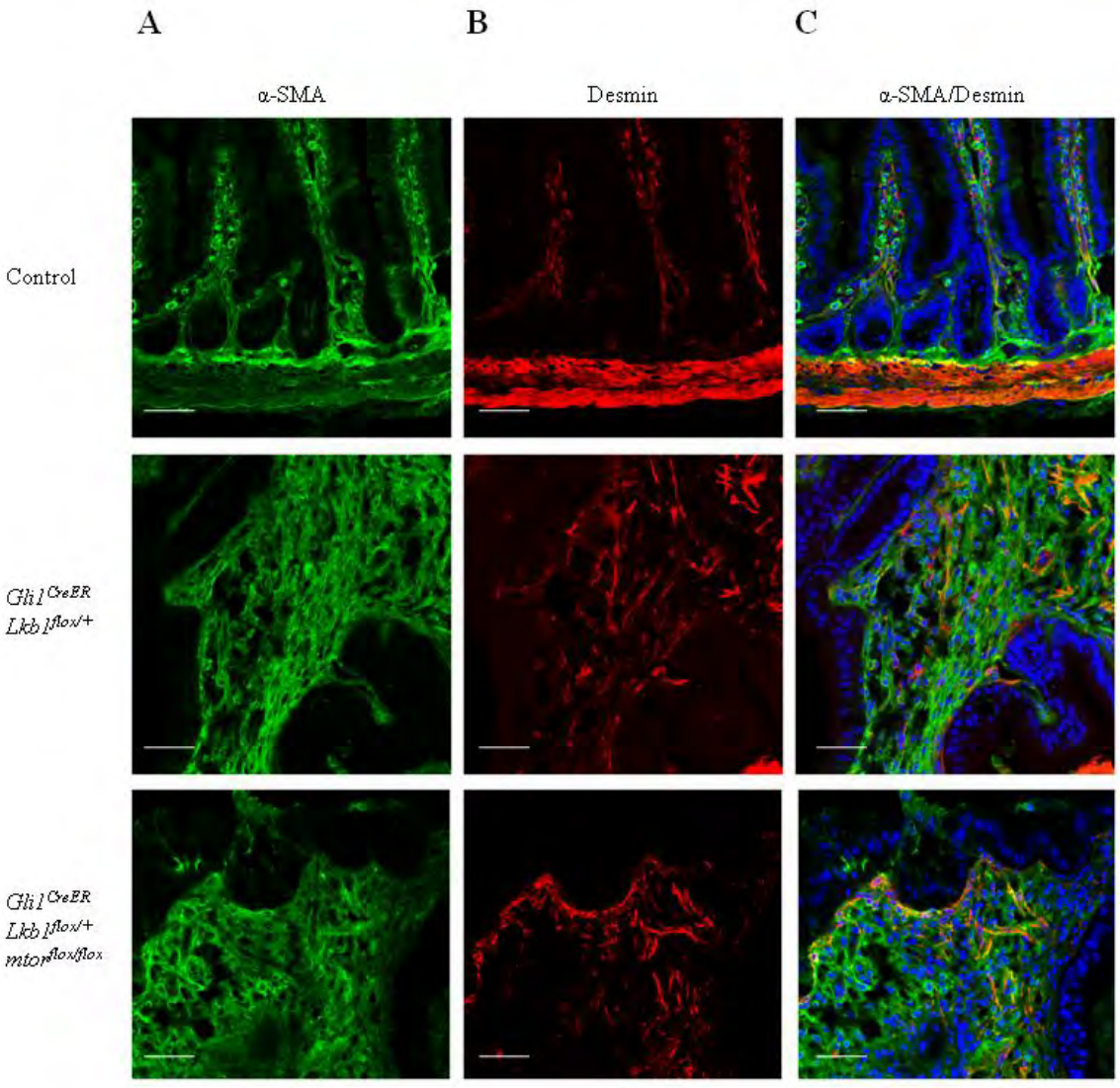


Figure B.7. *Gli1*^{CreER}*Lkb1*$^{flox/+}$*mtor*$^{flox/flox}$ polyps are comprised of an expanded myofibroblast population phenotypically consistent with *Gli1*^{CreER}*Lkb1*$^{flox/+}$polyps. *(A-C)* The expanded mesenchymal compartment in *Gli1*^{CreER}*Lkb1*$^{flox/+}$polyps and *Gli1*^{CreER}*Lkb1*$^{flox/+}$*mtor*$^{flox/flox}$ polyps is comprised of an α-SMA^{+}/Desmin^{-} myofibroblast population, as assayed through immunohistochemical staining. Scale Bar = 50 μM.

suppressing polyp growth independently of mTOR regulation. However, these data represents the first conclusive *in vivo* evidence excluding mTOR pathway activation in Peutz Jeghers polyps, and suggests that another molecular mechanism must be responsible for LKB1-deficient polyposis.

LKB1-mutant polyps require YAP/TAZ for polyp initiation and growth.

Recent reports have linked LKB1 to YAP/TAZ regulation and showed that human PJS polyp samples exhibited elevated YAP/TAZ nuclear protein levels (Mohseni et al., 2014; Nguyen et al., 2013). Therefore, I next decided to investigate YAP/TAZ, the transcriptional co-activators of the Hippo Pathway, in the LKB1-deficient polyps. I observed higher YAP/TAZ protein in both $Gli1^{CreER}Lkb1^{flox+}$ polyps as well as non-polyp adjacent intestine, as compared to control tissue *(Fig. B.8.A)*. I then investigated the tissue compartment expression of YAP, and found YAP protein expressed in the mesenchymal compartment of the $Gli1^{CreER}Lkb1^{flox+}$ polyps. From these data, I concluded that YAP/TAZ protein is elevated in myofibroblasts when LKB1 is knocked out.

To conclusively link YAP/TAZ to the molecular mechanism for polyposis in $Gli1^{CreER}Lkb1^{flox+}$ polyps, I again used mouse genetics. I crossed $Gli1^{CreER}Lkb1^{flox+}$ animals to animals carrying $Yap^{flox/flox}Taz^{flox/flox}$ alleles to generate $Gli1^{CreER}Lkb1^{flox/+}Yap^{flox/flox}Taz^{flox/flox}$ animals. I again induced Cre recombination at P30 and allowed animals to age to 12 months of age. I was excited to observe that genetic ablation of both YAP and TAZ in the gastrointestinal myofibroblast population not only

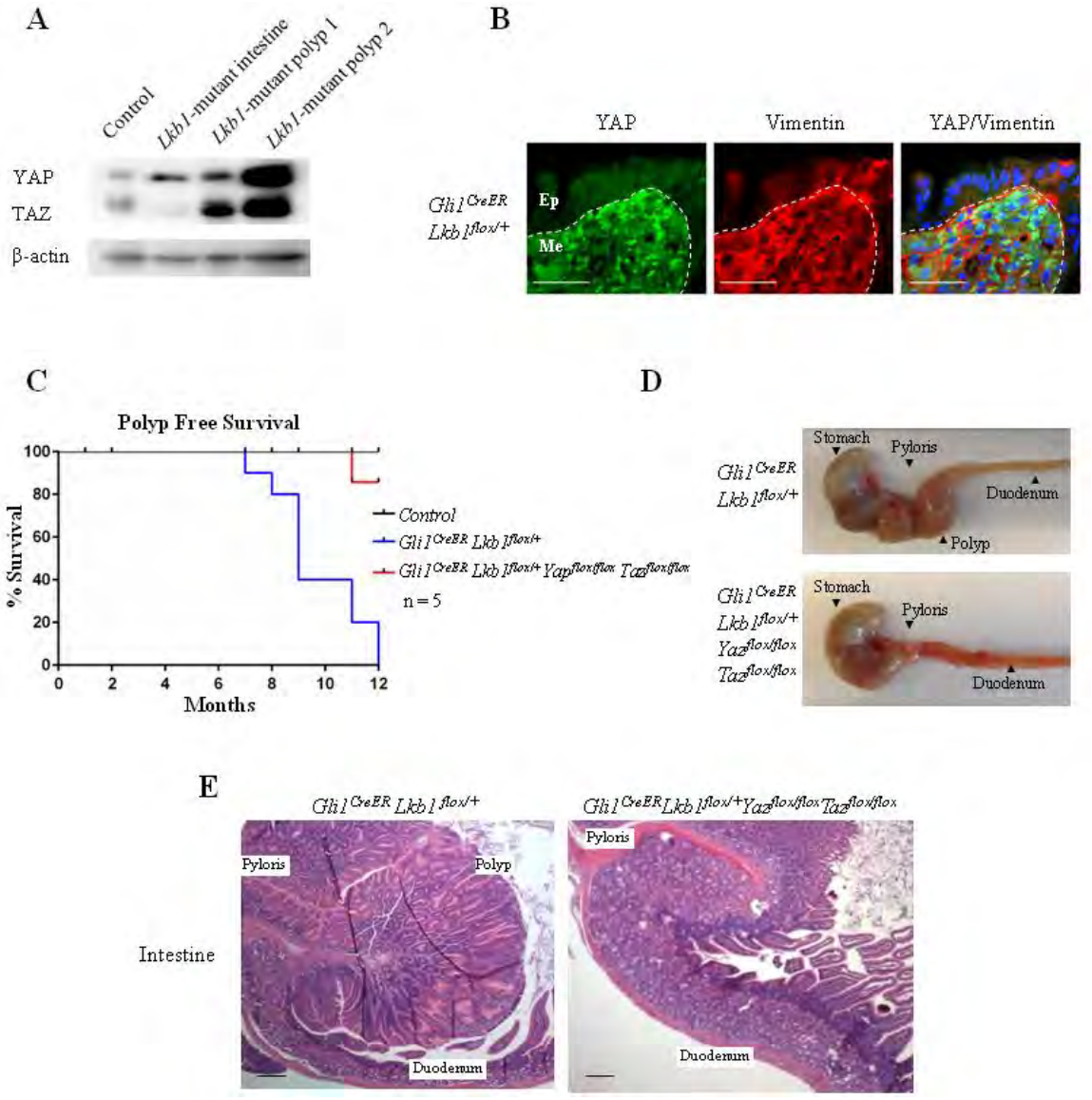


Figure B.8. LKB1-mutant polyps require YAP/TAZ for polyp initiation and growth. *(A)* Western blot showing protein expression of YAP and TAZ in two polyps isolated from *Gli1*^{CreER}*Lkb1*$^{flox/+}$animals, as well as non-polyp adjacent LKB1-deficient tissue, as compared to control tissue. *(B)* Mesenchymal tissue in *Gli1*^{CreER}*Lkb1*$^{flox/+}$polyps displays YAP protein expression, as assayed by immunofluorescence. *(C-E)* Homozygous genetic ablation of both *Yap* and *Taz*, in an *Lkb1*-deficient background, in the gastrointestinal myofibroblast cell population is sufficient to extend lifespan and prevent polyp formation in *Gli1*^{CreER}*Lkb1*$^{flox/flox}$*Yap*$^{flox/flox}$*Taz*$^{flox/flox}$ animals as compared to *Gli1*^{CreER}*Lkb1*$^{flox/+}$ animals. Scale Bar = 50 μM.

extended overall survival but also was sufficient to inhibit LKB1-deficient polyposis in *Gli1*^{CreER}*Lkb1*$^{flox+}$*Yap*$^{flox/flox}$*Taz*$^{flox/flox}$ animals *(Fig. B.8.C-E).*

Therefore, these data corroborate the previously published findings linking LKB1 and YAP/TAZ, and expand them to conclusively show that in Peutz Jeghers Syndrome, YAP/TAZ activity is critical for polyp initiation and growth (Mohseni et al., 2014; Nguyen et al., 2013). This study represents the first time that this finding has been shown conclusively *in vivo*, and suggests that inhibiting YAP/TAZ in patients with Peutz Jeghers Syndrome may be a viable treatment strategy.

Conclusions

This study represents the first *in vivo* evidence suggesting that genetically distinct inherited gastrointestinal hamartomatous polyposis syndromes arise from a shared cell of origin, and identifies that cell as a gastrointestinal myofibroblast. This work also represents the first reported mouse model for PTEN Hamartoma Syndrome/Cowden's Syndrome: the $Gli1^{CreER}Pten^{flox/flox}$ mouse model. Finally, this work also uses mouse genetics to conclusively show that in Peutz Jeghers Syndrome polyps, mutations in *Lkb1* initiate polyposis through aberrant regulation of YAP/TAZ activity, not through mTOR activity, as has been previously believed. Overall this work represents newly generated genetic models and critical findings for the field of gastrointestinal hamartomatous polyposis syndromes, and will hopefully yield clinically relevant information to help patients born with these syndromes.

Materials and Methods

Mouse Genetics

The *Gli1*^{CreER}, *R26*^{LacZ}, *Lkb1*^{flox}, *Pten*^{flox}, *Ampk1*^{flox}, *Ampk2*^{flox}, and *mtor*^{flox} mouse alleles were obtained from Jackson Laboratory. *Yap*^{flox} (Xin et al., 2011b) and *Taz*^{flox} (Xin et al., 2013) mice were a kind gift from Dr. EN Olson. The *R26*^{Smad7} mouse allele generation was described in **APPENDIX A.** To target the gastrointestinal myofibroblast population, *Gli1*^{CreER} mice were crossed to *R26*^{LacZ}, *Lkb1*^{flox}, *Pten*^{flox}, *Ampk1*^{flox}, *Ampk2*^{flox}, *mtor*^{flox}, *Yap*^{flox} and *Taz*^{flox} alleles. Cre recombination was induced by intraperitoneal injection of 120 mg/kg tamoxifen dissolved in corn oil. Overall survival was determined as the age of animal when it became moribund.

Tissue Collection and Histology

Animals were humanely euthanized with CO2 and cervical dislocation. Tissue was dissected and fixed in 10% NBF at 4°C. For paraffin sectioning, tissue was dehydrated in 70% EtOH and embedded in paraffin wax. Frozen sections were dehydrated in 30% sucrose and embedded in OCT media. Paraffin sections were cut at 6 μm on a microtome and frozen sections were cut at 12 μm on a cryotome. Hematoxylin & eosin reagents were used for routine staining of paraffin sections, and X-GAL staining reagents were used for routine lacZ staining of frozen sections. Histological analyses of mouse tissues were conducted by Dr. Zhong Jiang, Department of Pathology, UMass Medical School.

Western Blotting

Mouse tissue was dissected and minced in lysis buffer using tissue pestles. Protein lysates were probed with the following primary antibodies: YAP/TAZ (Cell Signaling) and β-actin (Genescript). HRP-conjugated Secondary antibodies were from Jackson Laboratories.

Immunohistochemistry / Immunofluorescence

For IHC, paraffin slides were first dewaxed in xylene and then rehydrated in serial dilutions of ethanaol. Slides were incubated in 10mM sodium citrate buffer pH 6.0 for 30 minutes in a rice cooker to expose antigens via heat-induced epitope retrieval. Endogenous peroxidase was blocked for 20 minutes in 3% H_2O_2-MeOH and then blocked for 1 hour at room temperature in 5% BSA, 1% goat serum, 0.5% Tween-20. Slides were incubated in primary antibody overnight at 4°C, and then secondary antibody for 1 hour at room temperature. Antibodies were diluted in either blocking buffer or SignalStain® Antibody Diluent (Cell Signaling). Signal was detected using the Vectastain Elite ABC kit and the DAB Peroxidase Substrate kit (Vector Laboratories) according to manufacturer protocols. Primary antibodies for IHC were: pS6 (Cell Signaling), pAktS473 (Cell Signaling), Ki67 (Abcam), and α-smooth muscle actin (αSMA) (Abcam).

For IF, slides were blocked in 5% BSA, 1% goat serum, 0.5% Tween-20 for 1 hour at room temperature, and then incubated in primary antibody overnight at 4°C. The next day, slides were incubated in secondary antibody for 1 hour at room temperature,

and then mounted with DAPI. Secondary antibodies were AlexaFluor-488, AlexaFluor-568, and AlexaFluor-647 conjugated antibodies (ThermoFisher) diluted in blocking buffer. Antibodies for IF were: β-galactosidase (Abcam), α-smooth muscle actin (αSMA) (Abcam), Desmin (ThermoFisher), and YAP (Cell Signaling).

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