NOVEL ROLES FOR THE TUMOR SUPPRESSOR APC THROUGH REGULATION OF GSK-3

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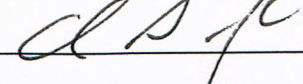
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ABSTRACT

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Adenomatous Polyposis Coli (APC) is a tumor suppressor and essential negative

regulator of the Wnt signaling pathway. Wnt signaling is crucial for proper

patterning and cell fate specification during development and regulates stem cell

homeostasis throughout adulthood. Mutations in *Apc* are strongly linked to

human colorectal cancers and these mutations aberrantly activate Wnt signaling.

How APC regulates the Wnt pathway and how oncogenic *Apc* mutations activate

Wnt signaling and promote tumorigenesis are not fully understood. To address

these questions, we utilized *in vitro* reconstitution assays, as well as *Apc*

knockdown or mutation in human cells, zebrafish, and mice. We find APC directly

enhances activity of Glycogen Synthase Kinase-3 (GSK-3), an essential

regulator of diverse signaling pathways. Furthermore, APC loss of function

mimics GSK-3 inhibition by reducing phosphorylation of Glycogen Synthase and

by activating mechanistic Target of Rapamycin in Complex 1 (mTORC1). Thus

we identify novel roles for APC as a regulator of GSK-3 activity and GSK-3

dependent signaling. Wnts reduce GSK-3 activity through an unknown

mechanism to activate downstream signaling. We find Wnts induce rapid APC

dissociation from the GSK-3/Axin complex, suggesting a new mechanism for how

Wnts reduce GSK-3 activity. GSK-3 also regulates mTORC1, and we find

oncogenic *Apc* mutations activate mTORC1. Furthermore, many *Apc* mutant

phenotypes are partially rescued by mTORC1 inhibition, while others are rescued

by combined mTORC1 and Wnt inhibition. These observations demonstrate roles

for mTORC1 activation downstream of *Apc* mutation. Taken together, our

findings provide new insight into APC function and the effects of oncogenic *Apc*

mutation.

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CHAPTER 1: INTRODUCTION

1-1. APC is a tumor suppressor and essential negative regulator of the Wnt pathway

Adenomatous Polyposis Coli (APC) is a large, multi-functional protein that plays critical roles in cell migration, apoptosis, cell cycle progression, axon outgrowth and guidance, cell-cell adhesion, regulation of microtubule dynamics, and many other important cellular processes (Hanson and Miller, 2005). APC is also an essential negative regulator of Wnt signaling, but how it performs this function is not well understood. Wnt signaling is essential during embryonic development for patterning, cell fate specification, and stem cell regulation. Wnt signals continue to influence stem cell fate decisions and tissue regeneration throughout adulthood (Clevers, 2006; MacDonald et al., 2009).

Loss of function studies in model organisms demonstrate a requirement for APC in development and maintenance of many tissues and organs, often through its role in regulating Wnt signaling. In mice, homozygous *Apc* mutation results in lethality during gastrulation (Fodde et al., 1994). Tissue-specific *Apc* loss of function demonstrates essential roles for APC in development and maintenance of mouse liver (Decaens et al., 2008), mammary glands (Gallagher et al., 2002), intestine (Radulescu et al., 2010; Sansom et al., 2004), bone (Miclea et al., 2009), ocular lens (Martinez et al., 2009), and heart (Hasegawa et

al., 2002). Tissue-specific APC loss of function also causes nuclear β-catenin localization (a hallmark of active Wnt signaling) and activation of Wnt target genes in each of the respective tissues. In contrast to mice, *Apc* mutant zebrafish complete gastrulation due to the presence of maternal wild type APC. However, these fish also have many developmental defects and die a few days after fertilization (Hurlstone et al., 2003). These defects include unlooped hearts, hyperproliferation of endocardial cushions resulting in failure to form functional heart valves, reduced blood circulation, failure to form livers (or enlarged livers in heterozygous mutants), fin bud arrest, intestinal hyperproliferation, aberrant body curvature resulting from defects in morphogenesis of the anterior-posterior axis, failure to form inner ear structures and failure to form swim bladders (Goessling et al., 2008; Hurlstone et al., 2003). Nuclear β-catenin localization and upregulation of Wnt target genes are also observed in many tissues in *Apc* mutant zebrafish including the heart, eye, and liver (Goessling et al., 2008; Hurlstone et al., 2003; Stephens et al., 2010). In *Drosophila*, mutation of *Apc2* disrupts denticle patterning consistent with hyperactive Wingless (Wnt) signaling (McCartney et al., 1999). Mutation of *Apc1* causes retinal photoreceptor apoptosis and pigment cell hypertrophy also as a result of hyperactive Wingless signaling (Ahmed et al., 1998). Combined loss of function of *Apc1* and *Apc2* results in ubiquitous activation of Wingless signaling and lethality during larval stages (Ahmed et al., 2002). In *C. Elegans,* knockdown of *apc-related-1 (apr-1)* results in failure to form endoderm (Rocheleau et al., 1997). These data indicate

that APC is essential for the development of many organs and tissues.

Mutations that aberrantly activate Wnt signaling, including mutations in *Apc*, are associated with human cancers (Anastas and Moon, 2012). APC functions as a tumor suppressor, and *Apc* mutations are strongly linked to colorectal cancer. Colorectal cancer is responsible for over 600,000 deaths worldwide each year, including 50,000 annually in the United States. It is the third most common cancer and second leading cause of cancer related death in the U.S. (U.S. Cancer Statistics Working Group, 2013). Thus, better understanding of APC function and the consequences of oncogenic *Apc* mutation* is critical.

Apc mutations are found in approximately 80 percent of sporadic colorectal cancers, and patients with germline *Apc* mutations develop Familial Adenomatous Polyposis (FAP), a condition marked by the formation of hundreds to thousands of intestinal adenomatous polyps. A small percent of these adenomas inevitably progress to invasive carcinomas and thus colonic resection is almost always required (Groden et al., 1991; Kinzler et al., 1991; Kinzler and Vogelstein, 1996; Miyoshi et al., 1992a). Mice and rats with germline *Apc* mutations also develop many intestinal adenomas. Homozygous *Apc* mutation results in early embryonic lethality. Therefore the human patients, mice, and rats that develop intestinal adenomas are heterozygous at the *Apc* locus in almost all cells. However, intestinal cells undergo loss of heterozygosity resulting in the

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*Throughout this dissertation "oncogenic *Apc* mutations" refer to truncating mutations within the mutation cluster region of *Apc* as described in section 1-3.

formation of tumors that are homozygous for the *Apc* mutation. Wnt signaling is aberrantly active in these tumors, consistent with the role of APC as a negative regulator of Wnt signaling (Amos-Landgraf et al., 2007; Kinzler and Vogelstein, 1996; Su et al., 1992). Furthermore Wnt signaling promotes growth and maintenance of *Apc* mutant tumors (Foley et al., 2008; Roh et al., 2001; Scholer-Dahirel et al., 2011).

Interestingly, development of intestinal adenomas may begin prior to activation of Wnt signaling in some cases. Despite homozygous *Apc* mutation, nuclear β-catenin localization (a requirement for active Wnt signaling) and activation of Wnt target genes are not detected in all early adenomas in patients with FAP, microadenomas in a rat model of FAP, and sporadic human polyps (Amos-Landgraf et al., 2007; Anderson et al., 2002; Blaker et al., 2004; Caldwell et al., 2010; D'Orazio et al., 2002; Kobayashi et al., 2000).

The importance of Wnt/β-catenin signaling downstream of *Apc* mutation is well established. However, the multitude of Wnt-independent APC functions, the prevalence of mutations in *Apc* compared to other Wnt pathway inhibitors in colorectal cancer, and the possibility of adenoma formation in the absence of active Wnt/β-catenin signaling led us to hypothesize that oncogenic *Apc* mutations may also function through additional mechanisms.

1-2. The Wnt signaling pathway

APC loss of function causes constitutive activation of the Wnt signaling

pathway, indicating APC is an essential negative regulator. Wnt signaling is crucial for establishing body axes and for patterning many tissues and organs during development, as well as regulating stem cell homeostasis and tissue maintenance in adults. Inhibition or ectopic activation of the pathway early on in development is lethal and later on can result in a wide variety of severe developmental defects. Improper regulation of the Wnt pathway is also implicated in human diseases including bipolar disorder and cancer. Mutations in Wnt pathway components that activate Wnt signaling have been identified in many cancers, including liver, prostate, breast, pancreatic, and most notably colorectal cancers, highlighting the importance of understanding Wnt signaling mechanisms (MacDonald et al., 2009).

In unstimulated cells, APC, Glycogen Synthase Kinase-3 (GSK-3) and the transcriptional co-activator β-catenin bind directly to the scaffolding protein Axin in a cytoplasmic complex (Figure 1-1A). GSK-3 phosphorylates β-catenin within this complex, targeting β-catenin for ubiquitination and proteasome mediated degradation. APC also promotes β-catenin phosphorylation and degradation through an unknown mechanism. Wnt ligands bind to Frizzled receptors inducing formation of a complex with the essential co-receptor low density lipoprotein receptor-related protein 6 (LRP6) at the plasma membrane (Wehrli et al., 2000). LRP6 becomes phosphorylated which promotes direct interaction with Axin, thus recruiting the Axin complex to the membrane (Figure 1-1B). Subsequently, GSK-3 is inhibited within the Axin complex through an unknown mechanism, allowing

Figure 1-1
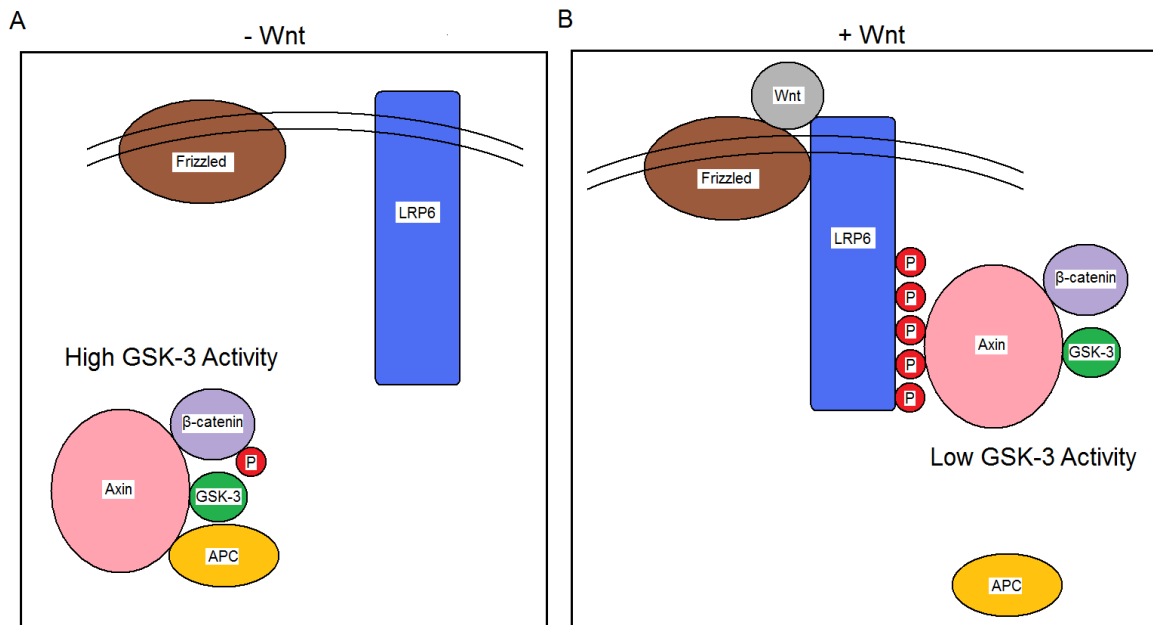
Figure 1-1. Wnt signaling inhibits GSK-3 within the Axin complex. (A) In
unstimulated cells, GSK-3 phosphorylates and destabilizes β-catenin within the
Axin complex. (B) Wnt signaling recruits Axin to phosphorylated LRP6, reducing
GSK-3 activity and stabilizing β-catenin. We propose APC enhances GSK-3
activity and Wnts induce APC dissociation from the Axin complex.

stabilization and accumulation of β-catenin. β-catenin then translocates into the nucleus and interacts with lymphocyte enhancer factor/T-cell factor (LEF/TCF) transcription factors to activate transcription of Wnt target genes (Clevers, 2006; MacDonald et al., 2009).

Several hypotheses have been proposed to explain how Wnts stabilize β-catenin. The E3 ubiquitin ligase β-TrCP interacts with the Axin complex to promote β-catenin ubiquitination and degradation, and β-TrCP dissociates from the complex following Wnt stimulation (Li et al., 2012). However, this event has not been observed until after β-catenin has accumulated. Similarly, Wnts induce Axin degradation and sequestration of GSK-3 inside multivesicular endosomes, but these events occur several hours after Wnt stimulation whereas GSK-3 is inhibited and β-catenin accumulates within minutes (Cook et al., 1996; Ding et al., 2000; Taelman et al., 2010). GSK-3 sequestration is also blocked by β-catenin knockdown and induced by expression of stabilized β-catenin, suggesting GSK-3 sequestration occurs downstream of β-catenin stabilization during Wnt signaling. Therefore, while these events may contribute to long-term β-catenin stabilization, they do not explain initial β-catenin accumulation.

Alternatively, phosphorylated LRP6 may directly inhibit GSK-3 once the Axin complex is recruited to the Wnt/Frizzled/LRP6 complex at the membrane. The LRP6 intracellular domain, which contains the Wnt-dependent phosphorylation sites, reduces phosphorylation of GSK-3 substrates in *in vitro*

kinase assays for GSK-3 activity, and mutating the phosphorylation sites on

LRP6 to alanines blocks this effect (Cselenyi et al., 2008; Piao et al., 2008; Wu et

al., 2009). However, GSK-3 also phosphorylates the LRP6 intracellular domain,

so reduced phosphorylation of other GSK-3 substrates used to assay GSK-3

activity may be simply due to substrate competition. It is also noteworthy that

micromolar concentrations of the LRP6 intracellular domain are required to

reduce phosphorylation of other GSK-3 substrates in these reactions. Since Wnts

induce recruitment of the Axin/GSK-3 complex to phosphorylated LRP6, further

experiments are needed to determine whether pre-phosphorylated LRP6 can

directly inhibit GSK-3.

We propose an alternative hypothesis in which APC directly enhances

GSK-3 activity and Wnts induce APC dissociation from the Axin complex to

reduce GSK-3 activity and activate downstream signaling (Figure 1-1B). The

rationale for this hypothesis will be discussed below.

1-3. Direct APC interaction with Axin antagonizes Wnt signaling

Axin and APC interact directly through a regulator of G-protein signaling

(RGS) domain within Axin and through three serine-alanine-methionine-proline

(SAMP) repeats within APC (Behrens et al., 1998; Kishida et al., 1998). Axin and

APC enhance β-catenin phosphorylation by GSK-3 *in vitro*, and adding them

simultaneously enhances β-catenin phosphorylation even further. In the

presence of APC, an Axin mutant lacking the RGS domain (ΔRGS-Axin) reduces β-catenin phosphorylation by GSK-3 compared to wild type Axin (Hinoi et al., 2000; von Kries et al., 2000). These data suggest that direct interaction between Axin and APC promotes β-catenin phosphorylation by GSK-3. In *Xenopus* embryos, ventral injection of ΔRGS-Axin but not wild type Axin causes axis duplication, indicating aberrant Wnt pathway activation (Hedgepeth et al., 1999; Sokol et al., 1991; Zeng et al., 1997). ΔRGS-Axin binds and sequesters GSK-3 in complexes that do not contain APC, suggesting direct Axin/APC interaction antagonizes Wnt signaling *in vivo*. ΔRGS-Axin also reduces phosphorylation of tau protein by GSK-3 in *Xenopus* oocytes, suggesting interaction with APC promotes GSK-3 activity towards multiple substrates (Hedgepeth et al., 1999).

Further evidence of the importance of Axin/APC interaction comes from mutations identified in cancers. *Apc* is mutated in approximately 80 percent of sporadic colorectal cancers, and hundreds of different mutations have been identified which cluster in a region that has been designated the Mutation Cluster Region (MCR). Almost all of these mutations result in expression of a truncated APC fragment that lacks the C-terminal half. Germline *Apc* mutations that cause Familial Adenomatous Polyposis are also truncating mutations located in the MCR (Hart et al., 1998; Kinzler and Vogelstein, 1996; Miyoshi et al., 1992b; Munemitsu et al., 1995; Nakamura et al., 1998). Interestingly, the remaining N-terminal APC fragment is essential for colorectal cancer cell proliferation. Knocking down expression of this fragment reduces proliferation of colorectal

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cancer cell lines and blocks tumor formation when cells from one of these lines are injected into nude mice (Chandra et al., 2012).

Human APC protein contains 2843 amino acids and the MCR comprises amino acids approximately 1100 - 1500. The C-terminal end of the MCR is less than 100 amino acids N-terminal to the first SAMP repeat (Figure 1-2A). Thus the location of the MCR in relation to the SAMP repeats suggests truncating *Apc* mutations must remove the SAMP repeats and disrupt interaction with Axin to promote tumorigenesis. These truncating *Apc* mutations abolish direct Axin/APC interaction, resulting in reduced β-catenin phosphorylation by GSK-3 and thus reduced β-catenin ubiquitination and degradation which leads to β-catenin accumulation and constitutive activation of Wnt target genes. Wnt signaling is strongly activated in tumors resulting from truncating *Apc* mutation in humans, mice, rats, and zebrafish (Amos-Landgraf et al., 2007; Haramis et al., 2006; Hart et al., 1998; Miyoshi et al., 1992b; Munemitsu et al., 1995; Nakamura et al., 1998). β-catenin knockdown reduces tumor cell proliferation and reduces the number of intestinal adenomas in *Apc* mutant mice (Foley et al., 2008). β-catenin knockdown also blocks growth of colorectal tumor xenografts harboring *Apc* mutations (Roh et al., 2001; Scholer-Dahirel et al., 2011). These data suggest aberrant Wnt pathway activation contributes to tumorigenesis downstream of *Apc* mutation.

Importantly, reintroducing wild type APC into *Apc* mutant colorectal cancer cells or expressing fragments of APC that contain the SAMP repeats (SAMP

Figure 1-2
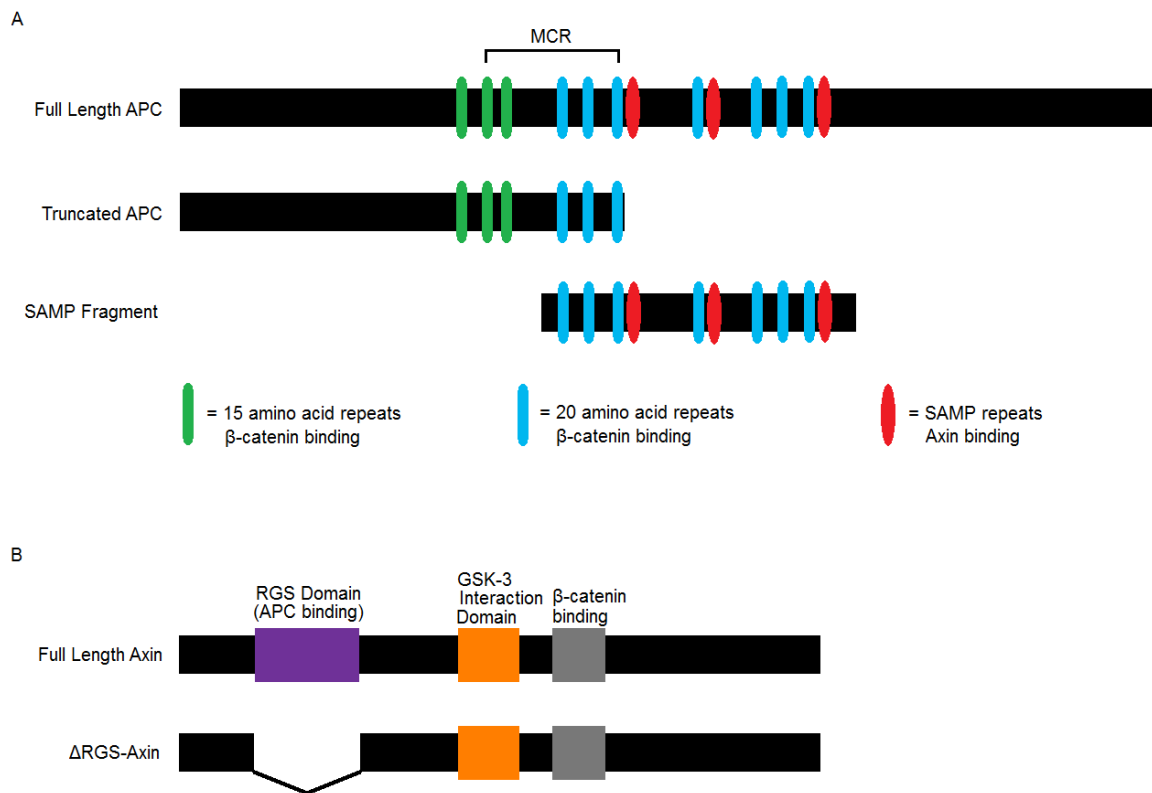
Figure 1-2. Domain structure of APC and Axin. (A) The mutation cluster region (MCR) within APC is immediately N-terminal to the first Axin binding SAMP repeat. Oncogenic *Apc* mutations truncate within the MCR, removing the SAMP repeats and abolishing direct interaction with Axin. An example of a fragment containing the SAMP repeats (SAMP fragment) that rescues aberrant Wnt signaling in *Apc* mutant colorectal cancer cells is also shown. (B) Full length Axin binds APC, GSK-3 and β-catenin through distinct domains. ΔRGS-Axin lacks the APC binding domain and therefore cannot directly interact with APC.

fragments, Figure 1-2A) rescues β-catenin phosphorylation by GSK-3, as well as β-catenin ubiquitination and degradation, and aberrant Wnt target gene activation (Kawahara et al., 2000; Yang et al., 2006). Deleting or mutating the SAMP repeats in APC blocks the rescue (Kawahara et al., 2000; von Kries et al., 2000). These data suggest that direct Axin/APC interaction is essential for promoting β-catenin phosphorylation by GSK-3 to downregulate β-catenin and keep the Wnt pathway inactive. Mutations in the APC-binding domain of Axin have also been identified in colorectal cancers, medulloblastomas, hepatoblastomas, hepatocellular carcinomas, and prostate cancers (Salahshor and Woodgett, 2005; Yardy et al., 2009; Zucman-Rossi et al., 2007). Taken together, these data led us to hypothesize that Wnts could regulate the Axin/APC interaction to stabilize β-catenin and activate downstream signaling.

1-4. GSK-3 and Wnt signaling regulate mTORC1

Mechanistic Target of Rapamycin (mTOR) is an atypical serine/threonine kinase found in two distinct complexes termed mTOR Complex 1 (mTORC1) and mTOR Complex 2 (mTORC2). mTORC1 is a nutrient and energy sensor that integrates a variety of signals to regulate translation, energy metabolism, cell growth and proliferation, and cell survival decisions. mTORC1 is aberrantly active in human diseases including type 2 diabetes and many cancers (Laplante and Sabatini, 2012; Ma and Blenis, 2009; Zoncu et al., 2011). mTORC1 activation is

frequently observed in sporadic cancers and inherited polyposis syndromes including Tuberous Sclerosis Complex, Peutz-Jeghers syndrome, and the PTEN-related hamartoma syndromes. These syndromes are caused by mutations in negative regulators of mTORC1 (*Tsc1/Tsc2, Lkb1*, and *Pten* respectively) (Inoki et al., 2005). The mTORC1 inhibitor Everolimus was recently approved by the U.S. Food and Drug Administration for treatment of Tuberous Sclerosis Complex (Laplante and Sabatini, 2012), highlighting the importance of mTORC1 activation.

mTORC1 activity is regulated by many upstream factors including the TSC1-TSC2-TBC1D7 complex. Tuberous Sclerosis Complex 2 (TSC2) is a GTPase activating protein for the small GTPase Rheb which activates mTORC1. Thus TSC2 negatively regulates mTORC1 (Dibble et al., 2012; Inoki et al., 2003; Tee et al., 2003). Active mTORC1 directly phosphorylates several downstream effectors, including S6 kinase 1 which is activated by phosphorylation and in turn phosphorylates the ribosomal protein S6 to promote translation (Ma and Blenis, 2009).

TSC2 is also regulated by multiple upstream factors including ERK, Akt, AMPK, and GSK-3. GSK-3 directly phosphorylates and activates TSC2 and therefore GSK-3 also negatively regulates mTORC1 (Figure 1-3A). GSK-3 knockdown or inhibition activates mTORC1, indicating GSK-3 is essential for keeping mTORC1 inactive (Huang et al., 2009; Inoki et al., 2006). TSC2 can physically associate with the Axin complex, and Axin knockdown also

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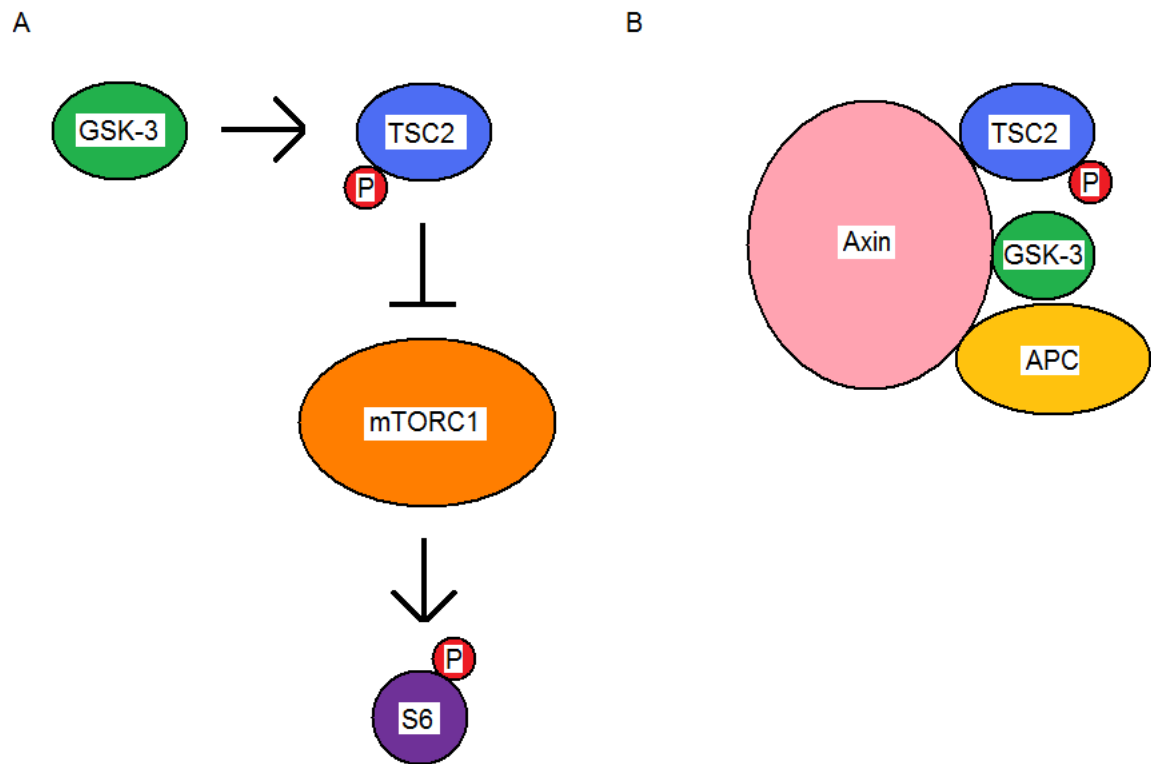
Figure 1-3

Figure 1-3. GSK-3 negatively regulates mTORC1. (A) TSC2 negatively regulates mTORC1 and GSK-3 phosphorylates and activates TSC2. Thus GSK-3 also negatively regulates mTORC1. mTORC1 activation promotes S6 phosphorylation. (B) TSC2 can associate with the Axin complex. Wnt signaling and Axin knockdown activate mTORC1.

activates mTORC1 (Inoki et al., 2006; Mak et al., 2005). Furthermore, Wnt ligands inhibit GSK-3 in the Axin complex, reduce TSC2 phosphorylation, and activate mTORC1. These data suggest mTORC1 is regulated by Wnts and GSK-3 in the Axin complex. mTORC1 activation by Wnts occurs within minutes, correlating with GSK-3 inhibition (Cook et al., 1996; Inoki et al., 2006). Regulation of mTORC1 by Wnts is independent of β-catenin as β-catenin knockout or overexpression does not affect mTORC1 activation (Inoki et al., 2006). The role of Wnt signaling, GSK-3, and Axin in regulating mTORC1 led us to hypothesize that APC negatively regulates mTORC1 and also led us to ask whether oncogenic APC mutations activate mTORC1.

1-5. GSK-3 is an essential regulator of diverse signaling pathways

GSK-3 inhibits Wnt signaling by directly phosphorylating and destabilizing β-catenin, and inhibits mTORC1 by directly phosphorylating and activating TSC2. GSK-3 also phosphorylates over 100 other putative substrates including p53, tau, amyloid precursor protein (APP), c-Myc, BCL-3, Cyclin D1, Cyclin E, Hypoxia-inducible factor-1 (HIF-1), and c/EBPβ. Through phosphorylation of its substrates, GSK-3 regulates many signaling pathways including ERK, Notch, Sonic Hedgehog, Bone Morphogenetic Protein (BMP), and Insulin/growth factor signaling through PI3K and Akt (Kaidanovich-Beilin and Woodgett, 2011; Kim and Snider, 2011; Kockeritz et al., 2006). For example, GSK-3 phosphorylates

and destabilizes the Notch signaling effector Notch intracellular domain (NICD). Inhibition or deletion of GSK-3 increases NICD levels and activates Notch target genes (Jin et al., 2009; Kim et al., 2009). Similarly, GSK-3 phosphorylates the Sonic Hedgehog pathway effector Ci/Gli, promoting proteolytic processing of Ci/Gli to generate a truncated repressor. GSK-3 loss of function causes accumulation of full length Ci/Gli and activates Sonic Hedgehog target genes (Forde and Dale, 2007; Jia et al., 2002; Price and Kalderon, 2002).

GSK-3 is a constitutively active and ubiquitously expressed serine/threonine kinase encoded by two separate genes: *Gsk-3α* and *Gsk-3β*. The kinase domains of GSK-3α and GSK-3β are 98 percent identical, and the two isoforms function redundantly in the Wnt pathway (Doble et al., 2007; Doble and Woodgett, 2003). GSK-3 autophosphorylates at a tyrosine residue within its activation loop during its chaperone-mediated folding and maturation. This phosphorylation enhances serine/threonine kinase activity of the mature GSK-3 (Cole et al., 2004; Lochhead et al., 2006). GSK-3 can also be inhibited by phosphorylation at an N-terminal serine residue (serine 21 on GSK-3α and serine 9 on GSK-3β), which creates a competitive pseudosubstrate inhibition motif (Dajani et al., 2001).

Upstream signals in general inhibit GSK-3 to activate downstream signaling. Insulin and other growth factors activate phosphatidylinositol-3-kinase (PI3K) which phosphorylates and activates Akt. Akt in turn phosphorylates GSK-3 on serine 9/21, thus inhibiting GSK-3 and allowing activation of downstream

effectors such as Glycogen Synthase, the eponymous substrate for GSK-3 (Cross et al., 1995; McManus et al., 2005; Proud, 2006). Importantly, neither Wnts nor Insulin/Akt induce serine 9/21 phosphorylation of GSK-3 in the Axin complex, and Wnt signaling is unaffected by mutation of serine 9/21 to alanine (Ding et al., 2000; McManus et al., 2005; Ng et al., 2009). These data suggest Wnts inhibit GSK-3 by a mechanism other than serine 9/21 phosphorylation, and that Wnt-regulated and Insulin/Akt-regulated GSK-3 represent two distinct subcellular pools.

Misregulation of GSK-3 activity is implicated in human diseases including bipolar disorder, schizophrenia, and Alzheimer's Disease. The GSK-3 inhibitor lithium has been used to treat bipolar disorder for over 50 years, and GSK-3 has been shown to be the relevant target of lithium in regulating mammalian behaviors, suggesting misregulation of GSK-3 contributes to bipolar disorder (Valvezan and Klein, 2011). Mutations in the *disrupted in schizophrenia 1* (*Disc1*) gene have been linked to schizophrenia, and DISC1 is an endogenous GSK-3 inhibitor, suggesting misregulation of GSK-3 may contribute to schizophrenia as well (Mao et al., 2009; Millar et al., 2000; Valvezan and Klein, 2011). Two major pathological hallmarks of Alzheimer's disease are aggregates of insoluble amyloid beta peptide (amyloid plaques) and neurofibrillary tangles composed mainly of the GSK-3 substrate tau hyperphosphorylated at the GSK-3 phosphorylation sites. GSK-3 promotes processing of the amyloid precursor protein (APP) to generate amyloid beta peptide. GSK-3 inhibition or loss of

function reduces amyloid plaque and neurofibrillary tangle formation and improves cognitive function in mouse models of Alzheimer's disease (Hernandez et al., 2009; Hurtado et al., 2012; Phiel et al., 2003). Thus GSK-3 is an essential regulator of many substrates and signaling pathways, and improper regulation of GSK-3 is implicated in human diseases, highlighting the importance of understanding how GSK-3 activity is regulated.

1-6. Parallels in APC and GSK-3 function

Interestingly, APC and GSK-3 are involved in many of the same developmental and cellular processes, and their gain and loss of function phenotypes are often similar. For example, knockdown of *apc-related-1 (apr-1)* in *C. Elegans* results in ectopic pharyngeal tissue and failure to form endoderm (Rocheleau et al., 1997), and these phenotypes are also observed when *gsk-3* is knocked down (Schlesinger et al., 1999). Loss of function mutations in *Drosophila Gsk-3β (zw-3)* phenocopy a hypomorphic *APC2* mutation which disrupts localization of nuclei to the cortex of syncytial *Drosophila* embryos (McCartney et al., 2001). *Zw-3* knockdown also disrupts denticle patterning and causes anterior gut expansion, like APC2 knockdown (Yu et al., 1999). Deletion of *Apc* or *Gsk-3* in mouse intestine blocks cell cycle arrest in response to mitotic spindle disruption, indicating APC and GSK-3 contribute to the mitotic spindle assembly checkpoint (Radulescu et al., 2010). GSK-3 knockdown in mouse bone

marrow causes short term expansion and long term depletion of hematopoietic

stem cells (HSCs) (Huang et al., 2009). Similarly, conditional APC deletion in

mouse bone marrow causes short term expansion and long term HSC depletion

(Qian et al., 2008). APC and GSK-3 are both essential for spindle formation

during mitosis. GSK-3 localizes to centrosomes and spindle microtubules, and

GSK-3 inhibitors disrupt spindle orientation, resulting in chromosomal

misalignment (Wakefield et al., 2003). APC also localizes to centrosomes and

spindle microtubules and APC loss of function disrupts spindle orientation and

causes chromosome misalignment (Caldwell and Kaplan, 2009; Hanson and

Miller, 2005; Lui et al., 2011). GSK-3 phosphorylates Histone H1.5 to regulate

chromatin condensation during mitosis, and APC is required for compaction of

mitotic chromatin (Dikovskaya et al., 2012; Happel et al., 2009).

     APC and GSK-3 also play similar roles in neurite outgrowth and the

establishment of axonal-dendritic polarity. Immature neurons extend several

small neurites, one of which is selected to become the mature axon. The Par

complex including mammalian partition defective 3 (mPar3), which is essential

for polarization of many cell types, accumulates at the tip of the neurite which will

become the mature axon. mPar3 and the Par complex are essential for axon

specification and establishment of neuronal polarity (Chen et al., 2013). APC is

required for mPar3 localization and axon specification. Furthermore, APC

overexpression results in mPar3 localization in two or more neurites which grow

and express axonal markers, suggesting APC promotes mPar3 accumulation at

neurite tips and axon specification. Similarly, GSK-3 inhibitors block mPar3

accumulation and neurite outgrowth (Shi et al., 2004). Taken together, these data

suggest APC and GSK-3 have many similar functions and led us to hypothesize

that APC and GSK-3 functionally interact in Wnt-independent settings.

1-7. Research summary

Wnt signaling is essential for patterning during embryonic development,

stem cell fate decisions in adults, and progression of many cancers. The

functions of key players in the Wnt pathway, and the mechanisms controlling key

steps in Wnt signal transduction are not understood. APC inhibits Wnt signaling

by promoting phosphorylation and degradation of β-catenin. Wnts stabilize β-

catenin by reducing its phosphorylation. How APC and Wnts regulate β-catenin

phosphorylation is unknown. To investigate the role of APC in regulation of β-

catenin, we tested a role for APC in regulating GSK-3 activity. We tested this in *in

vitro* kinase assays for GSK-3 activity and by knocking down APC in human cells.

We find that APC directly enhances GSK-3 activity, and thus promotes

phosphorylation of β-catenin and other substrates by GSK-3. Interestingly, we

also identify new roles for APC as a negative regulator of Glycogen Synthase

and mTORC1, like GSK-3. Furthermore, we find Wnt signaling induces APC

dissociation from the Axin/GSK-3 complex, suggesting a novel mechanism for

how Wnts inhibit GSK-3. These data suggest APC promotes β-catenin

phosphorylation by enhancing GSK-3 activity, and that Wnts reduce GSK-3 activity and β-catenin phosphorylation by causing APC to dissociate from Axin. These data are presented in Chapter 2 as published in Valvezan et al. (Journal of Biological Chemistry, 2012).

Truncating *Apc* mutations are strongly linked to colorectal cancers. Therefore it is essential to identify signaling mechanisms that act downstream to mediate the effects of *Apc* mutation. Constitutive activation of Wnt/β-catenin signaling is widely accepted as the predominant mechanism responsible for *Apc* mutant phenotypes. APC negatively regulates mTORC1 and mTORC1 activation promotes development and progression of many cancers. We hypothesized that truncating *Apc* mutations activate mTORC1 which also contributes to resulting phenotypes. We tested this hypothesis in *Apc* mutant zebrafish and mice. We found mTORC1 is strongly activated in many tissues in *Apc* mutant zebrafish and in intestinal polyps in *Apc* mutant mice. mTORC1 inhibition rescues several *Apc* mutant phenotypes, and combined mTORC1 and Wnt inhibition rescues additional phenotypes. These data indicate a role for mTORC1 downstream of truncating *Apc* mutation, and are presented in Chapter 3.

Taken together, our findings identify novel roles for APC in the Wnt pathway and other GSK-3 dependent signaling pathways. Our work also establishes mTORC1 as a critical effector downstream of oncogenic *Apc* mutations. Conclusions, implications, and future directions will be discussed in Chapter 4.

CHAPTER 2: ADENOMATOUS POLYPOSIS COLI (APC) REGULATES MULTIPLE SIGNALING PATHWAYS BY ENHANCING GLYCOGEN SYNTHASE KINASE-3 (GSK-3) ACTIVITY

2-1. Summary

Glycogen synthase kinase-3 (GSK-3) is essential for many signaling pathways and cellular processes. As Adenomatous Polyposis Coli (APC) functions in many of the same processes, we investigated a role for APC in the regulation of GSK-3-dependent signaling. We find that APC directly enhances GSK-3 activity. Furthermore, knockdown of APC mimics inhibition of GSK-3 by reducing phosphorylation of glycogen synthase and by activating mTOR, revealing novel roles for APC in the regulation of these enzymes. Wnt signaling inhibits GSK-3 through an unknown mechanism, and this results in both stabilization of β-catenin and activation of mTOR. We therefore hypothesized that Wnts may regulate GSK-3 by disrupting the interaction between APC and the Axin-GSK-3 complex. We find that Wnts rapidly induce APC dissociation from Axin, correlating with β-catenin stabilization. Furthermore, Axin interaction with the Wnt co-receptor LRP6 causes APC dissociation from Axin. We propose that APC regulates multiple signaling pathways by enhancing GSK-3 activity, and that

Wnts induce APC dissociation from Axin to reduce GSK-3 activity and activate downstream signaling. APC regulation of GSK-3 also provides a novel mechanism for Wnt regulation of multiple downstream effectors, including β - catenin and mTOR.

2-2. Introduction

Glycogen Synthase Kinase-3 (GSK-3) plays critical roles in a wide variety of essential biological processes during development and throughout adulthood, including tissue patterning, glucose metabolism, apoptosis, stem cell homeostasis and cell cycle regulation (Kockeritz et al., 2006). GSK-3 has over 40 known direct substrates, and regulates many signaling pathways including the Wnt, MAPK/ERK, BMP, mTOR, and Insulin pathways (Fuentealba et al., 2007; Fukuda et al.; Inoki et al., 2006; Kockeritz et al., 2006; Wang et al., 2006). Misregulation of GSK-3 has been proposed to play important roles in human disorders such as bipolar disorder, schizophrenia, Alzheimer's disease, and cancer (Hernandez et al., 2009; Jope and Roh, 2006; Luo, 2009). Therefore understanding the mechanisms that control GSK-3 activity is essential to understanding many biological processes as well as human disorders.
 GSK-3 is a constitutively active kinase that typically inhibits downstream signaling events; thus extracellular signals in general inhibit GSK-3 to induce intracellular signaling events. For example, Insulin promotes N-terminal

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phosphorylation of GSK-3 (Ser9/21), creating a pseudosubstrate motif that inhibits GSK-3 and allows activation of Glycogen Synthase (Cross et al., 1995; Dajani et al., 2001; McManus et al., 2005). Wnt signaling also inhibits GSK-3 activity, but through a mechanism that is independent of ser9/21 phosphorylation (Cook et al., 1996; Ding et al., 2000; McManus et al., 2005). The mechanism of GSK-3 inhibition by Wnts remains poorly understood.

The Wnt signaling pathway is essential for patterning and cell fate specification during embryonic development, and plays critical roles in stem cell homeostasis and tissue regeneration throughout adulthood (Clevers, 2006; MacDonald et al., 2009). Ectopic activation of the Wnt pathway causes many human cancers, including breast, prostate, liver, and most notably colorectal cancers (Paul and Dey, 2008; Salahshor and Woodgett, 2005). In the absence of Wnt ligands, GSK-3, the transcription factor β-catenin, and the tumor suppressor Adenomatous Polyposis Coli (APC) bind directly to the scaffolding protein Axin in a robust complex (Peterson-Nedry et al., 2008) that facilitates destabilizing phosphorylation of β-catenin by GSK-3. Canonical Wnt ligands bind to their receptor Frizzled and induce phosphorylation of the co-receptor LRP5/6, promoting direct interaction between LRP6 and Axin. This reduces GSK-3 activity, allowing stabilization of ß-catenin which then enters the nucleus to activate transcription (Clevers, 2006; MacDonald et al., 2009). Several mechanisms have been proposed to explain Wnt-induced inhibition of GSK-3, including Axin degradation (Lee et al., 2003; Tolwinski et al., 2003) and

sequestration of GSK-3 within multivesicular endosomes (Taelman et al., 2010). However, these events occur several hours after stimulation, whereas reduced GSK-3 activity and β-catenin stabilization are detected within minutes (Cook et al., 1996; Ding et al., 2000). In addition, the LRP6 intracellular domain can directly inhibit GSK-3, suggesting an alternative and plausible mechanism for inhibition of GSK-3 once it is recruited to the receptor/co-receptor complex (Cselenyi et al., 2008; Piao et al., 2008; Wu et al., 2009). However, micromolar concentrations of the LRP6 intracellular domain peptide are required to inhibit GSK-3, and the peptide itself is a GSK-3 substrate and thus may simply compete with the substrates that were assayed for phosphorylation by GSK-3.

Similar to GSK-3, APC also negatively regulates the Wnt pathway by promoting β-catenin phosphorylation and degradation (Hinoi et al., 2000; Su et al., 2008; Yang et al., 2006). APC interaction with Axin requires Ser-Ala-Met-Pro (SAMP) repeats on APC (Behrens et al., 1998). Mutations in *Apc* that delete the SAMP repeats and eliminate Axin binding are strongly linked to colorectal cancers, and cause constitutively elevated β-catenin levels and constitutive activation of Wnt target genes (Munemitsu et al., 1995). These effects can be rescued by reintroduction of wild type APC or fragments of APC that bind Axin through SAMP repeats, but not by SAMP mutant fragments that do not bind Axin (Kawahara et al., 2000; Munemitsu et al., 1995; von Kries et al., 2000). Therefore APC interaction with Axin is critical for downregulating β-catenin and maintaining the Wnt pathway in an inactive state, and thus parallels the function

of GSK-3 in Wnt signaling.

In addition to their parallel roles in Wnt signaling, APC and GSK-3 have similar functions in processes such as cell cycle regulation and apoptosis (Forde and Dale, 2007; Hanson and Miller, 2005). For example, APC and GSK-3 are both required for proper spindle formation during mitosis (Caldwell and Kaplan, 2009; Hanson and Miller, 2005; Wakefield et al., 2003). As APC promotes phosphorylation of β-catenin, a direct GSK-3 substrate, and is involved in many of the same processes as GSK-3, we hypothesized that APC regulates additional GSK-3 substrates and GSK-3 dependent signaling pathways. We find that APC directly regulates GSK-3 activity. Thus APC promotes phosphorylation of the direct GSK-3 substrate Glycogen Synthase independently of Wnt signaling. We also find that APC is required for GSK-3-dependent negative regulation of mTOR. This led us to hypothesize that a key function of APC in the Axin complex is to enhance GSK-3 activity and that Wnts reduce GSK-3 activity by causing dissociation of APC from the Axin complex. In support of this hypothesis, we find that Wnts rapidly disrupt the endogenous APC-Axin interaction. We propose that APC regulates multiple signaling pathways by enhancing GSK-3 activity, and that Wnts cause APC to dissociate from the Axin complex in order to reduce GSK-3 activity and activate downstream signaling.

2-3. Results

2-3-1. APC regulates Glycogen Synthase through GSK-3

APC promotes phosphorylation of the direct GSK-3 substrate β-catenin, and APC and GSK-3 function in many of the same cellular processes (Hanson and Miller, 2005; Kockeritz et al., 2006); we therefore asked whether APC promotes phosphorylation of additional GSK-3 substrates. Glycogen Synthase (GS), the eponymous substrate for GSK-3, is expressed in 293T cells and is constitutively phosphorylated by GSK-3 to inhibit its activity. Knocking down APC with siRNA reduced site-specific phosphorylation of GS, similar to treating with the GSK-3 inhibitor LiCl (Figure 2-1A) or alternative small molecule GSK-3 inhibitors (Supplemental Figure S2-1). These results were confirmed using an additional non-overlapping APC siRNA (Supplemental Figure S2-2). Insulin and other growth factors reduce GS phosphorylation through phosphorylation of Akt and GSK-3 (Alessi et al., 1996; Cross et al., 1995; McManus et al., 2005). Interestingly, knocking down APC did not affect phosphorylation of Akt or GSK-3, indicating APC does not regulate GS by activating Insulin signaling upstream of GSK-3 (Figure 2-1A). GS phosphorylation was rescued after APC knockdown by expressing a large fragment of APC (SAMP fragment) that also rescues β-catenin levels in colorectal carcinoma cells that lack wild type APC (Figure 2-1B). Importantly, LiCl blocked the rescue of GS phosphorylation by APC-SAMP, showing that the rescue is dependent on GSK-3 activity (Figure 2-1B). Knocking down APC in these cells also activated Wnt signaling, as assessed by activity of

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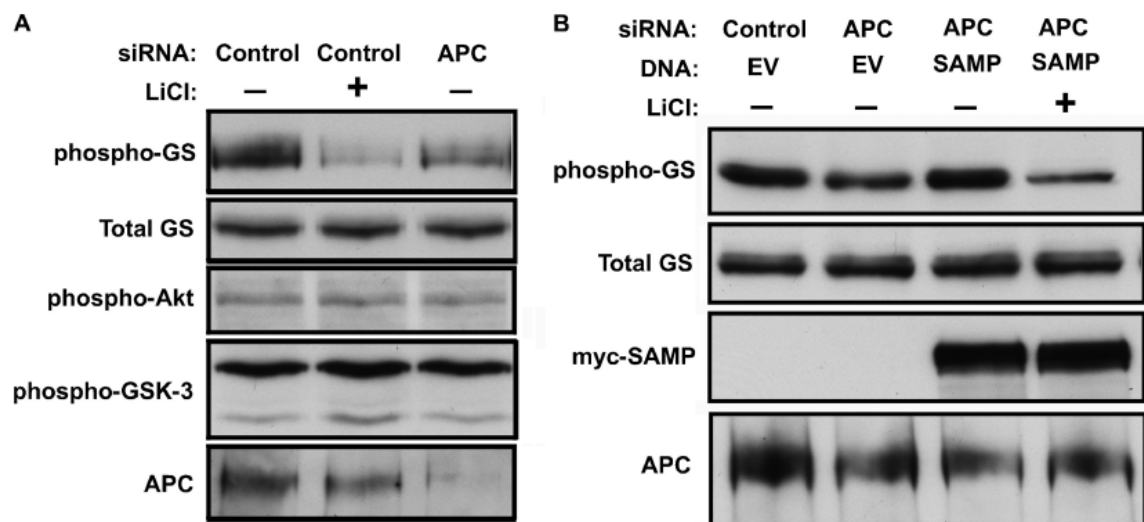
Figure 2-1. APC regulates Glycogen Synthase through GSK-3. (A) APC was knocked down by siRNA in 293T cells and phosphorylation of Glycogen Synthase (GS) at an established GSK-3 target site (GS-Ser641) was assessed by immunoblotting with a phospho-specific antibody. The same samples were immunoblotted for total GS (loading control), phospho-Akt (Ser473), phospho-GSK-3 (Ser9/21), and APC. Cells in lane 2 were also treated with15mM LiCl. Cells were also transfected with a scrambled siRNA (control). APC knockdown reduced APC protein levels and endogenous GS phosphorylation without affecting total GS or phosphorylation of Akt or GSK-3. (B) To rescue GS phosphorylation in APC knockdown cells, the myc-tagged SAMP fragment of APC was co-transfected with scrambled (control) or APC siRNAs. "EV" indicates empty vector control. The SAMP fragment of APC rescues phosphorylation of GS in APC knockdowns, and this rescue is blocked by LiCl.

a β-catenin responsive reporter (Supplemental Figure S2-3A), consistent with the known role for APC as a negative regulator of the Wnt pathway (Clevers, 2006; MacDonald et al., 2009). To rule out the possibility that GS phosphorylation was indirectly regulated by Wnt signaling downstream of the APC/Axin degradation complex, we expressed a stabilized and hence constitutively active form of β-catenin. Although stabilized β-catenin potently activated the Wnt reporter, it had no effect on phospho-GS (Supplemental Figure S2-3B), demonstrating that the reduction in GS phosphorylation in response to APC knockdown is not due to downstream activation of the Wnt pathway. These results suggest a novel role for APC in the regulation of GSK-3 activity.

We also asked whether APC knockdown affects GS regulation by insulin. We generated an HEK293 cell line that stably expresses an siRNA targeting APC (Schneikert and Behrens, 2006). Stable knockdown of APC reduces GS phosphorylation (Supplemental Figure S2-4), similar to Figure 2-1. Insulin also reduces GS phosphorylation in the parental HEK293 line (Alessi et al., 1996), but combined APC knockdown and insulin treatment do not further reduce GS phosphorylation (Supplemental Figure S2-4). Insulin did not induce phosphorylation of GSK-3 stably associated with the endogenous APC/Axin complex (Supplemental Figure S2-5), consistent with previous observations that the Axin-associated/Wnt-regulated subcellular pool of GSK-3 is distinct from insulin-regulated GSK-3 (Ding et al., 2000; McManus et al., 2005). Stable association of APC and GSK-3 independent of the Axin complex has not been

demonstrated to our knowledge.

2-3-2. APC regulates mTOR through GSK-3

GSK-3 negatively regulates mammalian Target of Rapamycin (mTOR), and either pharmacological or genetic inhibition of GSK-3 activates mTOR *in vivo* (Huang et al., 2009; Inoki et al., 2006). If APC promotes phosphorylation of other substrates by GSK-3, in addition to GS and β-catenin, then knockdown of APC may also activate mTOR. In support of this, knockdown of APC activated mTOR, as assessed by increased phosphorylation of ribosomal protein S6, similar to inhibiting GSK-3 with LiCl (Figure 2-2A), other small molecule GSK-3 inhibitors (Supplemental Figure S2-1), or knocking down *Gsk3* expression (Huang et al., 2009; Inoki et al., 2006). This was also confirmed using a non-overlapping APC siRNA (Supplemental Figure S2-2), and rescued by expressing the SAMP fragment of APC. This rescue was also blocked by LiCl (Figure 2-2B). The effect of APC knockdown on S6 phosphorylation was blocked by the mTOR inhibitor rapamycin (Figure 2-2D), further supporting our conclusion that APC functions upstream of mTOR. As above, expression of stabilized β-catenin had no effect on S6 phosphorylation (Supplemental Figure S2-3), indicating that mTOR activation resulting from APC knockdown is not due to activation of Wnt signaling downstream of ß-catenin.

Wnts regulate the GSK-3/APC/Axin complex to inhibit GSK-3 and activate

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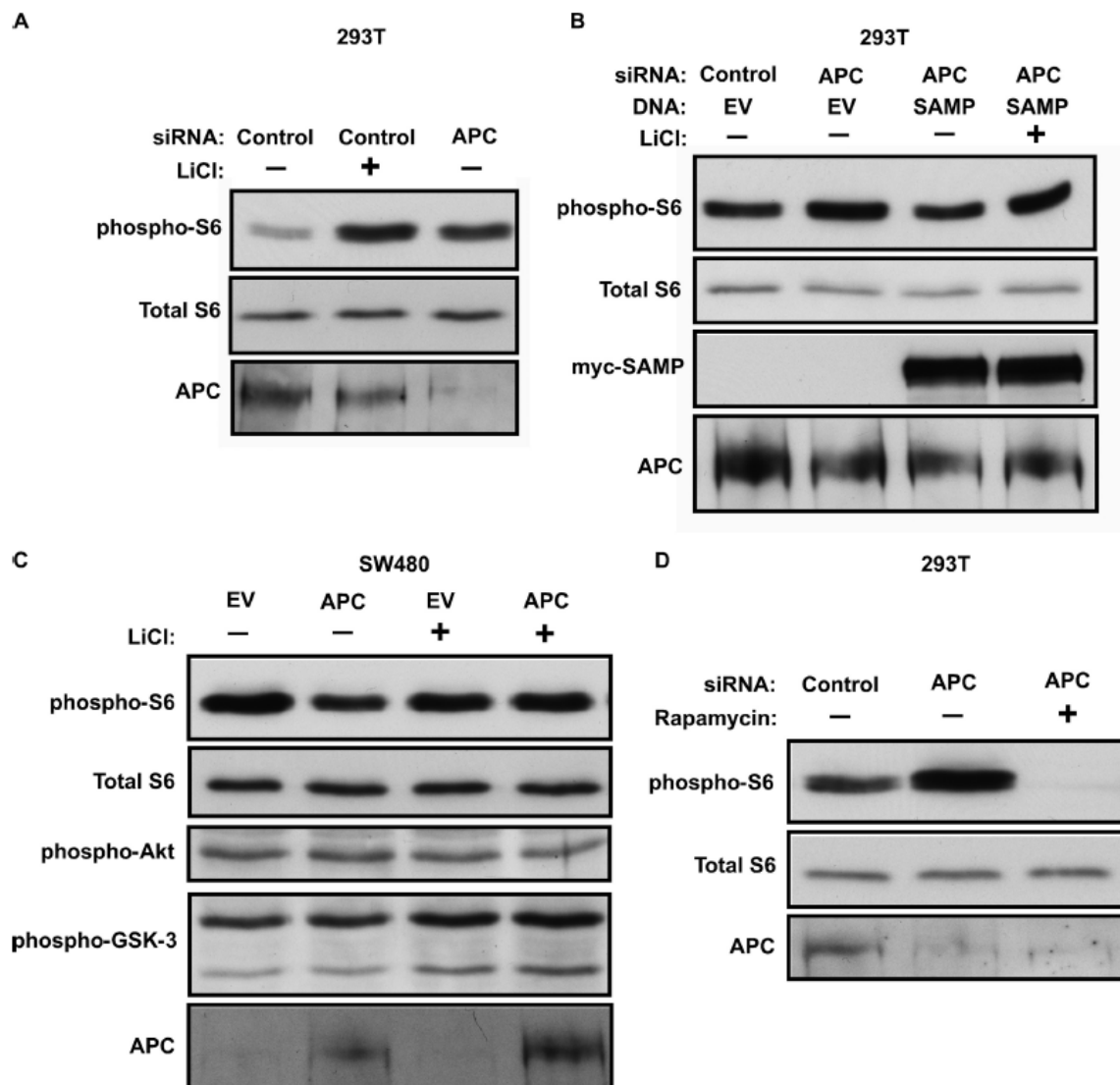
Figure 2-2. APC regulates mTOR through GSK-3. (A) APC was knocked

down by siRNA, as in Figure 2-1, and mTOR activity was assessed as

phosphorylation of ribosomal protein S6 (Ser235/236, upper panel). Inhibition of

GSK-3 with LiCl also activated mTOR. Total S6 (middle panel) is shown as a

loading control. The cell lysates in panels A and B are the same as those used in

Figure 2-1. (B) APC knockdown increased S6 phosphorylation and this effect

was rescued by the SAMP fragment of APC. (C) Expression of full length APC

reduces S6 phosphorylation in SW480 colorectal carcinoma cells, which only

express a truncated APC that lacks the SAMP repeats. Transfection efficiency

was estimated at 30% based on expression of YFP (data not shown). LiCl

increased S6 phosphorylation in cells expressing wild type APC. (D) Rapamycin

(10nM) blocks S6 phosphorylation after APC knockdown.

β-catenin and mTOR (Inoki et al., 2006; MacDonald et al., 2009). As APC promotes Glycogen Synthase phosphorylation by GSK-3, we asked whether Wnts affect Glycogen Synthase phosphorylation. L cells were treated with control or Wnt3a conditioned medium for up to two hours, but no change in GS phosphorylation was detected (Supplemental Figure S2-6A). Furthermore, Axin knockdown activates mTOR as observed previously (Inoki et al., 2006), but does not affect GS phosphorylation (Supplemental Figure S2-6B). Taken together, these data suggest Wnts and Axin regulate mTOR but not GS.

The SW480 human colorectal carcinoma cell line lacks wild-type APC, and instead expresses a truncated APC that fails to interact with Axin or to downregulate β-catenin (Kishida et al., 1998; Munemitsu et al., 1995). Phosphorylation and degradation of β-catenin can be restored in these cells by reintroduction of wild type APC (or the SAMP fragment) (Hinoi et al., 2000; Munemitsu et al., 1995; Su et al., 2008; Yang et al., 2006). Interestingly, S6 phosphorylation readily detected in untreated SW480 cells was not further increased by LiCl, in contrast to the effects observed in 293T cells, which express wild type APC (Figure 2-2C, compare lanes 1,3). We hypothesized that mTOR is aberrantly active in these cells due to the truncating APC mutations. Consistent with this idea, reintroduction of wild type APC reduces S6 phosphorylation, suggesting a novel role for APC as a negative regulator of mTOR. Furthermore, when wild-type APC was introduced into SW480 cells, LiCl activated mTOR (Figure 2-2C, compare lanes 2,4), indicating that restoring wild-

type APC restores GSK-3 activity and mTOR sensitivity to LiCl. Restoring wild type APC in SW480 cells did not affect Akt or GSK-3 phosphorylation. These data support a signaling pathway in which APC, acting through GSK-3, suppresses mTOR activity.

2-3-3. APC directly enhances GSK-3 activity.

Knocking down APC reduced phosphorylation of GS, a direct GSK-3 substrate, without affecting known signaling mechanisms upstream of GSK-3. To test whether APC regulates GSK-3 activity directly, we performed *in vitro* kinase reactions using purified recombinant proteins. Surprisingly, addition of the SAMP fragment of APC increases GSK-3-dependent phosphorylation of both Tau protein (Figure 2-3A lanes 2,3) and β-catenin (Figure 2-3B lanes 3,4) by approximately 5-7 fold. GSK-3 dependent phosphorylation of ß-catenin normally occurs within the Axin complex, and requires a priming phosphorylation of ß-catenin by Casein Kinase I alpha (CKIα) (Liu et al., 2002). Thus, we added Axin and Casein Kinase I (CKI) to the *in vitro* kinase reaction and again observed that SAMP further enhanced β-catenin phosphorylation by GSK-3. Thus, APC directly enhances GSK-3 activity towards multiple substrates both in the presence and absence of the Axin complex.

We also asked whether restoring wild type APC in APC-deficient colorectal carcinoma cells could enhance GSK-3 activity. We used an inducible

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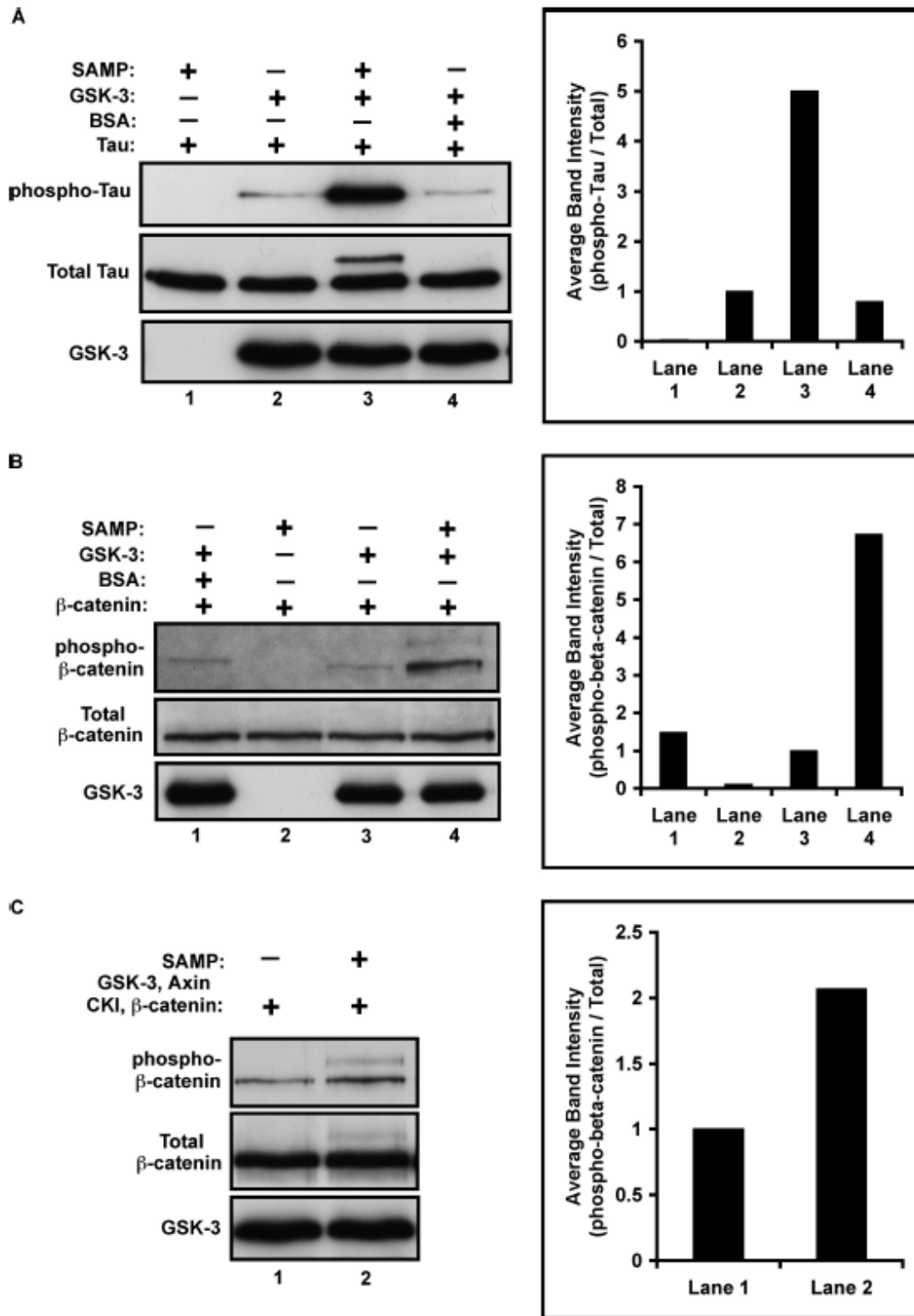
Figure 2-3

Figure 2-3. APC directly enhances GSK-3 activity. *In vitro* kinase reactions were carried out using purified recombinant proteins. (A) Addition of the SAMP fragment (80nM) of APC, but not BSA, directly enhances Tau phosphorylation (Ser 396/404) by GSK-3 by approximately 5 fold. For image quantification, phospho-Tau was normalized to total Tau and then all lanes were normalized to Lane 2. (B) SAMP, but not BSA, directly enhances β-catenin phosphorylation (Ser33/37/41) by GSK-3 by approximately 7 fold. For image quantification, phospho-β-catenin was normalized to total β-catenin and then all lanes were normalized to Lane 3. (C) SAMP enhances β-catenin phosphorylation by GSK-3 in the presence of Axin and Casein Kinase I (CKI) by approximately 2 fold. For image quantification, phospho-β-catenin was normalized to total β-catenin and then Lane 2 was normalized to Lane 1.

APC cell line derived from HT29 cells, human colorectal carcinoma cells that express a truncated APC similar to SW480 cells (Morin, 1999). HT29-APC cells express full length APC under control of the zinc-inducible metallothionein promoter and control HT29-ß-gal cells express ß-galactosidase (Supplemental Figure S2-7C). We immunoprecipitated endogenous GSK-3 from untreated or zinc treated cells and measured phosphorylation of a peptide derived from Glycogen Synthase (GS-2) by incorporation of ^{32}P (Supplemental Figure S2-7). APC induction enhances GS-2 phosphorylation, whereas zinc treatment in HT29-ß-gal cells has no effect. The GSK-3 inhibitor SB216763 blocks GS-2 phosphorylation in all groups indicating that GS-2 is phosphorylated primarily by GSK-3 in this assay. These data confirm that wild type APC enhances the activity of GSK-3 recovered from APC-deficient colorectal carcinoma cells.

2-3-4. Wnt3a induces Axin/APC dissociation

In the canonical Wnt pathway, β-catenin is constitutively phosphorylated by GSK-3 within the Axin complex and targeted for degradation. APC interaction with Axin is required for β-catenin phosphorylation and destabilization. Binding of Wnts to the surface receptor complex leads to inhibition of GSK-3 activity through an as yet poorly characterized mechanism (Clevers, 2006; MacDonald et al., 2009). As our data show that APC enhances GSK-3 activity in general and can specifically enhance GSK-3 phosphorylation of ß-catenin within the Axin complex

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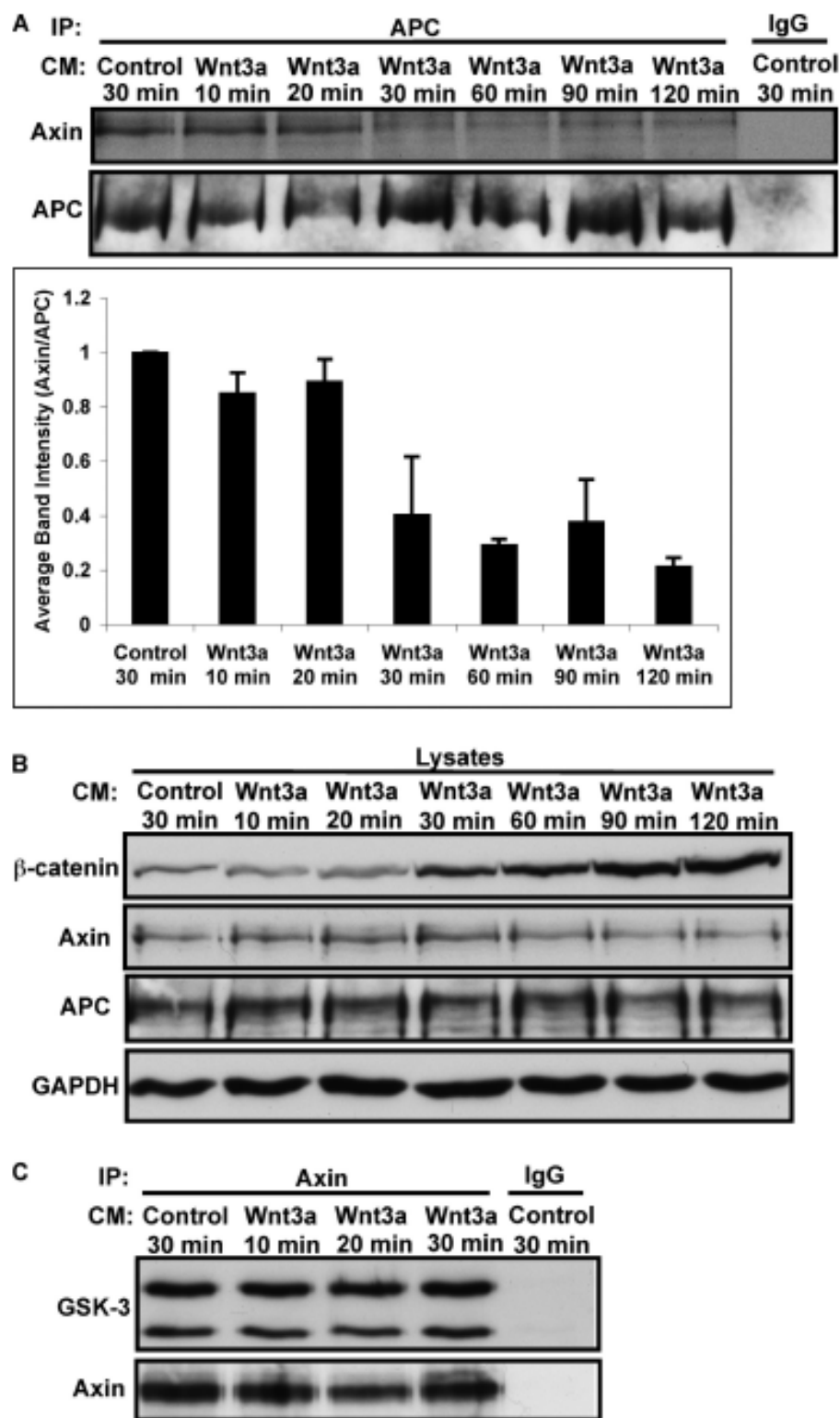
Figure 2-4

Figure 2-4. Wnt signaling disrupts the interaction between APC and Axin. L cells were treated with Control or Wnt3a conditioned medium (CM) for indicated times. (A) APC was immunoprecipitated from control and Wnt3a treated cells and samples were immunoblotted for Axin or APC. For image quantification, Axin was normalized to APC and then all lanes were normalized to control (Lane 1). Error bars represent standard error of the mean of four independent experiments. (B) Lysates from panel A were immunoblotted for β-catenin, Axin, APC and GAPDH. β-catenin accumulation in response to Wnt activation correlates with Axin/APC dissociation in panel A. (C) Wnt3a does not affect GSK-3 association with Axin.

(Figure 2-3), we hypothesized that Wnt signaling may reduce GSK-3 activity by inducing dissociation of APC from the Axin complex. To test this, we treated L cells with control or Wnt3a conditioned medium for up to 2 hours, immunoprecipitated endogenous APC, and detected endogenous APC-associated Axin. The amount of Axin associated with APC was dramatically reduced by 30 minutes, and interaction was not restored even after 2 hours (Figure 2-4A). Similar results were obtained when Axin was immunoprecipitated, when purified recombinant Wnt3a was used instead of Wnt3a conditioned medium, and when 293T cells were treated with Wnt3a (data not shown). Importantly, observation of Wnt-induced Axin/APC dissociation was sensitive to lysis and IP conditions, especially the pH of the lysis buffer (see methods). The amount of GSK-3 associated with Axin did not change under these conditions (Figure 2-4C). Interestingly, the onset of β-catenin accumulation correlated with APC/Axin dissociation (Figure 2-4B). Taken together, these results show that activation of the Wnt pathway causes APC/Axin dissociation, and this may contribute to reduced GSK-3 activity and subsequent β-catenin stabilization.

2-3-5. Axin binding to LRP6 causes APC dissociation

The Wnt co-receptor LRP6 is phosphorylated in response to canonical Wnt ligands, and LRP6 phosphorylation promotes direct interaction with Axin (Mao et al., 2001; Tamai et al., 2004; Zeng et al., 2005). As Wnt3a causes

dissociation of APC from Axin, we hypothesized that the recruitment of Axin to phosphorylated LRP6 may displace APC from the Axin complex. Deletion of the N-terminal extracellular domain of LRP6 results in a constitutively active LRP6 fragment (CA-LRP6) that is phosphorylated by GSK-3, interacts with Axin, and results in β-catenin stabilization and activation of Wnt target genes (Tamai et al., 2004; Zeng et al., 2005), (Supplemental Figure S2-8). We found that co-expression of CA-LRP6 and Axin markedly reduces the relative amount of APC associated with Axin (Figure 2-5A, compare lanes 3,4). In addition, inhibition of GSK-3 with LiCl reduced the Axin-CA-LRP6 interaction and restored APC-Axin interaction (Figure 2-5A, lanes 3-5), consistent with the known role for GSK-3 in phosphorylation and activation of LRP6. Conversely, LiCl had no effect on Axin-APC association in the absence of the CA-LRP6 fragment (Figure 2-5D). Importantly, the nonfunctional LRP6 mutant LRP6ΔN1Ab did not recruit Axin or activate Wnt signaling, as shown previously (Tamai et al., 2004; Zeng et al., 2005), (Supplemental Figure S2-8), and had no effect on Axin-APC interaction (Figure 2-5C). Importantly, there was no detectable APC association with CA-LRP6 despite significant Axin association with CA-LRP6 (Figure 2-5A, lane 2). These results show that recruitment of Axin to the activated form of LRP6 displaces APC from the Axin complex, and suggest that Wnt induced Axin-LRP6 interaction causes APC dissocation.
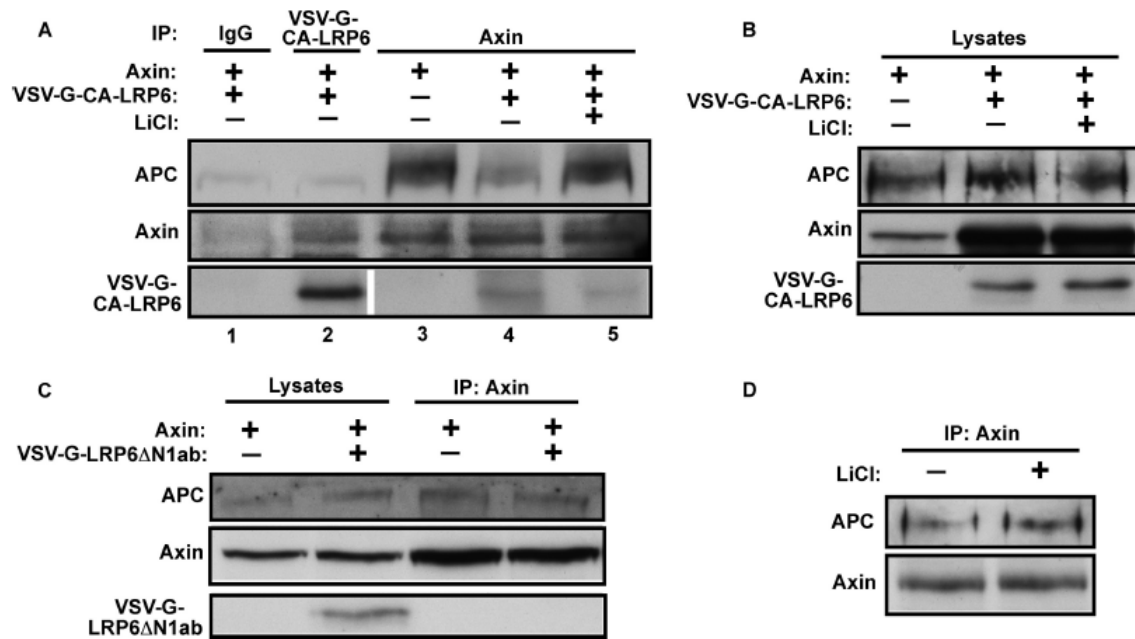
Figure 2-5

Figure 2-5. LRP6 binding to Axin causes APC dissociation. (A) A constitutively active form of LRP6 (VSV-G-CA-LRP6) was expressed in 293T cells with Axin, and cell lysates were immunoprecipitated with antibodies to VSV-G, Axin, or control antibody, and then immunoblotted for APC, Axin, or VSV-G-CA-LRP6. Axin IP samples were adjusted for equal loading of Axin. Constitutively active LRP6 reduces APC association with Axin (compare lanes 3,4). LiCl (15mM) reduces LRP6/Axin association and restores APC/Axin association (Lane 5). APC association with CA-LRP6 was not detected (Lane 2). (B) Western Blot analysis of APC, Axin and CA-LRP6 in lysates used for immunoprecipitation in Panel A. Co-expression of Axin and CA-LRP6, but not LRP6ΔN1ab, increased Axin levels for unclear reasons. (C) The unphosphorylatable VSV-G-LRP6ΔN1ab does not associate with Axin and does not affect Axin/APC interaction. (D) 15mM LiCl alone does not affect Axin/APC interaction.

2-4. Discussion

In this study, we defined a role for APC in the regulation of GSK-3 and GSK-3 dependent pathways, including Glycogen Synthase, mTOR, and Wnt signaling (Figure 2-6A).

APC mutations associated with colorectal cancer strongly activate Wnt signaling, and here we show that these mutations also activate mTOR, a metabolic sensor that is aberrantly activated in many cancers (Zoncu et al., 2011). As GSK-3 suppresses both Wnt and mTOR pathways (Inoki et al., 2006; MacDonald et al., 2009), our demonstration that APC enhances GSK-3 activity provides a mechanistic link between APC loss of function and activation of these two downstream signaling pathways. In support of this mechanism, tumor formation in mice with similar APC mutations is blocked by mTOR inhibitors (Fujishita et al., 2008; Koehl et al., 2010). Furthermore, bladder specific β-catenin stabilization synergizes with PTEN deletion, which activates mTOR, to promote bladder cancer in mice (Ahmad et al., 2011). Taken together, these data support the hypothesis that mTOR activation is required for APC-mediated tumor formation and suggest mTOR as a potential therapeutic target for treatment of cancers resulting from APC mutations.

We also show that APC promotes phosphorylation of Glycogen Synthase by GSK-3, implicating APC in glucose metabolism and glycogen synthesis for the first time. Interestingly, addition of Wnt ligands or knockdown of Axin activates β-

catenin and mTOR but does not affect GS phosphorylation. These data suggest APC and GSK-3 regulation of GS is independent of the Axin complex, unlike regulation of β-catenin and mTOR. Axin knockdown in *Drosophila* S2 cells increases glycogen levels but this may be through regulation of the *Drosophila* c-Cbl Associated Protein (DCAP) rather than GSK-3 and GS (Yamazaki and Yanagawa, 2003).

In addition to regulating Wnt, Glycogen Synthase, and mTOR signaling, APC and GSK-3 both promote mitotic spindle formation, negatively regulate MAPK/ERK and BMP signaling (Figure 2-6A), and regulate apoptosis (Forde and Dale, 2007; Fuentealba et al., 2007; Fukuda et al., 2010; Hanson and Miller, 2005; Miclea et al., 2011; Park et al., 2006; Wang et al., 2006). Taken together, these observations suggest a widespread role for APC in the regulation of GSK-3 dependent signaling. Future studies are needed to determine whether APC regulates additional GSK-3 dependent pathways such as the Notch, Hedgehog, and NF-κB pathways.

The finding that APC enhances GSK-3 activity prompted us to ask whether APC is also regulated by Wnt signaling to reduce GSK-3 activity. Canonical Wnts reduce GSK-3 activity through unknown mechanisms, and this is critical for β-catenin stabilization (Cook et al., 1996; Ding et al., 2000). We find that Wnts rapidly induce APC dissociation from Axin, in close temporal correlation with β-catenin accumulation. Furthermore, Axin interaction with activated LRP6, the Wnt co-receptor, causes APC dissociation. Therefore we

Figure 2-6
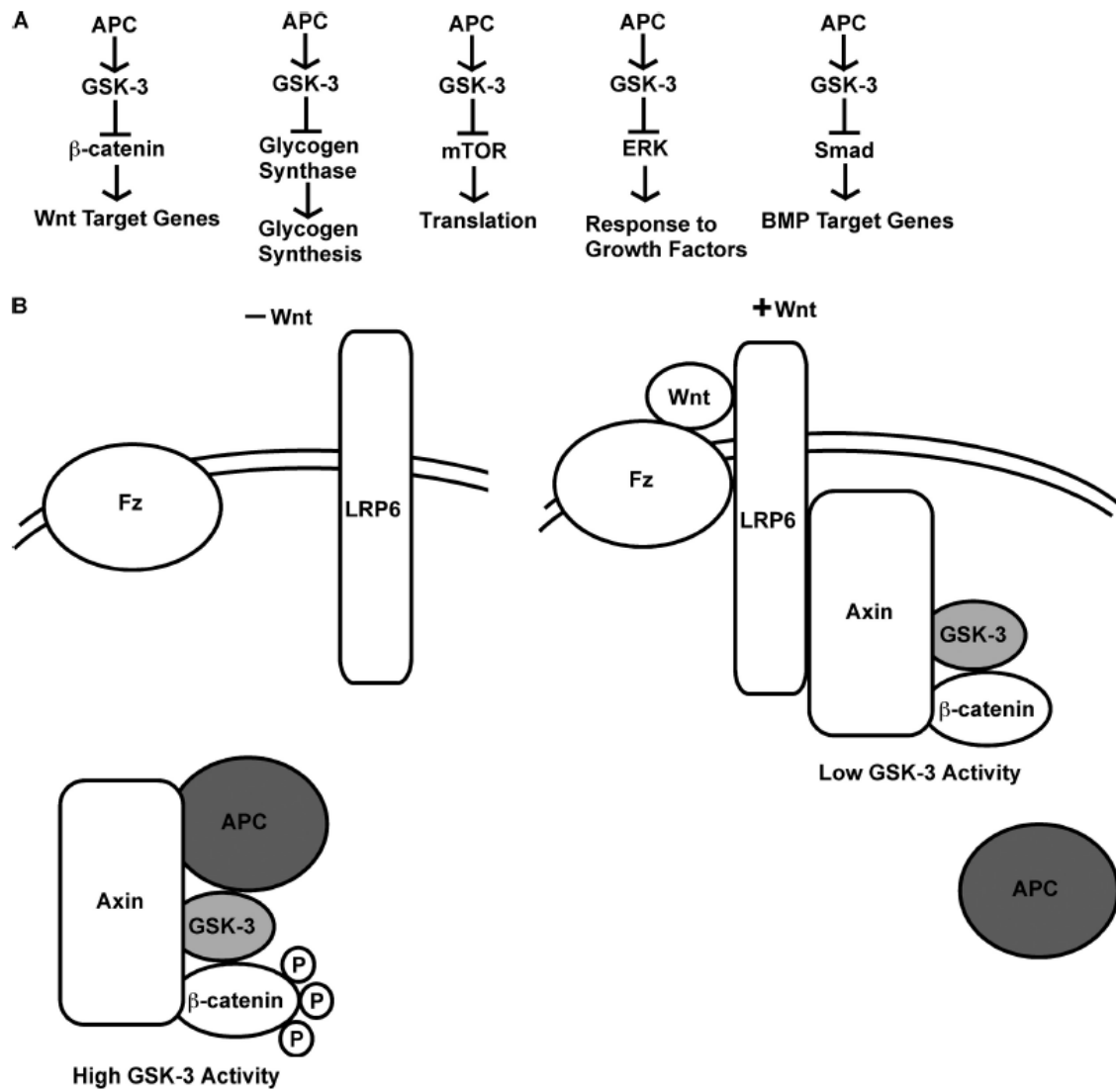
Figure 2-6. Model. (A) APC negatively regulates multiple signaling pathways, including the Wnt, Glycogen Synthase, mTOR, ERK, and BMP pathways by enhancing GSK-3 activity. (B) Wnts disrupt the Axin/APC interaction to reduce GSK-3 activity and thus activate downstream signaling

propose that Wnt-induced Axin recruitment to LRP6 displaces APC, reducing GSK-3 activity and activating downstream signaling (Figure 2-6B). Consistently, Wnt-1 induces membrane localization of Axin and GSK-3, but not APC (Chen et al., 2006).

Previous work showed APC is required for β-catenin phosphorylation and degradation, and this was attributed to APC recruiting β-catenin to the Axin complex (Hinoi et al., 2000; Yang et al., 2006). While this work is compatible with our findings, we also find that APC enhances GSK-3 activity towards multiple substrates, including β-catenin. Consistently, deletion of the RGS domain of Axin, which binds APC, reduces GSK-3 activity and activates Wnt signaling (Hedgepeth et al., 1999). Interestingly, APC also enhances activity of topoisomerase IIα (Wang et al., 2008), possibly suggesting a novel widespread function for APC in regulating enzymatic activity.

APC also blocks dephosphorylation of β-catenin by protein phosphatase-2A (PP2A) (Su et al., 2008). Therefore Wnt induced APC-Axin dissociation may stabilize β-catenin by both reducing GSK-3 activity and allowing PP2A mediated β-catenin dephosphorylation. Interestingly, this mechanism parallels Glycogen Synthase activation by Insulin which induces both inhibition of GSK-3 and dephosphorylation of Glycogen Synthase by protein phosphatase-1 (Brady and Saltiel, 2001).

Recent work has shown that Wnt signaling induces sequestration of the GSK-3 into multivesicular bodies, and this was suggested as a mechanism for

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attenuating phosphorylation and degradation of β-catenin (Taelman et al., 2010). However, that work examined GSK-3 several hours after stimulation with Wnts, whereas we observe dissociation of APC from Axin within 30 minutes, closely correlated with accumulation of β-catenin protein. Because of the different time scales, the work of Taelman et al is not incompatible with our findings, and may represent a later consequence of Wnt signaling.

GSK-3 negatively regulates mTOR by phosphorylating Tuberous Sclerosis Complex 2 (TSC2). TSC2 associates with the Axin complex, and canonical Wnts activate mTOR by inhibiting GSK-3 and reducing TSC2 phosphorylation (Inoki et al., 2006). As we show APC enhances GSK-3 activity and negatively regulates mTOR, Wnt-induced APC dissociation from Axin also provides a mechanism for how Wnts activate mTOR.

Since APC regulates multiple GSK-3 dependent processes, and is regulated by Wnts, additional studies are needed to determine whether APC can be regulated to reduce GSK-3 activity in other contexts. For example, during hippocampal development, axonal-dendritic polarity is established by selection of one of several minor neurites to grow into the mature axon (Dotti et al., 1988). This process involves localized inhibition of GSK-3 through unknown mechanisms (independent of S9/21 phosphorylation), and concomitant spatial relocation of APC (Gartner et al., 2006; Jiang et al., 2005; Shi et al., 2004). Thus, APC sequestration might reduce GSK-3 activity to promote hippocampal neurite outgrowth.

In conclusion, we demonstrate novel roles for APC as a negative regulator of both Glycogen Synthase and mTOR, like GSK-3, and uncover a novel ability of APC to directly enhance GSK-3 activity. We also show that Wnts induce APC-Axin dissociation, suggesting a new mechanism for how Wnts decrease GSK-3 activity. Further studies are needed to investigate a role for APC in additional GSK-3 dependent processes, and to determine whether APC is regulated in other contexts to reduce GSK-3 activity.

2-5. Materials and Methods

2-5-1. Cell culture, conditioned medium and transfections

HEK293 cells (ATCC #CRL-1573), HEK293T OT-Luciferase cells (Phiel et al., 2001), L cells (ATCC #CRL-2648), L-Wnt3a cells (ATCC #CRL-2647), and SW480 cells (ATCC #CCL-228) were cultured in Dulbecco's Modified Eagle Medium (GIBCO #11965) supplemented with 10% Fetal Bovine Serum (Hyclone #SH30071.03) and 1% penicillin/streptomycin (GIBCO #15140). Insulin was purchased from Sigma (#I9278). Control and Wnt3a conditioned media were prepared from L and L-Wnt3a cells respectively according to ATCC instructions. Cells were transfected 24 hours after plating using Lipofectamine 2000 (Invitrogen #11668) for plasmids or Lipofectamine RNAiMax (Invitrogen #13778) for siRNAs according to manufacturer's instructions. Transfections were done in

Opti-MEM Reduced Serum Medium (GIBCO #31985).

2-5-2. Plasmids and siRNAs

Myc-SAMP (APC 1211-2075) in pEF-BOS vector was provided by Akira Kikuchi (Hinoi et al., 2000). VSV-G-LRP6DN1 (constitutively active LRP6) and VSV-G-LRP6ΔN1ab (nonfunctional LRP6) both in pCS2+ vector were provided by Xi He (Tamai et al., 2004). Axin in pCS2+MT vector was described previously (Hedgepeth et al., 1999). APC in pCMV vector was provided by Bert Vogelstein and Kenneth Kinzler (Smith et al., 1994). Myc-tagged stabilized β-catenin (T41A/S45A) in pCS2+MT vector was provided by Ed Morrisey (Xu et al., 2000). Axin and GFP shRNAs expressed from pSUPER vector were provided by Xiao-Fan Wang (Guo et al., 2008). APC siRNA expressed from pSUPER vector (pH1iAPC729) was provided by Jean Schneikert (Schneikert and Behrens, 2006). The following siRNAs were purchased from Applied Biosystems and transfected at a final concentration of 75nM; Silencer Negative Control #1 (Catalog #AM4635), APC (siRNA ID #42812), APC (siRNA ID #s1435).

2-5-3. Western blotting, immunoprecipitations and luciferase assays.

Cells were lysed in buffer containing 20mM Tris pH 7.5, 140mM NaCl, 1mM EDTA, 10% glycerol, 1% Triton X-100, 1mM DTT, 50mM NaF, and

protease inhibitor cocktail (Sigma P8340), phosphatase inhibitor cocktail #1 (Sigma P2850) and #2 (Sigma P5726) or #2 and #3 (Sigma P0044) used 1:100 each. Lysis buffer used in Figure 2-4 contained 20mM Tris pH 8.0 instead of pH 7.5. Supernatants were collected after centrifugation at 13,000 rpm for 5 min at 4°C. For immunoprecipitation, lysates were incubated with antibodies to Axin (Kofron et al., 2001), APC (Santa Cruz #sc-896), VSV-G (Sigma #V4888) or control IgG (Thermo Scientific #31235) for 2 hrs, and then Protein G Agarose beads (Invitrogen #15920) were added for an additional 2 hrs. Antibody-bound beads were washed with lysis buffer 3 times for 5 min each and resuspended in standard 2X Laemmli Sample Buffer. All steps were done at 4°C. Samples were heated for 5 min at 95°C before SDS-PAGE and western blot analysis using the following antibodies from Cell Signaling: phospho-Glycogen Synthase (#3891), Glycogen Synthase (#3893), phospho-Akt (#4058), phospho-GSK-3 (#9331), Axin (#3323), phospho-S6 (#4858), total S6 (#2317), phospho-β-catenin (#9561), GAPDH (#2118), or antibodies to GSK-3 (Calbiochem #368662), APC (Santa Cruz #sc-896 or Abcam #ab58), myc (Sigma #C3956), β-catenin (BD Transduction Laboratories #610153), VSV-G (Sigma #V4888), Axin (Kofron et al., 2001), phospho-Tau (PHF-1 antibody provided by Peter Davies (Greenberg et al., 1992)), or total Tau (T14/46 antibodies provided by Virginia Lee). For luciferase assay, cells were lysed in 1X Promega Passive Lysis Buffer (Promega E194A) and luciferase assay was performed using the Promega Dual Luciferase Reporter Assay System (Promega #E1910) according to manufacturer's

instructions.

2-5-4. In vitro kinase reactions and image quantification

Purified recombinant proteins were incubated in kinase reaction buffer (100mM Tris pH 7.5, 5mM DTT, 10mM MgCl$_2$, 100µM ATP) for 10 min at 30°C. Reactions were stopped by adding standard 2X Laemmli Sample Buffer and incubating at 95°C for 5 min. The following protein concentrations were used; 80nM GST-SAMP, 100nM His-β-catenin, 100nM His-Axin, 100nM Tau, 80nM BSA (Sigma #A9647), 2 units/µl GSK-3 (New England Biolabs #P6040), 4 units/µl CKI (New England Biolabs #P6030). Western blot images were quantified using ImageJ software. For Supplemental Figure S2-7, cells were lysed in standard RIPA buffer, immunoprecipitated with anti-GSK-3 antibody, and GSK-3 kinase activity assay was performed as previously described (Klein and Melton, 1996).

2-6. Supplemental Material

Supplemental Figure S2-1

A

DMSO CHIR99021

phospho-GS

Total GS

phospho-S6

Total S6

B

DMSO   6-BIO

phospho-GS

Total GS

phospho-S6

Total S6
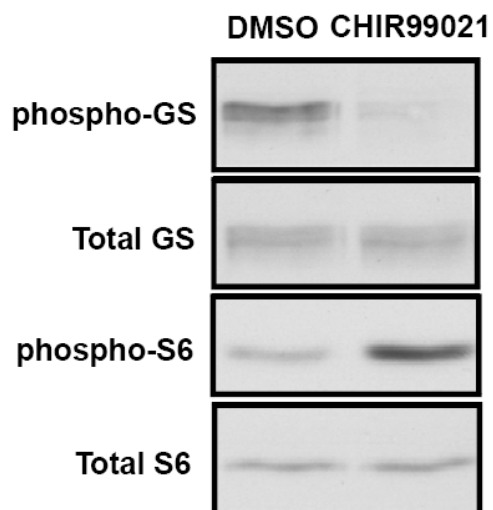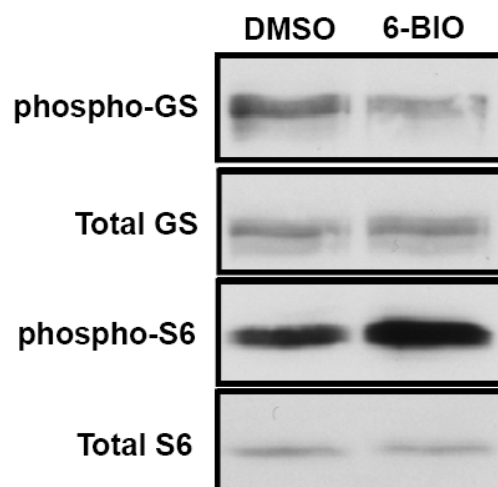
Supplemental Figure S2-1. GSK-3 inhibition reduces GS phosphorylation and activates mTOR. (A) CHIR99021 (6µM), or (B) 6-Bromoindirubin-3'-oxime (5µM) reduces GS phosphorylation (Ser641) and increases S6 phosphorylation (Ser235/236) in 293T cells.

Supplemental Figure S2-2
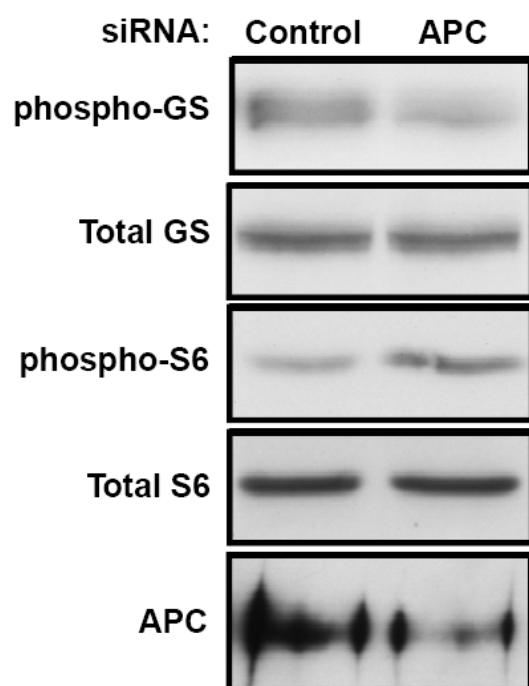
Supplemental Figure S2-2. APC knockdown with a second, non-overlapping siRNA reduces GS phosphorylation and activates mTOR. APC was knocked down in 293T cells by transfection of Applied Biosystems APC siRNA ID #s1435 which targets a non-overlapping sequence compared to Applied Biosystems APC siRNA ID #42812 used in Figures 2-1 and 2-2. APC knockdown with this second siRNA also reduces GS phosphorylation and increases S6 phosphorylation.

Supplemental Figure S2-3

A

B

59
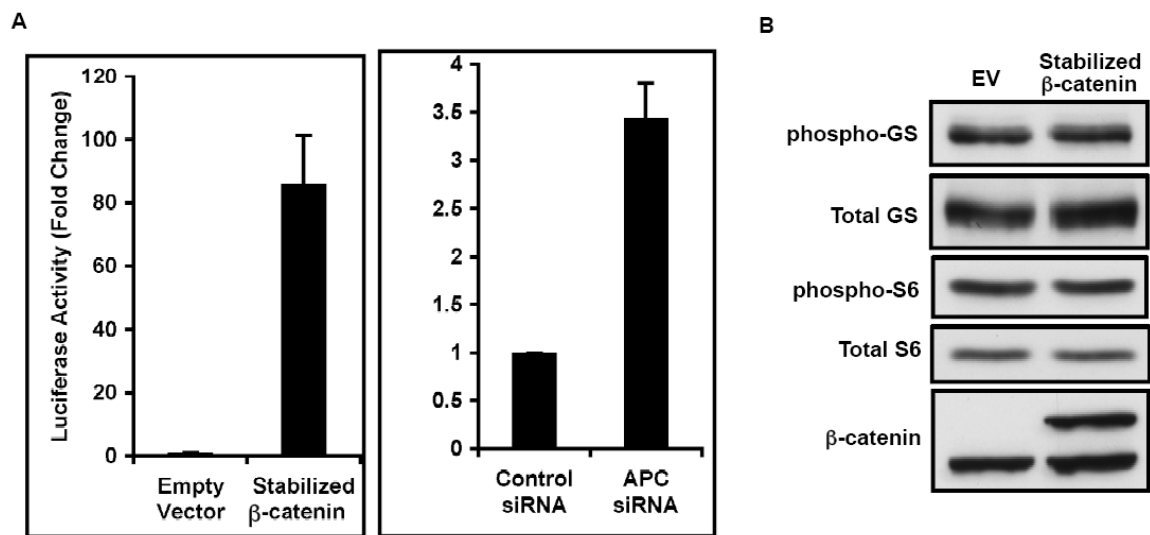
Supplemental Figure S2-3. Downstream Wnt pathway activation does not affect GS or S6 phosphorylation. Stabilized ß-catenin (T41A/S45A) expressed in 293T OT Luciferase cells robustly activated the TOPFLASH Wnt reporter (A), but did not affect phospho-GS (Ser641) or phospho-S6 (Ser235/236) (B). Error bars represent standard error of the mean.

Supplemental Figure S2-4
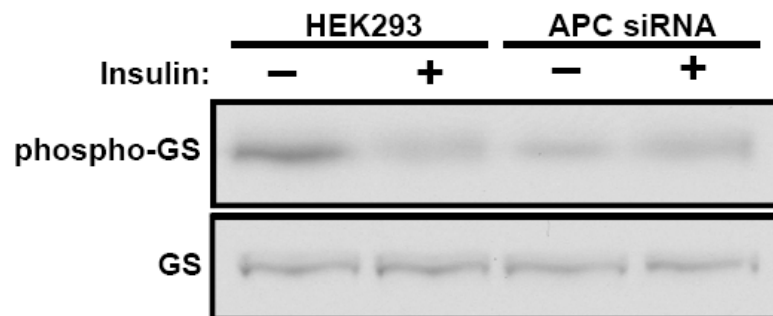
Supplemental Figure S2-4. Insulin-dependent inhibition of GS phosphorylation is not enhanced by concomitant APC knockdown. HEK293 cells stably expressing siRNA targeting APC and control HEK293 cells were treated with 2µM insulin for 30 min. APC knockdown reduces GS phosphorylation in the absence of insulin and insulin reduces GS phosphorylation in control HEK293 cells. Combined APC knockdown and insulin treatment does not further reduce GS phosphorylation.

Supplemental Figure S2-5

A
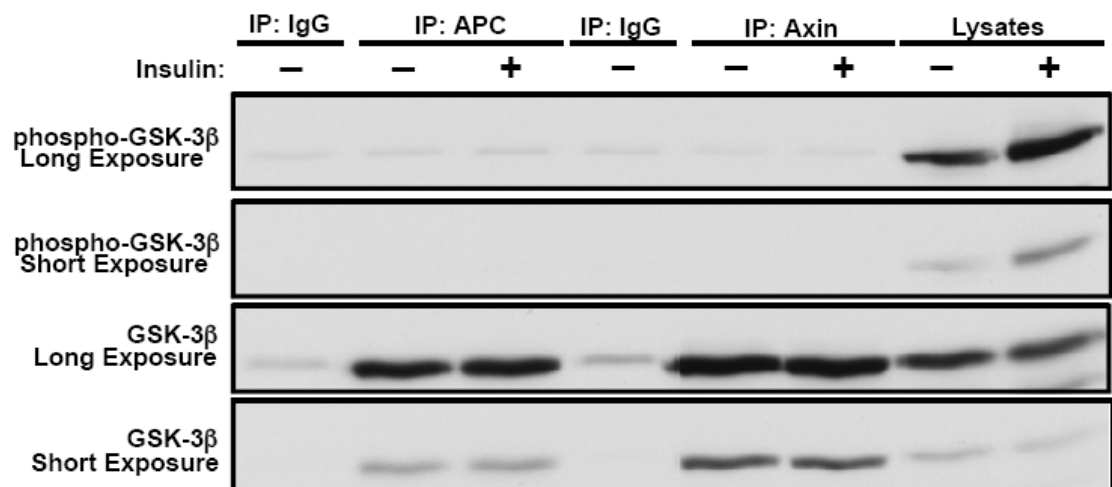
B
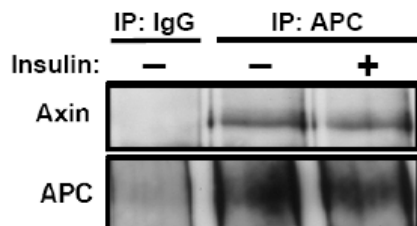
C

D
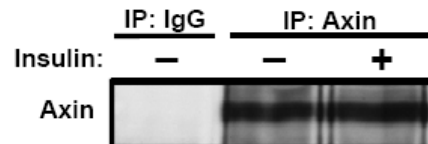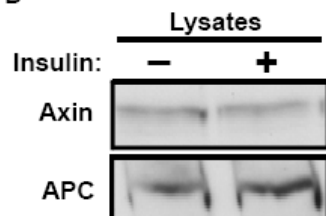
Supplemental Figure S2-5. Insulin does not induce phosphorylation of GSK-3 associated with the Axin/APC complex. 293T cells were treated with 2µM insulin for 30 min followed by immunoprecipitation of endogenous protein using control IgG, APC, or Axin antibodies. (A) Insulin induced serine-9 phosphorylation of GSK-3ß in whole cell lysates but not GSK-3ß associated with immunoprecipitated APC or Axin, which detects primarily the Axin/APC complex. The longer exposures show background GSK-3ß or phospho-GSK-3ß present in immunoprecipitates to make it clear that, at the limit of detection, there is no change in phosphorylation of GSK-3ß associated with this complex, as described previously (Ding et al., 2000). (B) Immunoblot of APC and Axin present in APC IPs. (C) Immunoblot of Axin in Axin IPs. (D) Insulin does not affect Axin or APC levels in the input lysates.

Supplemental Figure S2-6

A

B
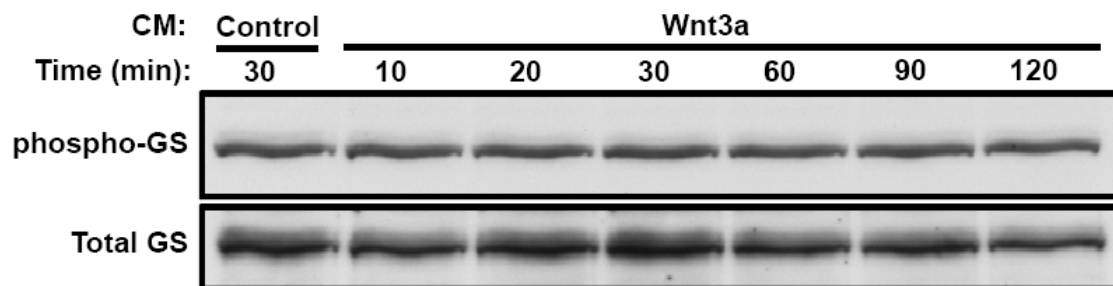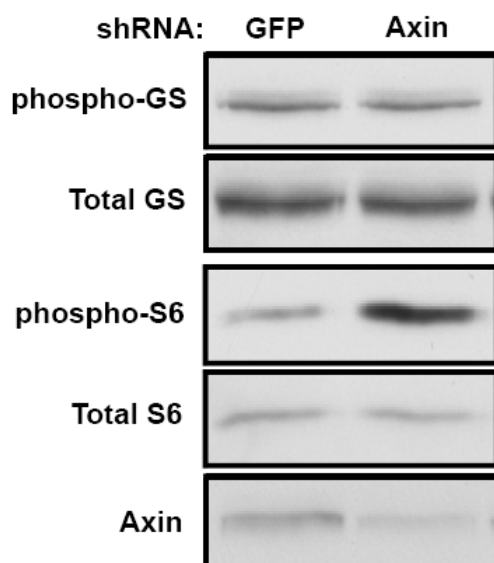
Supplemental Figure S2-6. Glycogen Synthase phosphorylation is not regulated by Wnt3a or Axin. (A) L cells were treated with control or Wnt3a conditioned medium for up to two hours. Samples are the same as those used in Figure 2-4B. Wnt3a stabilized β-catenin (see Figure 2-4B) but did not affect GS phosphorylation. (B) 293T cells were transfected with GFP or Axin shRNA. Axin knockdown increased S6 phosphorylation but did not affect GS phosphorylation.

Supplemental Figure S2-7

A
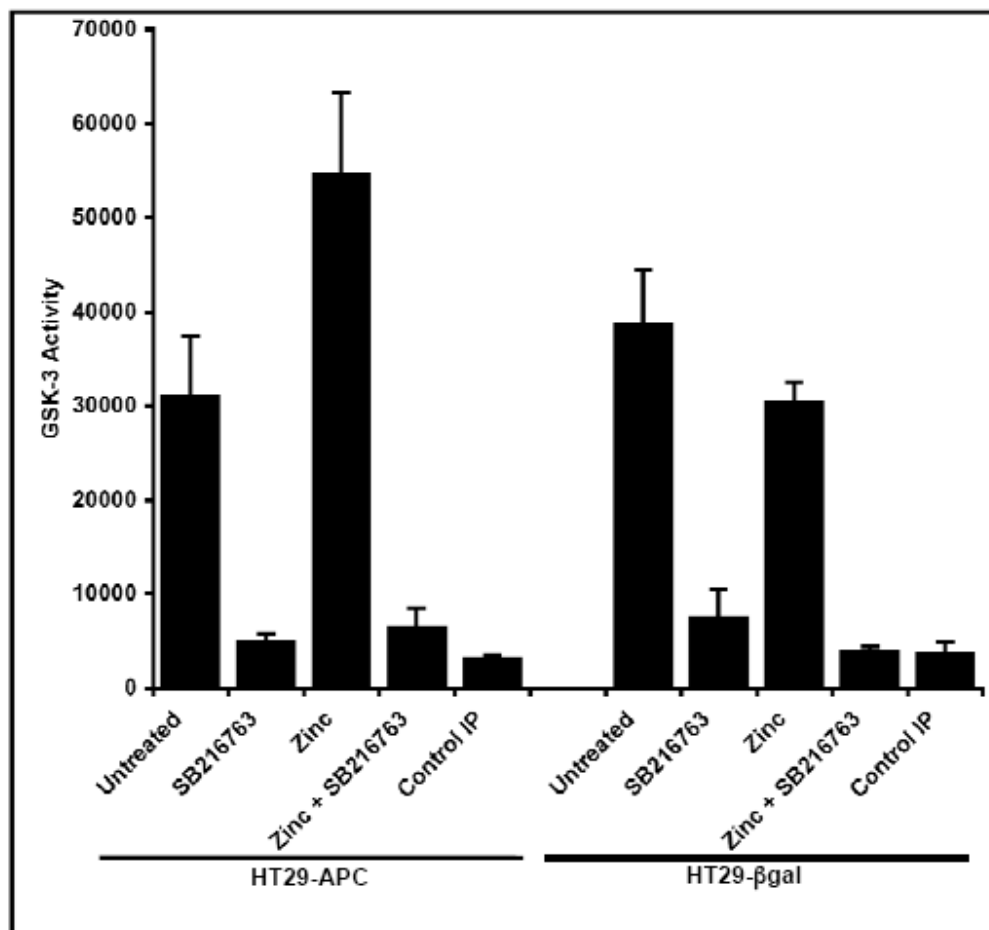
B
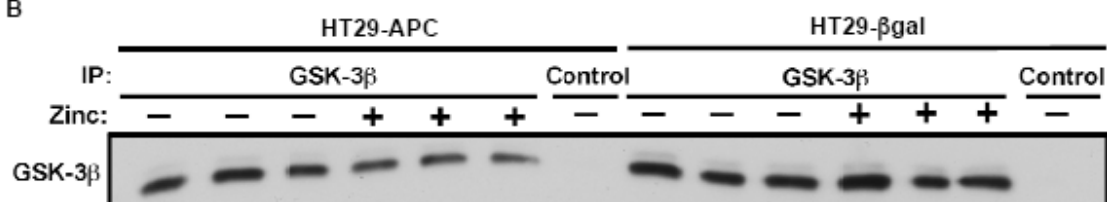
C
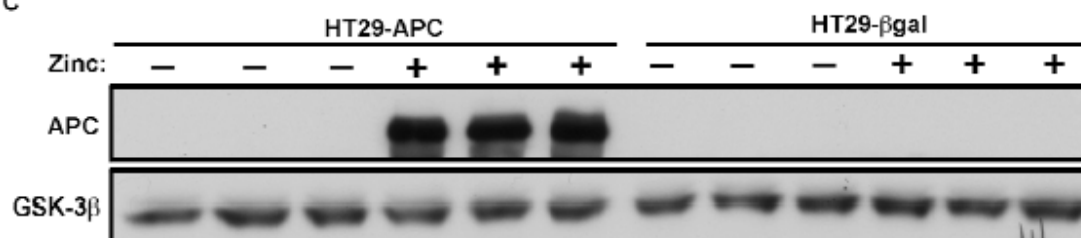
Supplemental Figure S2-7. Wild type APC expression enhances GSK-3 activity recovered from APC deficient colorectal carcinoma cells. (A) Endogenous GSK-3 was immunoprecipitated in triplicate from untreated or ZnCl2 treated (150µM, 24 hrs) HT29-APC and HT29-ßgal cells. Immunoprecipitates from each group were split and each sample was treated with buffer or the GSK-3 inhibitor SB216763 (10µM). A peptide derived from Glycogen Synthase (GS-2 peptide) was added to the immunoprecipitates (20 µM) and GSK-3 activity was assayed by ^{32}P -incorporation. Kinase reactions were run in triplicate and mean activity is shown for each group. Error bars represent standard error of the mean. "Control IP" indicates that antirabbit IgG antibody was used for immunoprecipitation. (B) GSK-3 present in the IPs (done in triplicate) used for the GSK-3 activity assay in panel A. (C) Zinc induced full length APC in HT-29-APC cells, as shown by immunoblot of whole cell lysates.

Supplemental Figure S2-8

A

B
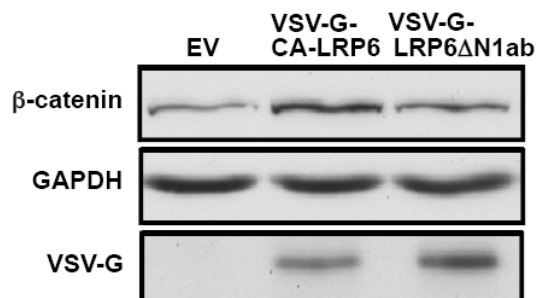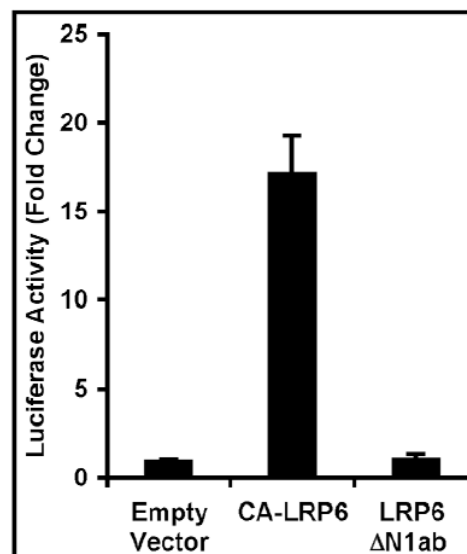
Supplemental Figure S2-8. Constitutively active LRP6 activates downstream Wnt signaling. Expression of VSV-G tagged constitutively active LRP6 (VSV-G-CA-LRP6), but not the nonfunctional, unphosphorylatable VSV-G-LRP6ΔN1ab fragment, elevated β-catenin levels (A) and activated the TOPFLASH Wnt reporter (B) in 293T OT Luciferase cells. Error bars represent standard error of the mean.

CHAPTER 3: ONCOGENIC MUTATIONS IN *ADENOMATOUS POLYPOSIS COLI (Apc)* ACTIVATE MECHANISTIC TARGET OF RAPAMYCIN COMPLEX 1 (mTORC1)

3-1. Summary

Adenomatous polyposis coli (APC) is a negative regulator of the Wnt pathway that is mutated in familial adenomatous polyposis (FAP) and most sporadic colorectal cancers. *Apc* mutations cause accumulation of the Wnt signaling effector ß-catenin and sustained activation of Wnt target genes. However, recent evidence suggests that additional downstream effectors may contribute to *Apc* mutant phenotypes. We previously identified a mechanism by which APC suppresses mTORC1 in cultured cells. As mTORC1 activation is associated with inherited polyposis syndromes such as Peutz-Jeghers and Cowden's syndromes, we hypothesized that oncogenic *Apc* mutations may activate mTORC1 and that this may contribute to *Apc* mutant phenotypes. Here we report marked mTORC1 activation in zebrafish and mice harboring oncogenic *Apc* mutations. We show that mTORC1 contributes to several *Apc* mutant phenotypes, as inhibition of mTORC1 improves survival, reverses liver hyperplasia, and partially rescues circulatory defects in the *Apc* mutant zebrafish.

Furthermore, combined inhibition of mTORC1 and Wnt/ß-catenin signaling rescues defects in anterior-posterior morphogenesis. Thus we identify widespread roles for aberrant mTORC1 activation in phenotypes resulting from *Apc* mutation. Simultaneous inhibition of Wnt and mTORC1 signaling may represent an effective strategy for treating colorectal cancers resulting from APC mutations.

3-2. Introduction

Colorectal cancer (CRC) is the third most common cancer and second leading cause of cancer related death in the United States, responsible for over 50,000 deaths annually in the US and over 600,000 worldwide (U.S. Cancer Statistics Working Group, 2013). 80% of sporadic CRCs result from mutations in Adenomatous Polyposis Coli (APC) (Kinzler and Vogelstein, 1996; Cancer Genome Atlas Network, 2012). Patients with germline APC mutations develop Familial Adenomatous Polyposis (FAP) which is marked by hundreds to thousands of adenomatous colon polyps and progression to invasive carcinomas (Groden et al., 1991; Kinzler et al., 1991; Miyoshi et al., 1992). APC mutations occur within a mutation cluster region (MCR) and result in expression of a truncated protein that lacks the C-terminal half (Miyoshi et al., 1992). Intestinal cells in humans, mice, and rats with these mutations undergo loss of heterozygosity, thus initiating tumor development (Amos-Landgraf et al., 2007;

Haramis et al., 2006; Kinzler and Vogelstein, 1996; Su et al., 1992).

As a negative regulator of the Wnt signaling pathway, APC is a core component of the degradation complex that mediates the turnover of ß-catenin. *Apc* mutations therefore stabilize and constitutively activate Wnt/ß-catenin signaling, a key step in the development of CRCs (Korinek et al., 1997; MacDonald et al., 2009; Morin et al., 1997; Munemitsu et al., 1995). Overexpression of ß-catenin in the colon leads to adenoma formation (Romagnolo et al., 1999) whereas knocking down ß-catenin reduces adenoma size and frequency (Foley et al., 2008; Scholer-Dahirel et al., 2011). In addition, CRCs with wild-type Apc frequently have stabilizing mutations in ß-catenin (Morin et al., 1997; Polakis, 2000), providing compelling evidence for the role of ß-catenin in colorectal carcinogenesis.

However, several groups have reported that nuclear localization of ß-catenin, which is required to activate Wnt signaling, is infrequently observed in early adenomas of patients with FAP, sporadic human polyps, and microadenomas in a rat model of FAP, despite *Apc* loss of heterozygosity (LOH) and elevated cytosolic ß-catenin (Amos-Landgraf et al., 2007; Anderson et al., 2002; Blaker et al., 2004; Kobayashi et al., 2000). In addition, defects in intestinal differentiation were observed in zebrafish *Apc^{mcr}* mutants without detectable nuclear ß-catenin or activation of a Wnt transcription reporter (Phelps et al., 2009b). While the absence of detectable nuclear ß-catenin in adenomas could also reflect the sensitivity of the detection methods, the observations have

nevertheless led to the identification of additional steps required for nuclear translocation of ß-catenin, including activation of Ras and Rac1 (Phelps et al., 2009b; Zhu et al., 2011).

These observations have also raised the possibility that additional effectors downstream of APC may contribute to *Apc* loss of function phenotypes (Phelps et al., 2009a; Phelps et al., 2009b). APC has Wnt/ß-catenin-independent roles including regulation of apoptosis, microtubule dynamics, regulation of retinoic acid biosynthesis, and cell-cell adhesion (Hanson and Miller, 2005; Phelps et al., 2009a). We previously found that APC directly enhances the activity of Glycogen Synthase Kinase-3 (GSK-3) (Valvezan et al., 2012). GSK-3 in turn negatively regulates mechanistic Target of Rapamycin Complex 1 (mTORC1) (Inoki et al., 2006) and thus we found that APC, through GSK-3, suppresses mTORC1 activity in cultured cells (Valvezan et al., 2012). mTORC1 is a metabolic sensor and translational regulator, that promotes cell growth and proliferation, and is aberrantly active in many cancers (Laplante and Sabatini, 2012). Thus we hypothesized that oncogenic *Apc* mutations may activate mTORC1, and that this activation may contribute to *Apc* loss of function phenotypes.

To investigate mTORC1 activity downstream of APC, we have examined oncogenic *Apc* mutations in zebrafish and mice. Homozygous *Apc* mutant mice die before gastrulation (Fodde et al., 1994), whereas homozygous mutant zebrafish (*apc^{mcr/mcr}* zebrafish) survive until early larval stages (Hurlstone et al.,

2003). *Apc* mutant zebrafish have multiple developmental defects including impaired circulation, hyperproliferation of the cardiac cushions, enlarged livers, and defects in morphogenesis of the anterior-posterior axis resulting in severe body curvature (Goessling et al., 2008; Hurlstone et al., 2003). These observations indicate a critical role for APC in development of multiple organs and tissues.

Here we show that mTORC1 is aberrantly activated in *Apc*[mcr/mcr] zebrafish and that mTORC1 inhibition attenuates multiple phenotypes in *Apc* mutants, indicating that mTORC1 activation contributes to these phenotypes. We also find that mTORC1 is robustly activated in intestinal adenomas in *Apc*[min] mice. Taken together, these data suggest that APC mutations function in part by activating mTORC1.

3-3. Results

3-3-1.Oncogenic Apc mutation activates mTORC1

We previously found that APC directly enhances the kinase activity of GSK-3 (Valvezan et al., 2012). GSK-3 inhibits mTORC1 activity (Inoki et al., 2006) and thus knocking down APC reduces GSK-3 activity and activates mTORC1 in cultured cells (Valvezan et al., 2012). We therefore hypothesized that oncogenic APC mutations should activate mTORC1 in vivo. For these

Figure 3-1
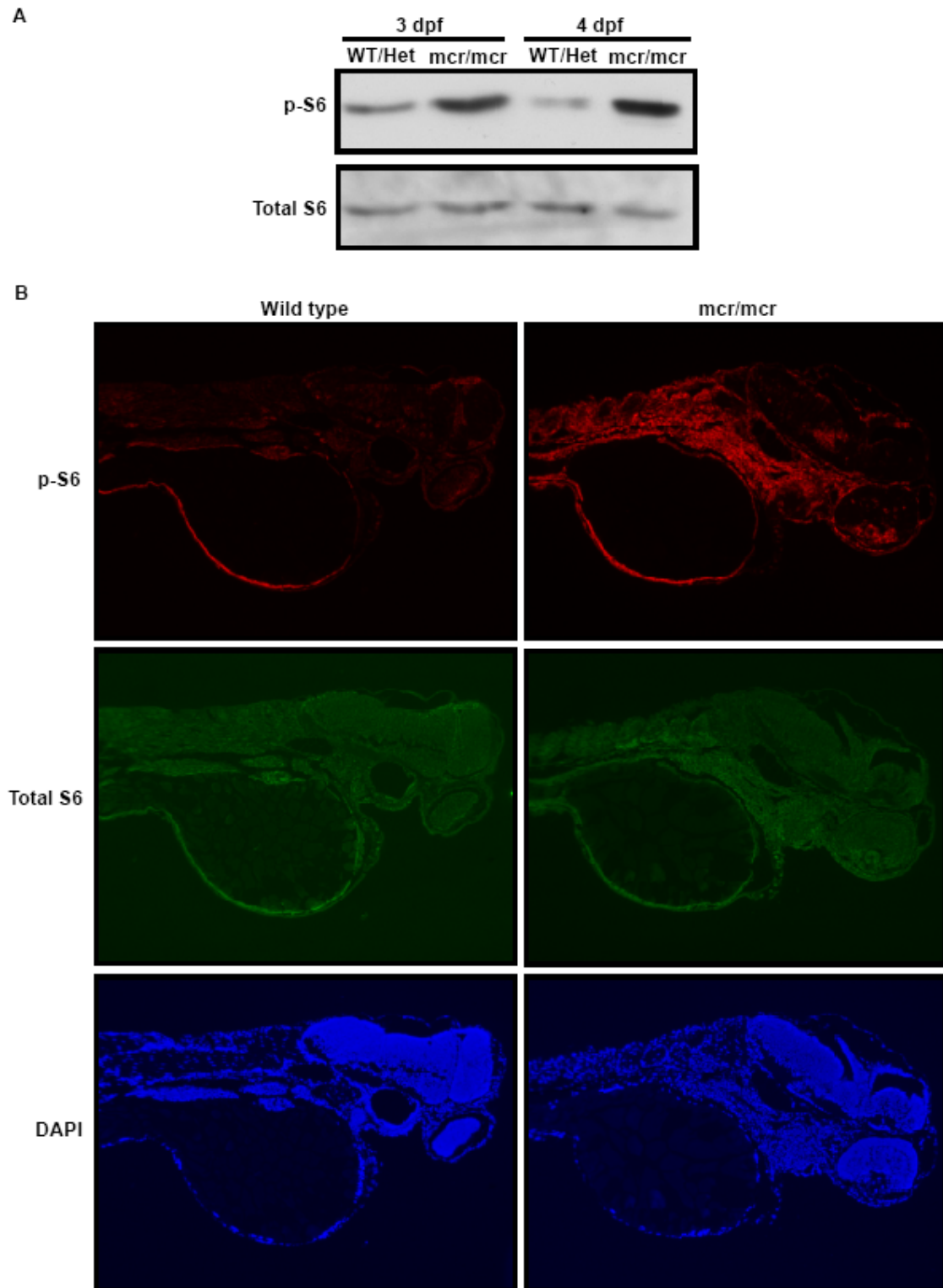
Figure 3-1. Activation of mTORC1 in *apc^mcr/mcr* zebrafish. Western blot

analysis using antibodies to phosphorylated S6, indicating mTORC1 activity, or

total S6 on whole embryo lysates from pooled wild type and heterozygous

apc^mcr/+ zebrafish or homozygous *apc^mcr/mcr* zebrafish at 3 or 4 days post

fertilization. mTORC1 activity is increased in the homozygous *apc^mcr/mcr* zebrafish.

(B) Immunohistochemical staining of sagittal sections from wild type or

homozygous *apc^mcr/mcr* zebrafish at 3 dpf using antibodies to phosphorylated or

total S6 and counterstained with DAPI. mTORC1 is aberrantly active in multiple

mesodermal and endodermal derivatives in the homozygous *apc^mcr/mcr* zebrafish.

studies we used Apc^{mcr} zebrafish, which contain a previously characterized

mutation that causes a premature stop codon within the mutation cluster region

(mcr) of *Apc*, analgous to truncating mutations typically found in human

colorectal cancers (Haramis et al., 2006; Hurlstone et al., 2003). We compared

mTORC1 activity in $apc^{mcr/mcr}$ zebrafish to wild type and heterozygous zebrafish.

Homozygous *Apc* mutation activates mTORC1, as assessed by western blotting

whole embryo lysates for phosphorylation of ribosomal protein S6, a well

established readout of mTORC1 activity (Laplante and Sabatini, 2012).

Phosphorylated S6 is increased in homozygous mutants at 3 and 4 days post

fertilization (dpf) compared to pooled wild type and heterozygous fish, without a

change in total S6 (Figure 3-1A). To assess tissue specific changes in mTORC1

activity, we also performed immunohistochemistry on larvae fixed at 3 dpf.

Immunostaining for phospho-S6 reveals broadly increased mTORC1 activity in

multiple mesodermal and endodermal derivatives (Figure 3-1B). These data

indicate that an oncogenic *Apc* mutation activates mTORC1.

3-3-2. mTORC1 inhibition extends the survival of $apc^{mcr/mcr}$ zebrafish

To test whether aberrantly active mTORC1 contributes to $apc^{mcr/mcr}$

phenotypes, we asked whether mTORC1 inhibition rescues phenotypes in these

fish. Torin1, a direct inhibitor of mTOR kinase activity (Thoreen et al., 2009), was

added to developing embryos at 24 hpf and survival was assessed daily. Vehicle

Figure 3-2

A

B

C

D

E
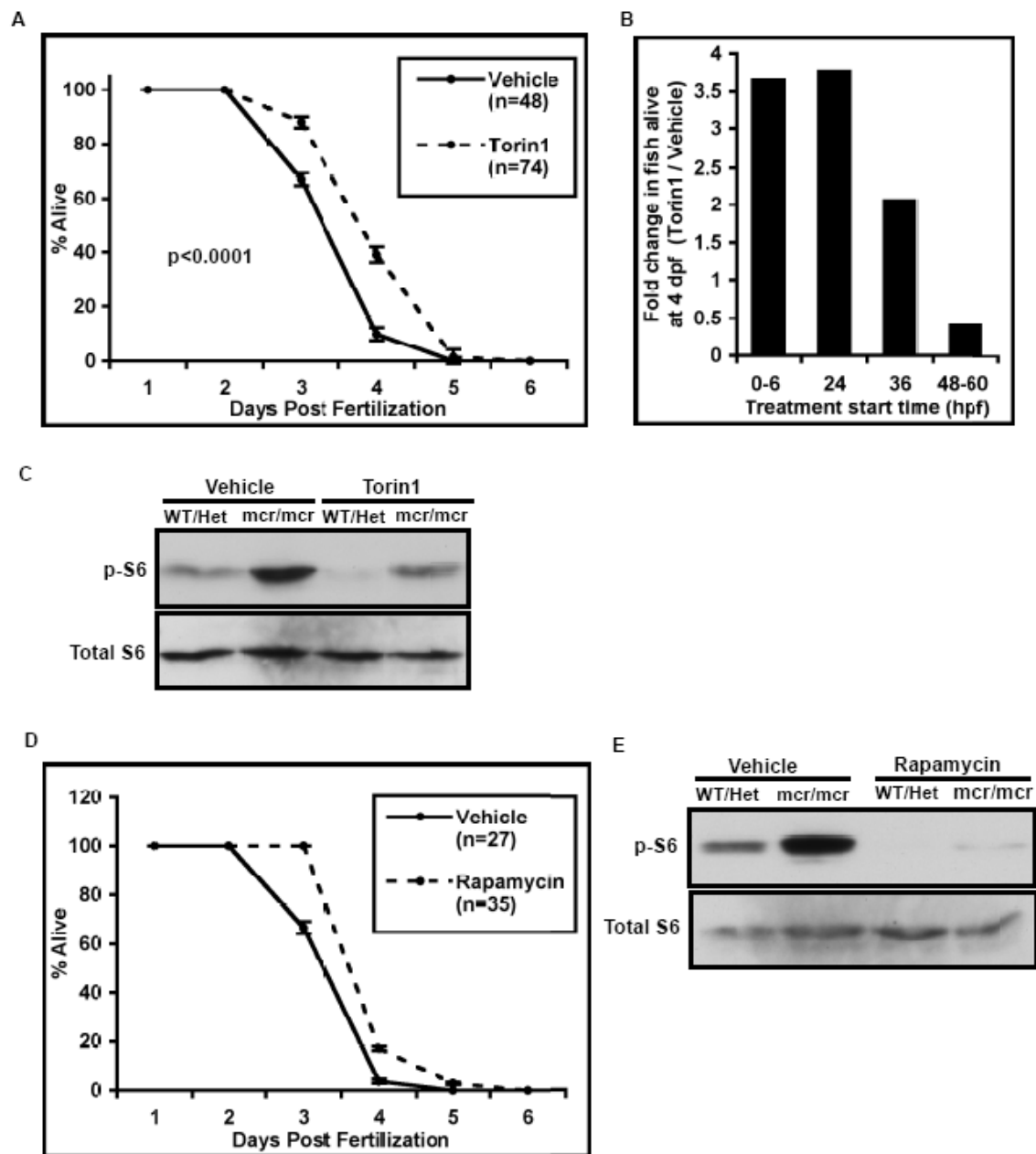
Figure 3-2. mTORC1 inhibition extends survival in *apc^mcr/mcr* zebrafish. (A)

apc^mcr/mcr zebrafish were treated with vehicle or Torin1 (250nM) starting at 24 hpf.

The percent of fish alive was recorded every 24 hours. (B) Torin1 (250nM) was

added between 0-6 hpf, at 24 hpf, at 36 hpf, or between 48-60 hpf. The percent

of Torin1 treated fish alive at 4 dpf was normalized to the percent of vehicle

treated fish alive at 4 dpf. (C) Western blot analysis using antibodies to phospho-

S6 or total S6 on whole embryo lysates from pooled 3 day old wild type and

heterozygous *apc^mcr/+* zebrafish or homozygous *apc^mcr/mcr* zebrafish treated with

vehicle or Torin1. Torin1 treatment under these conditions (250nM) reduces

mTORC1 activity in homozygous *apc^mcr/mcr* zebrafish to levels similar to vehicle

treated wild type/heterozygous fish. (D) Survival curves of *apc^mcr/mcr* zebrafish

treated with vehicle or Rapamycin (200nM) starting at 24 hpf. (E) Western blot

analysis as in (C) on fish treated with vehicle or Rapamycin. Error bars in (A,D)

represent standard error of the mean and p-values were calculated using the log-

rank (Mantel-Cox) test.

treated $apc^{mcr/mcr}$ zebrafish larvae die 3-4 days post fertilization (dpf) while

treatment with Torin1 extends their survival by up to 24 hours, with approximately

40% of Torin1 treated larvae surviving at 4 dpf compared to ~10% of vehicle

treated controls (Figure 3-2A; p<0.0001). We also assessed the effect of adding

Torin1 at different developmental stages. Adding Torin1 at 0-6 hpf or 24hpf had

similar effects on survival: Approximately 3.5 times as many Torin1 treated

$apc^{mcr/mcr}$ zebrafish were alive at 4 dpf compared to vehicle treated $apc^{mcr/mcr}$

zebrafish when treatment was started at either 0-6 hpf or 24 hpf (Figure 3-2B).

Treatment at 36 hpf or later was less effective (Figure 3-2B). Importantly, Torin1

treatment reduced mTORC1 activity in homozygous $apc^{mcr/mcr}$ zebrafish to levels

similar to vehicle treated wild type/heterozygous fish (Figure 3-2C), highlighting

the role for aberrant mTORC1 activation resulting from oncogenic *Apc* mutation.

Treatment with Rapamycin, an allosteric inhibitor of mTORC1 that acts through a

distinct mechanism (Sarbassov et al., 2006), also extends survival of $apc^{mcr/mcr}$

zebrafish (Figure 3-2D). These data suggest that aberrant mTORC1 activation

resulting from oncogenic *Apc* mutation contributes to early lethality in $apc^{mcr/mcr}$

zebrafish.

3-3-3. mTORC1 inhibition improves circulation in apc$^{mcr/mcr}$ zebrafish

$apc^{mcr/mcr}$ zebrafish have dramatically reduced circulation compared to wild

type fish at 2-3 dpf (Supplemental videos 3-1 through 3-4)

81
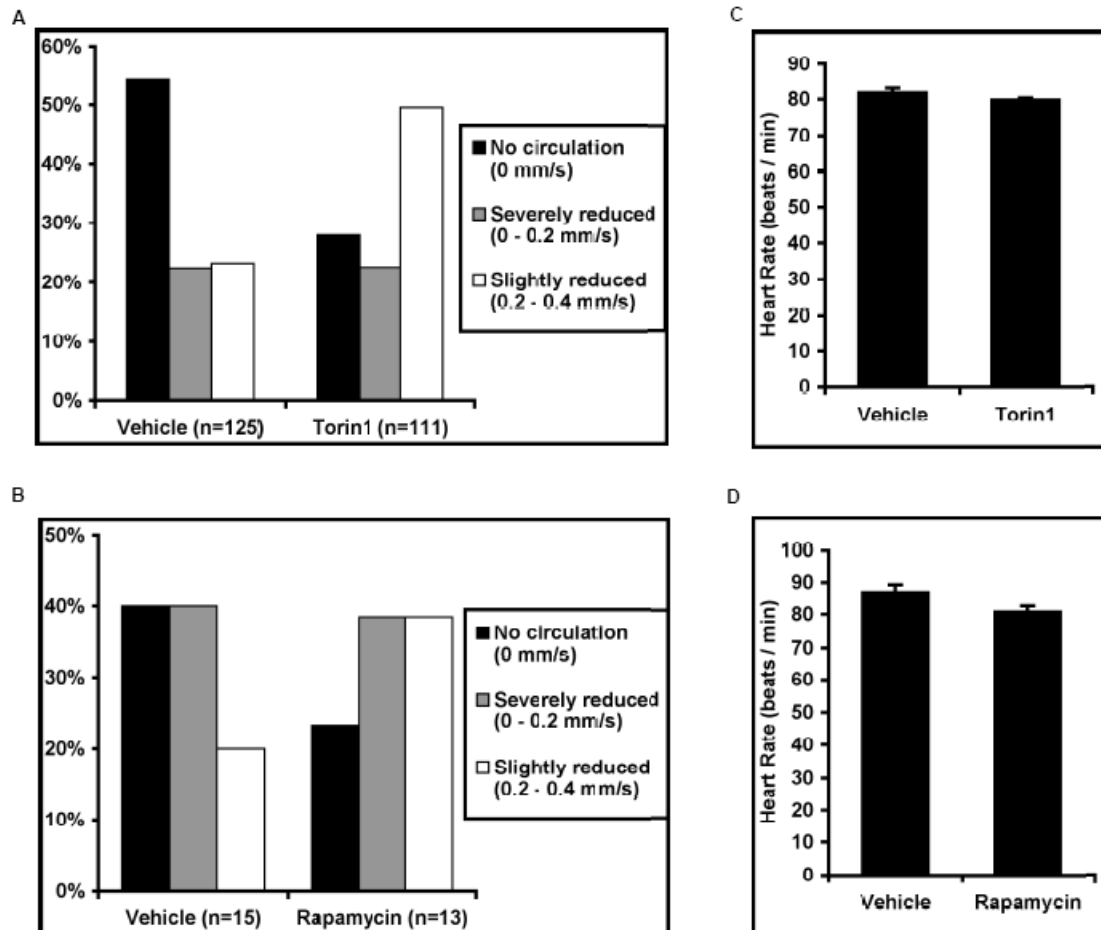
Figure 3-3

Figure 3-3. mTORC1 inhibition improves circulation in *apc^mcr/mcr* zebrafish.

(A) *apc^mcr/mcr* zebrafish were treated with vehicle or Torin1 (250nM) and the rate of blood flow through the dorsal aorta was measured using high speed videomicroscopy in unanesthetized larvae at 2-3 dpf. Blood flow phenotypes were categorized according to flow rate as indicated in the figure. Representative videos for each scoring category can be found in online supplemental material (Supplemental videos 1-4). (B) Rapamycin also improves circulation in *apc^mcr/mcr* zebrafish. (C,D) Torin1 and Rapamycin treatment do not increase the heart rate of *apc^mcr/mcr* zebrafish.

(Hurlstone et al., 2003). We used high speed videomicroscopy to measure blood flow through the dorsal aorta; approximately half of vehicle treated fish had no circulation through the dorsal aorta and approximately 75% had a flow rate of less than 0.2 mm/second (Figure 3-3A). Torin1 treatment improves circulation, resulting in a flow rate greater than 0.2 mm/second in approximately 50% of mutants, and reducing the percent of mutants with no circulation to approximately 25% (Figure 3-3A). Rapamycin similarly doubled the percent of mutants with flow greater than 0.2 mm/second and halved the percent of mutants with no circulation (Figure 3-3B). Representative videos showing blood flow through the dorsal aorta in wild type fish and each of the three categories used for scoring $apc^{mcr/mcr}$ zebrafish can be found in online supplemental material. Torin1 and Rapamycin did not increase the heart rate of the $apc^{mcr/mcr}$ zebrafish (Figure 3-3C,D). These data indicate that mTORC1 activation in $apc^{mcr/mcr}$ zebrafish contributes to the reduced circulation phenotype.

3-3-4. mTORC1 inhibition rescues liver hyperplasia in heterozygous $apc^{mcr/+}$ zebrafish

Heterozygous $apc^{mcr/+}$ zebrafish have enlarged livers by 3 dpf due to an increase in the number of hepatocytes (Figure 3-4) (Goessling et al., 2008). To ask whether this phenotype can be rescued by mTORC1 inhibition, we crossed heterozygous $apc^{mcr/+}$ zebrafish to *lfabp:RFP* fish, which express red fluorescent

Figure 3-4
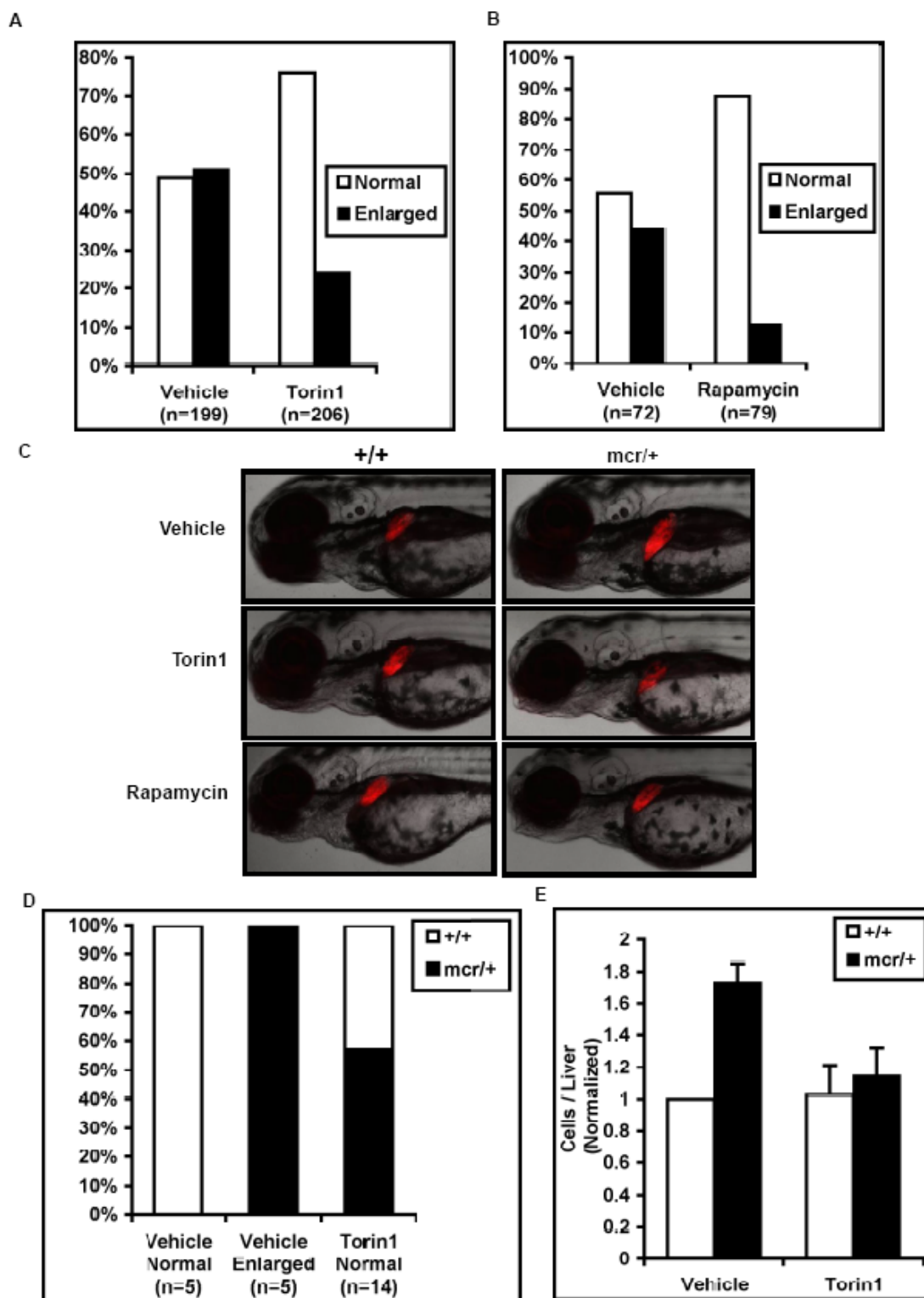
Figure 3-4. mTORC1 inhibition rescues liver hyperplasia in heterozygous *apc^{mcr/+}* **zebrafish.** *Lfabp:rfp* zebrafish, which express liver specific RFP, were mated with *apc^{mcr/+}* zebrafish. (A) Resulting progeny (expected 50% *apc^{+/+}*, 50% *apc^{mcr/+}*) were treated with vehicle or Torin1 and the percent of fish with normal or enlarged livers at 3-4 dpf was recorded. Approximately 50% of vehicle treated fish had enlarged livers as described previously (Goessling et al., 2008). Torin1 reduces the percent of fish with enlarged livers. (B) Rapamycin similarly rescues liver enlargement. (C) Representative pictures showing liver enlargement in vehicle treated heterozygous *apc^{mcr/+}* zebrafish but not in Torin1 or Rapamycin treated heterozygotes. (D) Liver size correlates closely with genotype in vehicle treated embryos as described previously (Goessling et al., 2008), but heterozygous *apc^{mcr/+}* embryos account for approximately half of Torin1 treated embryos with normal size livers, confirming Torin1 reduces liver size in heterozygous *apc^{mcr/+}* embryos. (E) The number of RFP positive cells per embryo was measured by flow cytometry. Vehicle treated *apc^{mcr/+}* zebrafish had approximately 75% more hepatocytes than vehicle treated wild type fish, and this was rescued by Torin1 treatment.

protein under control of the liver specific liver fatty acid binding protein (lfabp) promoter (Her et al., 2003). 50% of resulting progeny are expected to be heterozygous $apc^{mcr/+}$ and 50% are expected to be wild type at the *Apc* locus ($apc^{+/+}$). We scored liver size in the resulting RFP+ progeny and found approximately 50% had normal size livers and 50% had enlarged livers as previously reported (Goessling et al., 2008). Treatment with Torin1 or Rapamycin greatly reduced the percent of fish with enlarged livers and increased the percent with normal livers (Figure 3-4A,B). Genotyping confirmed strong correlation between heterozygous *Apc* mutation and liver enlargement in vehicle treated fish (Figure 3-4C,D). However, when Torin1 treated fish with normal size livers were genotyped, approximately half were found to be $apc^{mcr/+}$, confirming that Torin1 restores normal liver size in heterozygous *Apc* mutant zebrafish (Figure 3-4C,D). To quantify the effect of Torin1 treatment on liver hyperplasia, the number of RFP+ cells per liver was measured by flow cytometry. $apc^{mcr/+}$ fish had approximately 75% more cells per liver and this was restored to wild type levels by Torin1 (Figure 3-4E). Torin1 had no effect on the number of hepatocytes in wild-type zebrafish. These data demonstrate a critical role for mTORC1 in liver hyperplasia resulting from oncogenic *Apc* mutation.

3-3-5. Combined inhibition of mTORC1 and Wnt signaling reduces body curvature in apc$^{mcr/mcr}$ zebrafish

We have found that aberrant mTORC1 activation contributes to early lethality, reduced circulation, and liver hyperplasia resulting from oncogenic *Apc* mutation. *Apc* loss of function also activates Wnt/ß-catenin signaling, which plays a major role in *Apc* mutant phenotypes. Thus we asked whether combined inhibition of mTORC1 and Wnt/ß-catenin signaling could rescue additional phenotypes. Aberrant body curvature resulting from defects in morphogenesis of the anterior-posterior axis is one of the most prominent phenotypes in *apc$^{mcr/mcr}$* zebrafish (Figure 3-5) (Hurlstone et al., 2003). Treatment with either Torin1 or the Wnt inhibitor XAV939 (Huang et al., 2009b) alone did not affect body curvature, but combining Torin1 and XAV939 resulted in *apc$^{mcr/mcr}$* zebrafish with straight bodies similar to wild type (Figure 3-5A). Approximately 95% of vehicle, Torin1, or XAV939 treated *apc$^{mcr/mcr}$* zebrafish had curved body axes, whereas approximately 40% of *apc$^{mcr/mcr}$* zebrafish treated with Torin1 and XAV939 were straight (Figure 3-5B). Similarly, Rapamycin improved body curvature in combination with XAV939 but had no effect on its own (Figure 3-5C). Representative images of scoring categories used in panels B and C are show in panel D. These data highlight the importance of combined mTORC1 and Wnt/ß-catenin signaling downstream of *Apc* mutation and demonstrate that simultaneous inhibition of both pathways can rescue *Apc* mutant phenotypes that are not rescued by targeting either pathway alone.

Figure 3-5

89
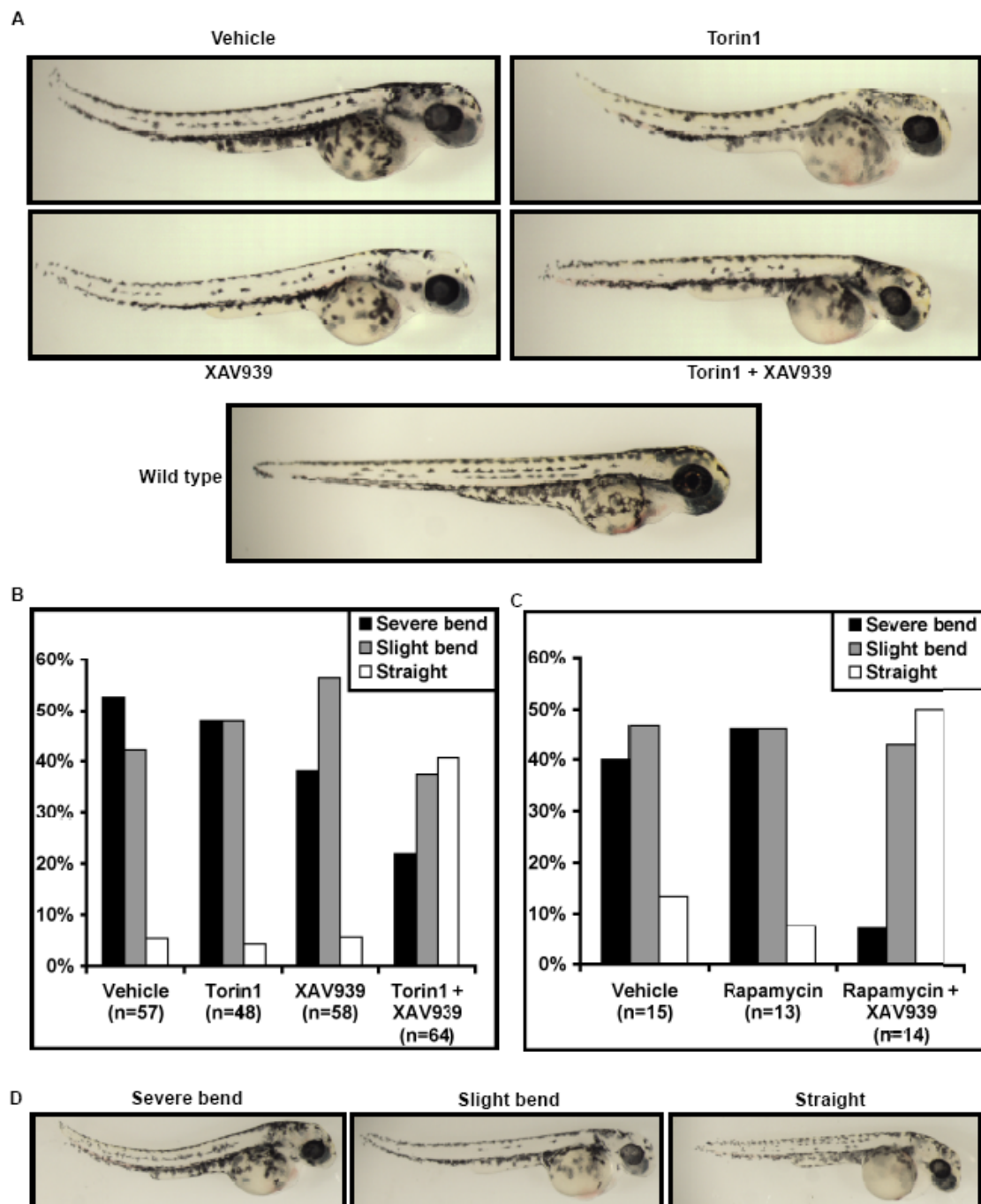
Figure 3-5. Combined inhibition of mTORC1 and Wnt signaling reduces body curvature in *apc^{mcr/mcr}* zebrafish. (A) Representative images of *apc^{mcr/mcr}* zebrafish at 2 dpf treated with vehicle, Torin1 (250nM), XAV939 (500nM), or Torin1 plus XAV939. Combined Torin1 and XAV939 treatment rescues body curvature whereas either treatment alone does not. (B) Zebrafish were scored between 2-3 dpf based on severity of body curvature and the percent of fish in each category is shown. Only combined Torin1 plus XAV939 treatment reduces body curvature. (C) Rapamycin also reduces body curvature in combination with XAV939, but not by itself. (D) Representative pictures of each of the three categories used to score body curvature in (B,C).

3-3-6. mTORC1 is strongly activated in Apc^{min} mouse intestinal polyps

To confirm our findings in zebrafish using an established mammalian model of polyposis, we examined GSK-3 and mTORC1 activity in *Apc^{min}* mice, which develop intestinal adenomas through loss of heterozygosity (LOH) for *Apc*. Phospho-S6 staining was strongly induced in polyps compared to adjacent normal tissue (Figure 3-6A), similar to previous reports (Fujishita et al., 2008; Metcalfe et al., 2010). Furthermore, phosphorylation of glycogen synthase, an endogenous GSK-3 substrate, was markedly reduced in polyps, but not normal epithelium, from *Apc^{min}* mice (Figure 3-6B). These data are consistent with a model in which loss of APC reduces GSK-3 activity, which in turn activates both mTORC1 and Wnt/ß-catenin signaling.

Figure 3-6
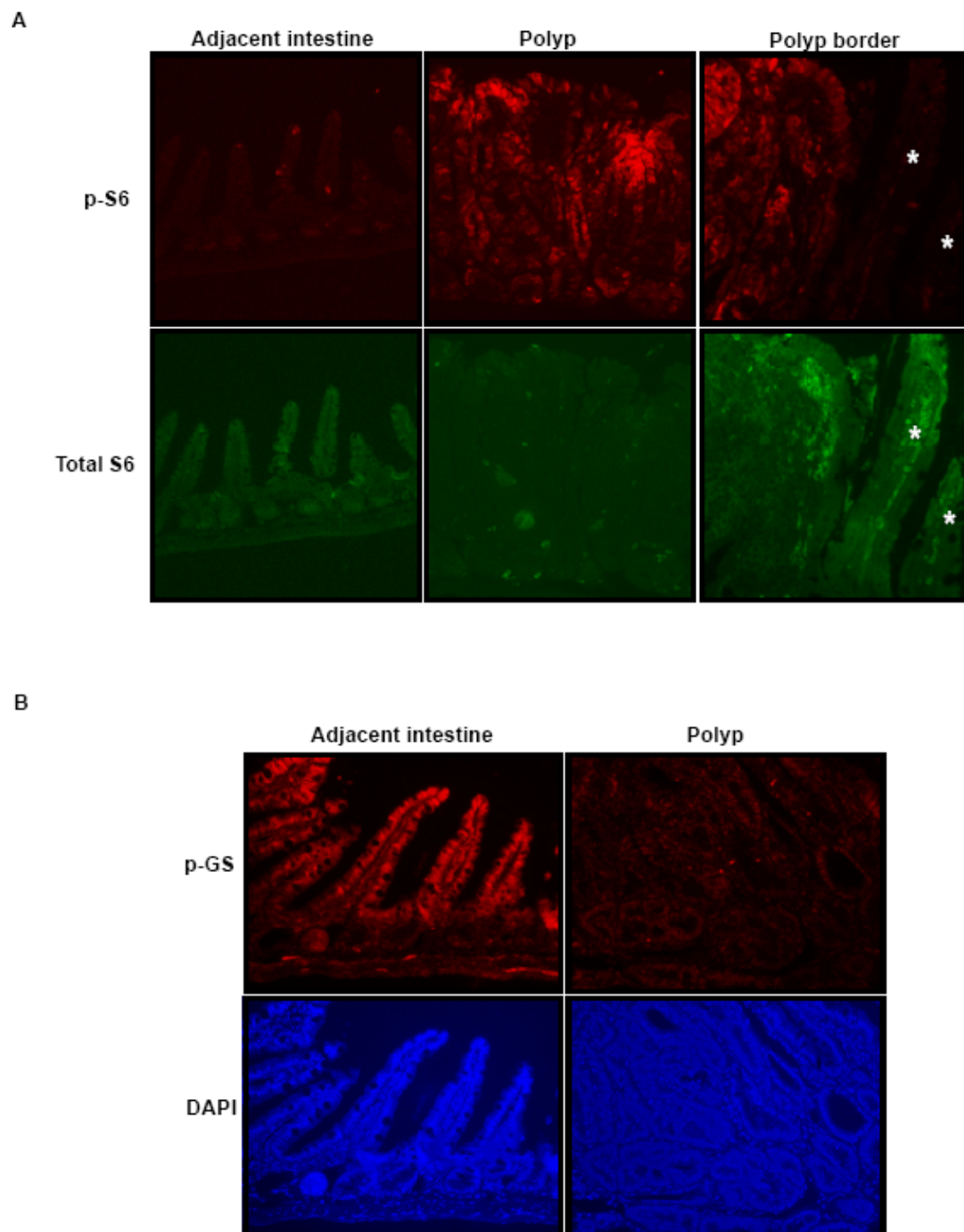
Figure 3-6. mTORC1 is strongly activated and Glycogen Synthase phosphorylation is reduced in *Apc^{min}* mouse intestinal polyps.

Immunostaining of intestinal sections from *Apc^{min}* mice. (A) Staining for phosphorylated S6, indicating mTORC1 activity, or total S6 reveals mTORC1 activity is dramatically increased in polyps compared to immediately adjacent normal tissue. Asterisks indicate normal villi immediately adjacent to a polyp. (B) Immunostaining using an antibody to Glycogen Synthase phosphorylated at the GSK-3 phosphorylation site and counterstained with DAPI. Glycogen Synthase phosphorylation is strongly reduced in polyps compared to immediately adjacent normal intestine.

3-4. Discussion

The importance of enhanced Wnt/ß-catenin activity as a mediator of *Apc* loss of function phenotypes is firmly established from studies in model organisms and in colorectal carcinomas in humans. However, reports that early neoplastic lesions may appear before nuclear localization of ß-catenin is detectable have prompted investigation into additional steps that may be required for the nuclear translocation and activation of ß-catenin (Phelps et al., 2009b; Rosenbluh et al., 2012; Zhu et al., 2011). These reports have also raised the possibility that APC regulates additional effectors that contribute to *Apc* loss of function phenotypes. We show that truncating mutations in *Apc*, which are homologous to those found in human colorectal cancers and FAP, cause marked activation of mTORC1 and that mTORC1 activation contributes to *Apc* mutant phenotypes in zebrafish, including lethality in the larva stage, impaired circulation, defects in morphogenesis, and liver hyperplasia. Thus we identify widespread roles for aberrant mTORC1 activation in phenotypes resulting from oncogenic mutations in *Apc*.

These findings are consistent with a signaling pathway in which APC directly enhances GSK-3 activity (Valvezan et al., 2012). We previously showed that APC directly facilitates GSK-3 activity in vitro and knockdown of APC reduces GSK-3 activity toward endogenous substrates, including glycogen synthase, in cultured cells. Inoki et al showed that GSK-3 suppresses mTORC1

activity (Inoki et al., 2006), and this was confirmed in vivo in mouse bone marrow (Huang et al., 2012; Huang et al., 2009a); thus our model predicts that inhibition of GSK-3 caused by *Apc* loss of function should activate mTORC1 in vivo. A number of findings here and in previous work strongly support this novel signaling module. Thus, we observe that GS phosphorylation is substantially reduced in intestinal polyps from Apc^{min} mice (Figure 3-6B). We also confirm that mTORC1 is markedly activated in *Apc* mutant polyps (Figure 3-6A), consistent with previous reports (Fujishita et al., 2008; Metcalfe et al., 2010). Furthermore, mTORC1 inhibitors reduce adenoma size and number, as well as mortality, in *Apc* mutant mice, demonstrating the importance of mTORC1 activation for tumorigenesis resulting from *Apc* mutation (Fujishita et al., 2008; Koehl et al., 2010; Metcalfe et al., 2010). mTOR expression is also increased in *Apc* mutant polyps which may also contribute to elevated mTORC1 activity (Fujishita et al., 2008).

      The importance of combined Wnt/ß-catenin and mTORC1 signaling in zebrafish development is also supported by our finding that aberrant body curvature in $apc^{mcr/mcr}$ zebrafish is rescued by combining mTORC1 inhibition with a Wnt pathway inhibitor, as neither inhibitor alone achieves rescue. We find that hyperplasia of the liver in heterozygous Apc^{mcr} zebrafish is partially rescued by mTORC1 inhibition, and previous work demonstrated that this phenotype is also partially rescued by ß-catenin knockdown (Goessling et al., 2008), again indicating a role for both ß-catenin and mTORC1 in this hyperplastic phenotype.

Taken together, these data suggest activation of both mTORC1 and Wnt/ß-catenin signaling contributes to multiple phenotypes resulting from oncogenic *Apc* mutation.

In further support of a role for mTORC1 downstream of APC, *Apc^{mcr}* zebrafish are partially phenocopied by knockdown of *Tsc1*, which causes similar defects in body curvature, and by genetic inactivation of *Tsc2*, which causes liver hyperplasia similar to *Apc^{mcr}* heterozygotes (DiBella et al., 2009; Goessling et al., 2008; Hurlstone et al., 2003; Kim et al., 2010). The *Tsc2* phenotype is not identical to *Apc^{mcr}*, however. This could be due to persistence of maternal Tsc2 gene expression relative to Apc as well as the important role of ß-catenin and possibly other effectors downstream of APC.

Extracolonic manifestations in FAP include polyps of the gastric fundus and small bowel, osteomas, lipomas, desmoid tumors, adrenal cortical adenomas, congenital hypertrophy of the retinal pigment epithelium (CHRPE) and hepatoblastomas (Rustgi, 2007), suggesting parallels with other hereditary disorders that cause neoplasias in multiple organs, including tuberous sclerosis, Peutz-Jeghers syndrome, and Cowden's syndrome (Crino et al., 2006; Inoki et al., 2005; Rustgi, 2007). Peutz-Jeghers and Cowden's syndromes are hamartomatous polyposis syndromes that involve multiple organ systems and are mediated in part through activation of mTORC1. Peutz-Jeghers syndrome is caused by mutations in LKB, an upstream regulator of AMPK and TSC2, and Cowden's syndrome is caused by mutations in PTEN that also activate mTORC1

(Inoki et al., 2005). While each of these syndromes is clinically and pathologically distinct, it is interesting to note that they all share mTORC1 activation, which may be an early molecular step in tumor formation in each case. In contrast, the differences in these syndromes may be due to the fact that APC, LKB, and PTEN each regulate multiple other effectors.

In conclusion, APC negatively regulates mTORC1 and oncogenic APC mutations disrupt this function, resulting in constitutive mTORC1 activation in both zebrafish and mammalian models. Several resulting phenotypes are partially rescued by mTORC1 inhibition, demonstrating a widespread role for mTORC1 activation downstream of APC mutation. Combined inhibition of mTORC1 and Wnt signaling rescues additional phenotypes, highlighting the importance of the unique combination of active mTORC1 and Wnt that results from oncogenic APC mutation. Combined inhibition of Wnt and mTORC1 signaling may represent an effective strategy for treatment of colorectal cancers resulting from APC mutation.

3-5. Materials and Methods

3-5-1. Zebrafish

Embryos were raised at 28.5°C in standard E3 medium (Westerfield, 1993). 30µg/ml pronase (Roche #10165921001) was added at 24 hours post

fertilization (hpf) to digest the chorion and washed out at 48 hpf. Apc^{mcr} zebrafish and primers used for genotyping were described previously (Hurlstone et al., 2003). *Lfabp:rfp* zebrafish were described previously (Her et al., 2003). Rapamycin (LC Laboratories #R-5000), Torin1 (Tocris Bioscience #4247) and/or XAV939 (Sigma #X3004) were added directly into the fish medium starting at 24 hpf unless otherwise indicated. Medium was changed and fresh treatments were added every 24 hours. Videomicroscopy and quantification of blood flow rate was performed in unanesthetized zebrafish larvae at 60 hpf as previously described (Hoage et al., 2012). Flow cytometry was performed as previously described (Goessling et al., 2008) except embryos were dissociated in 0.25% trypsin (Westerfield, 1993). For immunofluorescence, 3 dpf zebrafish embryos were fixed in 4% paraformaldehyde for 2 hours at room temperature, washed in PBST, dehydrated in an ascending ethanol series and submitted to the Perelman School of Medicine Cancer Histology Core at the University of Pennsylvania for paraffin embedding and sectioning. Immunofluorescence staining for S6 and phospho-S6 was performed as in Apc^{min} mouse intestinal sections (see below). Zebrafish husbandry and egg procurement were carried out in accordance with the guidelines of the University of Pennsylvania Institutional Animal Care and Use Committee.

3-5-2. Lysis and western blots

Zebrafish embryos were lysed on ice in 5µl/embryo of buffer containing

1% NP-40, 20mM Tris pH 8.0, 50mM NaCl, 2.5mM EDTA, 1mM DTT, protease

inhibitor cocktail (Sigma #P8340) diluted 1:100, phosphatase inhibitor cocktails

#2 (Sigma #P5726) and #3 (Sigma #P0044) also diluted 1:100 each. Equal

volume of standard 2X Laemmli Sample Buffer was added and samples were

heated at 95°C for 5 minutes and then centrifuged at 14,000 rpm for 5 minutes at

4°C. Supernatants were collected for SDS-PAGE and western blot analysis using

the following antibodies purchased from Cell Signaling Technology: S6 ribosomal

protein (#2317), phospho-S6 ribosomal protein Ser235/236 (#4858).

3-5-3. Apc^{min} mice

Apc^{min} mice were purchased from The Jackson Laboratory (stock

#002020) and described previously (Moser et al., 1993). For

immunofluorescence, intestines were fixed in 10% phosphate buffered formalin

overnight, washed in PBST, dehydrated in an ascending ethanol series and

submitted to the Perelman School of Medicine Molecular Pathology and Imaging

Core at the University of Pennsylvania for paraffin embedding and sectioning.

Paraffin embedded sections were washed in xylene and rehydrated in a

descending ethanol series followed by boiling in 10mM sodium citrate pH 6.0 and

blocking in Starting Block buffer (Thermo Scientific #37539). Antibodies to S6,

phospho-S6 Ser235/236, or phospho-Glycogen Synthase Ser641 (Cell Signaling

Technology #3891) were added overnight at 4°C followed by incubation with Cy2 or Cy3 conjugated secondary antibodies.

3-6. Supplemental Video Legends

Online Supplemental Video 3-1. Wild type circulation. Representative video showing circulation through the dorsal aorta of a wild type zebrafish at approximately 60 hpf. Circulation is rapid (mean flow rate > 0.4 mm/sec) and pulsatile.

Online Supplemental Video 3-2. Slightly reduced circulation. Representative video showing circulation through the dorsal aorta of an *apc$^{mcr/mcr}$* zebrafish scored as "Slightly reduced" in Figure 3 at approximately 60 hpf. Mean flow for this group was 0.2- 0.4 mm/sec and was still pulsatile.

Online Supplemental Video 3-3. Severely reduced circulation. Representative video showing circulation through the dorsal aorta of an *apc$^{mcr/mcr}$* zebrafish scored as "Severely reduced" in Figure 3 at approximately 60 hpf. This category was defined as mean flow rate of 0-0.2 mm/sec.

Online Supplemental Video 3-4. No Circulation. Representative video showing lack of circulation through the dorsal aorta of an $apc^{mcr/mcr}$ zebrafish scored as having "No circulation" in Figure 3 at approximately 60 hpf. No circulating cells are visible.

CHAPTER 4: CONCLUSIONS AND FUTURE DIRECTIONS

4-1. Overview

The Wnt pathway is essential for patterning and cell fate specification during embryogenesis and for tissue maintenance and regeneration in adults. Aberrant Wnt activation is critical for development and progression of many cancers. APC is an essential negative regulator of the Wnt pathway and mutations in *Apc* potently induce tumorigenesis. The mechanistic role of APC in the Wnt pathway and the downstream effectors of oncogenic *Apc* mutations are not known. APC promotes β-catenin phosphorylation by GSK-3 and has similar roles as GSK-3 in various cellular processes including mitotic spindle formation, cell migration, and axon specification. Furthermore, APC and GSK-3 loss of function phenocopy one another during development of many organs and tissues, and in stem cell regulation in adults. Thus we asked if APC can regulate GSK-3 and GSK-3 dependent signaling. To address this question we examined the effects of APC on GSK-3 activity in vitro, and the effects of APC loss of function on substrates and signaling pathways regulated by GSK-3 in human cells, mice, and zebrafish. We find APC directly enhances GSK-3 activity and thus regulates GSK-3 dependent signaling, including signaling through Glycogen Synthase (GS) and mTORC1. Our findings uncover new roles for APC and suggest a widespread role for APC in regulating GSK-3 dependent signaling. We

also find that Wnts induce APC dissociation from the GSK-3/Axin complex, suggesting a novel mechanism for how Wnts regulate GSK-3 activity.

We examined the effects of oncogenic *Apc* mutations on mTORC1 and find that these mutations activate mTORC1 which contributes to many resulting phenotypes. These data suggest a new model for colorectal cancer pathogenesis in which *Apc* mutations reduce GSK-3 activity, resulting in combined activation of Wnt/β-catenin and mTORC1 signaling.

Our work raises many intriguing questions, some of which will be discussed in this chapter. These questions include: How does APC enhance GSK-3 activity? How does APC regulate mTORC1? Does APC play widespread roles in GSK-3 dependent signaling and regulation of GSK-3 substrates? Do APC and GSK-3 interact independently of Axin? Is APC dissociation from Axin required for Wnt signal transduction? Does APC regulate embryonic development and stem cell homeostasis through GSK-3 and mTORC1? Is mTORC1 activation required for tumorigenesis downstream of *Apc* mutation? Finally, can combined inhibition of mTORC1 and Wnt/β-catenin signaling effectively treat colorectal cancer?

4-2. APC regulates GSK-3 activity and GSK-3 dependent signaling

We examined a role for APC in regulation of GSK-3 activity and found that APC enhances phosphorylation of tau and β-catenin by GSK-3. Surprisingly, this

occurs even in the absence of the Axin scaffold, suggesting APC directly interacts with and enhances GSK-3 activity. Future work is needed to elucidate the mechanism by which APC enhances GSK-3 activity. APC may induce conformational changes or stabilize active GSK-3 conformations. To explore this possibility, the crystal structure of GSK-3 can be examined and compared in the presence and absence of APC. Alternatively, APC may promote interaction between GSK-3 and its substrates. APC can directly bind β-catenin, but direct APC interaction with tau has not been reported. To test this possibility, we can disrupt the β-catenin binding sites on APC and see if it can still enhance β-catenin phosphorylation by GSK-3. We can also identify regions of APC that are required to enhance GSK-3 activity to provide insight into how it performs this function. This can be achieved through deletion analysis followed by *in vitro* kinase assays for GSK-3 activity in the presence or absence of APC fragments. An interesting potential candidate region is the recently described β-catenin inhibitory domain (CID) of APC. This region spans approximately 60 amino acids and is required for APC to downregulate β-catenin, but its mechanism of action is unknown (Kohler et al., 2009). It is located between two β-catenin binding sites and has no known interacting partners. The CID is included in the SAMP fragment of APC used in our in vitro assays, and could contribute to enhancing GSK-3 activity. APC also binds topoisomerase IIα and enhances its enzymatic activity (Wang et al., 2008), and binds the Rac1 and Cdc42 specific guanine nucleotide exchange factor Asef1 and enhances its GEF activity (Kawasaki et al.,

2000). Taken together, these data suggest APC directly enhances GSK-3 activity and may have a broader role in regulating enzymatic activity. There is little overlap between the APC fragment that enhances topoisomerase IIα activity and the SAMP fragment that enhances GSK-3 activity, and there is no overlap between either of these two fragments and the fragment that enhances Asef1 activity, suggesting APC may regulate activity of these enzymes through distinct domains.

Our finding that APC can enhance GSK-3 activity led us to ask whether APC regulates other GSK-3 substrates or GSK-3 dependent signaling pathways. APC is an essential negative regulator of Wnt signaling, and we find additional roles for APC as an essential negative regulator of GS and mTORC1. APC knockdown reduces inhibitory phosphorylation of GS by GSK-3 without activating upstream PI3K/Akt signaling, consistent with direct regulation of GSK-3 by APC. APC knockdown also mimics GSK-3 inhibition by activating mTORC1. Furthermore, mTORC1 is aberrantly active in many tissues in *Apc* mutant zebrafish, and we find reduced GS phosphorylation and aberrant mTORC1 activation in intestinal adenomas in *Apc* mutant mice. Importantly, activating downstream Wnt signaling by expressing constitutively active β-catenin does not affect GS or mTORC1 activity. These data identify novel roles for APC in the regulation of GS and mTORC1, and suggest APC regulation of GSK-3 activity is important in several signaling pathways.

Surprisingly, we observe strong mTORC1 activation in whole embryo

lysates from *apc^mcr/mcr* zebrafish compared to pooled wild type and heterozygous mutants by western blot analysis (Figure 3-1). Immunohistochemical staining also confirms nearly global mTORC1 activation in *apc^mcr/mcr* zebrafish (Figure 3-1). mTORC1 integrates many upstream signals that control its activity, and how decisions are made in the presence of competing signals is an area of intense investigation. Our observations suggest that inhibitory input from APC and GSK-3 may be required to inactivate mTORC1 even in the presence of other inhibitory signals. However it is also possible that APC regulates several factors that influence mTORC1, such as Akt or ERK. We find APC knockdown activates mTORC1 without affecting Akt phosphorylation or GSK-3 phosphorylation by Akt, suggesting APC does not regulate Akt activity (Figure 2-1).

Future work should continue to address the mechanism by which APC inhibits mTORC1. APC enhances GSK-3 activity and GSK-3 inhibits mTORC1 by phosphorylating TSC2 (Inoki et al., 2006). Thus we can determine whether APC loss of function reduces TSC2 phosphorylation by GSK-3. In contrast to our model, APC may not promote TSC2 phosphorylation by GSK-3, and could regulate mTORC1 through a different mechanism. This would prompt investigation into whether APC regulates other upstream factors that control mTORC1 activity, including ERK and AMPK. APC loss of function activates ERK (discussed further on pgs 107-108), and ERK activates mTORC1. Thus we could ask if ERK inhibition blocks mTORC1 activation.

APC may also regulate other GSK-3 dependent signaling pathways

through its ability to enhance GSK-3 activity. Bone morphogenetic protein (BMP)

signaling promotes phosphorylation of receptor regulated Smads (R-Smads)

which allows them to bind Smad4 and translocate to the nucleus to activate

transcription (ten Dijke, 2006). GSK-3 phosphorylates R-Smads at different sites

which promote R-Smad degradation. GSK-3 inhibition enhances the magnitude

of BMP target gene and BMP reporter activation in response to BMP ligands, as

well as the duration of the response (Fuentealba et al., 2007; Fukuda et al.,

2010). Interestingly, APC knockdown similarly increases activation of a BMP

reporter by BMP ligands (Miclea et al., 2011). Expression of stabilized,

constitutively active β-catenin has no effect on BMP signaling (Fuentealba et al.,

2007; Fukuda et al., 2010). These data suggest that both APC and GSK-3

negatively regulate BMP signaling in a β-catenin-independent manner. Future

work is needed to determine whether APC promotes R-Smad phosphorylation by

GSK-3.

    GSK-3 also regulates signaling through extracellular signal-related kinase

(ERK). Extracellular signals such as EGF activate RAS and induce activating

phosphorylation of MEK which subsequently phosphorylates and activates ERK.

GSK-3 knockdown increases ERK phosphorylation and expression of

downstream targets of ERK activation (Wang et al., 2006; Zhai et al., 2007).

Furthermore, treatment with GSK-3 inhibitors increases ERK phosphorylation

within 30 minutes (Wang et al., 2006). These data suggest GSK-3 negatively

regulates ERK activity. Similarly, ERK phosphorylation is induced by loss of APC

function, and expressing wild type APC decreases ERK phosphorylation in *Apc* mutant colorectal cancer cells. APC overexpression also blocks RAS-induced cell proliferation (Park et al., 2006). The mechanism by which GSK-3 regulates ERK is not clear, but may involve GSK-3 phosphorylating and stabilizing mitogen kinase phosphatase-1 (MKP-1) which de-phosphorylates and thus deactivates MEK (Wang et al., 2009b). Thus we can ask whether APC promotes MKP-1 phosphorylation by GSK-3.

APC has also been shown to promote phosphorylation of direct GSK-3 substrates. For example, APC promotes β-catenin and GS phosphorylation by GSK-3. GSK-3 phosphorylates the transcription factor CCAAT enhancer-binding protein β (C/EBPβ) (Zhao et al., 2005), and APC knockdown reduces C/EBPβ phosphorylation at the GSK-3 sites (Larabee et al., 2011). These data suggest APC also promotes C/EBPβ phosphorylation by GSK-3. *Bacillus anthracis* edema toxin (ET) activates GSK-3 in the nucleus, and GSK-3 inhibitors block effects of ET on gene expression and cell cycle progression (Larabee et al., 2008). Interestingly, ET increases levels of nuclear APC and promotes physical interaction between APC, GSK-3, and C/EBPβ in the nucleus. *Apc* knockdown or mutation also blocks effects of ET on gene expression, as does C/EBPβ knockdown (Larabee et al., 2011). These data suggest ET promotes nuclear association between APC and GSK-3 to promote C/EBPβ phosphorylation by GSK-3.

Taken together, these data suggest APC promotes phosphorylation of β-

catenin, GS, and C/EBPβ by GSK-3, and negatively regulates Wnt, mTORC1, BMP, and ERK signaling, like GSK-3. APC negatively regulates Wnt signaling by promoting phosphorylation of β-catenin by GSK-3. APC may regulate mTORC1, BMP, and ERK signaling by promoting phosphorylation of TSC2, R-Smads, and MKP-1 respectively by GSK-3 (Table 4-1).

Future work is also needed to determine whether APC regulates additional GSK-3 substrates and GSK-3 dependent signaling pathways. GSK-3 inhibits Notch signaling by phosphorylating and destabilizing the Notch intracellular domain (NICD) (Jin et al., 2009; Kim et al., 2009), and Hedgehog signaling by phosphorylating Ci/Gli to promote its proteolytic processing to generate a truncated repressor (Forde and Dale, 2007; Jia et al., 2002; Price and Kalderon, 2002). The role of APC in Notch and Hedgehog signaling or phosphorylation of NICD or Ci/Gli has not been examined. GSK-3 also phosphorylates over 100 putative substrates and a role for APC in regulating the vast majority of them has not been examined. Future work could determine whether APC loss of function reduces NICD and Ci/Gli phosphorylation by GSK-3, and activates Notch and Hedgehog target genes. We can also survey many GSK-3 substrates to see which are affected by APC loss of function. Phosphorylation-specific antibodies are available to detect phosphorylation of many GSK-3 substrates including p53, c-Myc, amyloid precursor protein, and cyclin E. Thus we can knockdown APC expression in cultured cells or induce *Apc* deletion in mice and western blot or immunostain to detect phosphorylation of GSK-3 substrates.

Table 4-1

Signaling pathway	GSK-3 function	APC function
Wnt	- Inhibits Wnt signaling - Phosphorylates β-catenin	- Inhibits Wnt signaling - Promotes β-catenin phosphorylation
Insulin/Glycogen Synthase	- Phosphorylates Glycogen Synthase	- Promotes Glycogen Synthase phosphorylation
mTORC1	- Inhibits mTORC1 - Phosphorylates TSC2	- Inhibits mTORC1
BMP	- Inhibits BMP signaling - Phosphorylates R-Smads	- Inhibits BMP signaling
ERK	- Inhibits ERK	- Inhibits ERK
C/EBPβ	- Phosphorylates C/EBPβ	- Promotes C/EBPβ phosphorylation

Table 4-1. GSK-3 and APC play similar roles in many signaling pathways.

GSK-3 is an essential negative regulator of diverse signaling pathways. APC

inhibits many of the same signaling pathways by promoting phosphorylation of

GSK-3 substrates.

4-3. Axin-independent APC/GSK-3 interaction

APC and GSK-3 physically associate through the scaffolding protein Axin which mediates β-catenin phosphorylation by GSK-3. GSK-3 suppresses mTORC1 activity by phosphorylating TSC2 which also associates with Axin (Inoki et al., 2006; Mak et al., 2005). Wnt signaling inhibits GSK-3 in the Axin complex, reduces TSC2 phosphorylation, and activates mTORC1 within minutes (Inoki et al., 2006; MacDonald et al., 2009). Axin knockdown also activates mTORC1 (Inoki et al., 2006, Supplemental Figure S2-6B). These data suggest TSC2 and mTORC1, like β-catenin, are regulated by GSK-3 in the Axin complex. To confirm this, additional experiment should test whether disrupting Axin/GSK-3 or Axin/TSC2 interaction reduces TSC2 phosphorylation and activates mTORC1.

APC and GSK-3 also negatively regulate BMP and ERK signaling (Fuentealba et al., 2007; Fukuda et al., 2010; Miclea et al., 2011; Park et al., 2006; Wang et al., 2006; Zhai et al., 2007). Treatment with Wnt ligands for 1 hour reduces R-Smad phosphorylation by GSK-3 and increases total R-Smad levels (Fuentealba et al., 2007). GSK-3 regulates ERK signaling by antagonizing phosphorylation of MEK and ERK. Wnt ligands induce phosphorylation of MEK and ERK within 30 minutes (Yun et al., 2005) and Axin knockdown similarly increases MEK and ERK phosphorylation (Jeon et al., 2007). Axin overexpression reduces MEK and ERK phosphorylation and blocks EGF induced reporter activation and EGF induced cell proliferation (Jeon et al., 2007). These

data suggest BMP and ERK signaling may also be regulated by GSK-3 and APC in the Axin complex.

GSK-3 also phosphorylates and inhibits Glycogen Synthase, but Wnt signaling and Axin knockdown do not affect GS phosphorylation (Supplemental Figure S2-6). As APC promotes GS phosphorylation by GSK-3 (Figure 2-1), these data suggest a functional Axin-independent APC/GSK-3 interaction and raise the question of whether APC and GSK-3 can physically associate in an Axin-independent manner.

Stable Axin-independent APC/GSK-3 association has not been reported to date. GSK-3 phosphorylates APC at multiple sites (Ikeda et al., 2000; Rubinfeld et al., 1996; Zumbrunn et al., 2001), and APC directly enhances GSK-3 activity *in vitro* (Figure 2-3), suggesting APC and GSK-3 can directly interact. However, stable direct APC/GSK-3 binding is not detected in vitro (Hinoi et al., 2000), and scaffolding proteins may be required for them to function together in cells. In support of this, APC and GSK-3 must each interact directly with the Axin scaffold for β-catenin to be efficiently phosphorylated by GSK-3 in cells. Cell fractionation studies indicate APC exists in at least two distinct cytoplasmic complexes, one of which was identified as the Axin/β-catenin destruction complex (Penman et al., 2005). The function of the other complex, and whether it contains GSK-3 have not been determined (Penman et al. proposed this complex interacts with and regulates microtubules based on the ability of APC in those fractions to bind microtubules in vitro (Penman et al., 2005)).

Additional experiments are needed to determine whether APC and GSK-3 stably interact in the absence of Axin. For example, APC can be immunoprecipitated from SNU475 cells which do not express Axin (Satoh et al., 2000), to see if GSK-3 is associated, and vice versa. Common interacting partners between APC and GSK-3 can also be identified to provide insight into potential scaffolding proteins and complexes that might bind both APC and GSK-3. If another scaffold or complex that binds both APC and GSK-3 is identified, we can ask whether deletion of the scaffold, or of proteins essential for complex formation, reduces GS phosphorylation by GSK-3. We can also test whether specific removal of APC or GSK-3 from the complex reduces GS phosphorylation.

4-4. Wnt signaling induces APC dissociation from the Axin complex

Wnt signaling inhibits GSK-3 through an unknown mechanism to stabilize β-catenin and activate downstream signaling. APC enhances GSK-3 activity and direct APC/Axin interaction is required to downregulate β-catenin and inhibit Wnt signaling. These data led us to hypothesize that Wnts disrupt APC interaction with Axin to reduce GSK-3 activity and stabilize β-catenin. We find Wnts rapidly induce APC dissociation from Axin without affecting GSK-3/Axin interaction. Consistent with our hypothesis, APC dissociation correlates closely with β-catenin stabilization. Wnt signaling recruits Axin to LRP6 at the plasma

membrane and we find Axin interaction with LRP6 causes APC dissociation. These data suggest Wnt-induced Axin recruitment to LRP6 promotes APC dissociation to reduce GSK-3 activity and activate downstream signaling.

Consistent with our model, Axin, LRP6, GSK-3, β-catenin, and the Axin binding protein microtubule actin cross-linking factor-1 (MACF-1), but not APC, are detected in isolated cell membranes following Wnt stimulation. Furthermore, MACF-1 immunoprecipitation from membrane fractions after Wnt stimulation reveals MACF-1 interaction with Axin, LRP6, GSK-3, and β-catenin, but not APC (Chen et al., 2006).

Tran and Polakis show that APC within the Axin complex is polyubiquitinated on lysine 63 (Tran and Polakis, 2012). Knocking down HectD1, the E3 ubiquitin ligase that polyubiquitinates APC on K63, disrupts APC/Axin interaction (Tran et al., 2013). Interestingly, Wnts reduce K63 polyubiquitination, correlating with APC dissociation and β-catenin stabilization (Tran and Polakis also immunoprecipitated APC and found that Wnts reduce the amount of associated Axin, thus reproducing our finding that Wnts induce APC dissociation from the Axin complex) (Tran and Polakis, 2012). Taken together, these data suggest Wnts induce APC dissociation from the Axin complex, possibly by reducing polyubiquitination at lysine 63 within APC.

While we demonstrate that Wnt signaling induces APC dissociation from Axin which correlates with β-catenin stabilization, additional experiments are needed to determine whether APC dissociation is required for, or contributes to,

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β-catenin stabilization. To prevent APC dissociation, an Axin-APC fusion protein can be expressed and the rate of β-catenin accumulation in response to Wnt ligands can be measured. Alternatively, previously described inducible dimerization systems (Bayle et al., 2006) can be employed to force APC interaction with Axin in the presence of Wnt ligands. The Axin complex can also be immunoprecipitated followed by in vitro kinase assay to determine whether APC dissociation from Axin contributes to reduced GSK-3 activity. We find Axin binding to LRP6 causes APC dissociation. To test whether Axin binding to LRP6 reduces K63 polyubiquitination, we can express a constitutively active LRP6 fragment that binds Axin, or control fragment that does not bind Axin (Figure 2-5) to see if they affect K63 polyubiquitination. Deubiquitylating enzymes can also be screened to identify ones that are required for Wnt-induced deubiquitination of APC. We can then determine whether those enzymes are required for Wnt-induced APC dissociation.

APC binds directly to the regulator of G-protein signaling (RGS) domain of Axin. G Protein-Coupled Receptor Kinase 2 (GRK2) contains a similar RGS domain which also directly binds APC. Interestingly, Wnt signaling enhances interaction between APC and the RGS domain of GRK2 (Wang et al., 2009a). These data suggest Wnts may induce APC dissociation from Axin allowing association with GRK2 instead. The functional significance of APC interaction with GRK2 is not known. To address this, future experiments can determine whether deletion of the RGS domain of GRK2 affects β-catenin stabilization or

activation of Wnt target genes.

APC enhances β-catenin phosphorylation by GSK-3 within the Axin complex, but also reduces β-catenin dephosphorylation by protein phosphatase 2A (PP2A). PP2A binds directly to Axin and is a component of the Axin/GSK-3/APC/β-catenin complex. β-catenin is rapidly dephosphorylated by PP2A in *Apc* mutant colorectal cancer cell extracts, and this is blocked by the addition of wild type APC. Similarly, APC blocks dephosphorylation of β-catenin by PP2A when purified proteins are assayed in vitro (in the absence of cell extracts) (Su et al., 2008). Thus APC prevents β-catenin dephosphorylation by PP2A. These data suggest Wnt-induced APC dissociation from the Axin complex reduces β-catenin phosphorylation by both reducing GSK-3 activity and allowing β-catenin dephosphorylation by PP2A. This parallels other signaling pathways in which reduced phosphorylation of an essential effector is achieved rapidly by simultaneous inhibition of the predominant kinase and activation of a phosphatase. For example, insulin activates Akt which phosphorylates and inhibits GSK-3 so it can no longer phosphorylate GS. Insulin also activates glycogen targeted protein phosphatase-1 which dephosphorylates GS (Brady and Saltiel, 2001).

4-5. Roles for APC through regulation of GSK-3 and mTORC1

GSK-3 suppresses mTORC1 activity (Inoki et al., 2006). We find APC

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enhances GSK-3 activity and negatively regulates mTORC1. These observations led us to ask whether APC regulation of GSK-3 and mTORC1 is important during embryonic development. mTORC1 is aberrantly activate in $apc^{mcr/mcr}$ zebrafish and we hypothesized that mTORC1 contributes to *Apc* mutant phenotypes. We find mTORC1 inhibition extends the survival of $apc^{mcr/mcr}$ zebrafish and partially rescues circulation defects. mTORC1 inhibition also rescues liver enlargement in heterozygous apc^{mcr} zebrafish, and rescues defects in morphogenesis of the anterior-posterior axis when combined with a Wnt inhibitor in homozygous mutants. These data suggest mTORC1 activation contributes to *Apc* mutant phenotypes, and highlight the importance of APC suppressing mTORC1 activity during development.

Early lethality in $apc^{mcr/mcr}$ zebrafish is likely the result of heart defects which include hyperproliferation of the endocardial cushions. Endocardial cushions in these fish do not form a proper valve and instead proliferate and eventually fuse, separating the ventricle from the atrium and blocking circulation (Hurlstone et al., 2003). We find mTORC1 inhibition improves circulation and extends the survival of $apc^{mcr/mcr}$ zebrafish, suggesting mTORC1 activation may contribute to cushion hyperproliferation and reduced cardiac function. However, circulation is an indirect measure of heart function and survival may be influenced by other factors. Therefore additional experiments are needed to directly address the role of APC and the effects of aberrant mTORC1 activation on heart development. Immunohistochemical staining for phosphorylated S6 can

be performed to determine whether mTORC1 is aberrantly active in the heart and more specifically, in the endocardial cushions in $apc^{mcr/mcr}$ zebrafish. IHC and in situ hybridization can also be used to see if mTORC1 inhibition reduces valve markers and proliferative markers which are upregulated in $apc^{mcr/mcr}$ zebrafish. In vivo imaging and high speed videomicroscopy can also be utilized to assess whether mTORC1 inhibition reduces cushion proliferation or rescues heart valve formation. Contractility and blood flow through the heart can be measured to see if mTORC1 inhibition improves cardiac function.

Wnt ligands are expressed in endocardial cushions and developing atrioventricular valves in mice, and a Wnt/β-catenin reporter is active in these tissues (Alfieri et al., 2009). Wnt/β-catenin signaling is also active in endocardial cushions in zebrafish. The Wnt inhibitor Dkk1 blocks cushion formation in zebrafish, indicating Wnt signaling plays a critical role in cushion and valve formation (Hurlstone et al., 2003). Cushion-specific overexpression of Wnt ligands in chick embryos also causes cushion hyperproliferation that blocks AV blood flow (Person et al., 2005). These data suggest aberrant β-catenin signaling in $apc^{mcr/mcr}$ zebrafish could contribute to the cushion and valve defects. Importantly, these data do not conclusively demonstrate a role for β-catenin, since Wnt signaling also activates mTORC1 (Inoki et al., 2006).

GSK-3β knockdown also results in hyperproliferation of endocardial cushions and failure to form a functional heart valve (Lee et al., 2007), as does a mutation in *Axin (mbl)* that disrupts its interaction with GSK-3 (Heisenberg et al.,

2001; Hurlstone et al., 2003). *apc*^mcr/mcr^ zebrafish hearts also fail to loop, and this is phenocopied by GSK-3β knockdown or *Axin mbl* mutation (Heisenberg et al., 2001; Hurlstone et al., 2003; Lee et al., 2007). Taken together, these data suggest a critical role for APC in regulating cardiac development by activating GSK-3 in the Axin complex and inhibiting β-catenin and mTORC1.

Wnt/β-catenin signaling is essential during many stages of heart development and is required for heart looping and formation of functional AV valves (Brade et al., 2006). However, β-catenin overexpression has not been shown to be sufficient to cause heart looping or valve defects. This could simply be because β-catenin has not been overexpressed in the endocardial cushions when they are proliferating to form the valves. β-catenin overexpression early during development induces an ectopic secondary axis, and later suppresses anterior development and can block heart formation (Ciani and Salinas, 2005; Kelly et al., 1995; Ueno et al., 2007). The presence of maternal wild type APC likely prevents these early Wnt activation phenotypes in *apc*^mcr/mcr^ zebrafish. Alternatively, the lack of heart looping or heart valve defects in response to β-catenin overexpression could indicate that activation of both β-catenin and mTORC1 signaling is important in *apc*^mcr/mcr^ zebrafish, GSK-3β morphants and *Axin mbl* mutants (and/or disruption of other overlapping functions of APC, GSK-3, and Axin). Similarly, TSC loss of function activates mTORC1 and mimics some *Apc* mutant phenotypes, but heart looping or heart valve defects have not been reported (DiBella et al., 2009; Kim et al., 2010).

To assess the relative contributions of Wnt/β-catenin and mTORC1 signaling to heart defects in *apc^mcr/mcr^* zebrafish, Wnt/β-catenin signaling can be inhibited alone and in combination with mTORC1 inhibition. Heart looping, endocardial cushion proliferation, valve formation, and cardiac function can be assessed using the techniques described earlier (pg 118). Transgenic zebrafish can also be made to express constitutively active β-catenin in the developing heart tube or endocardial cushions to see if β-catenin activation is sufficient to induce the heart defects seen in *apc^mcr/mcr^* zebrafish. Transgenic zebrafish can also be created to allow inducible TSC deletion, and thus mTORC1 activation, in the developing heart to see if mTORC1 activation phenocopies heart defects in *apc^mcr/mcr^* zebrafish. To provide evidence that *Apc* mutant phenotypes result from reduced GSK-3 activity, we can overexpress GSK-3 in *apc^mcr/mcr^* zebrafish to see if it rescues heart defects. Furthermore, if a region of APC that is essential for enhancing GSK-3 activity can be identified (as proposed in section 4-2, pg 104), we can see if expressing an APC mutant that lacks this region rescues *apc^mcr/mcr^* zebrafish. Rescue by wild type APC and not the APC mutant would suggest a requirement for APC enhancing GSK-3 activity.

Preliminary observations suggest combining the Wnt inhibitor XAV939 with mTOR inhibitors does not fully rescue heart looping defects in *apc^mcr/mcr^* zebrafish (although more thorough dose curves and treatment time courses are needed). These observations raise the possibility that additional functions of APC may contribute. For example, BMP signaling is active on one side on the

developing heart tube, establishing early asymmetry that is required for heart looping. Thus inhibition or ectopic activation of BMP signaling in the heart tube prevents looping (Breckenridge et al., 2001; Chen et al., 1997). APC and GSK-3 negatively regulate BMP signaling (Fuentealba et al., 2007; Fukuda et al., 2010; Miclea et al., 2011). These data suggest BMP signaling may be ectopically active in $apc^{mcr/mcr}$ zebrafish which contributes to failed heart looping.

APC regulation of GSK-3 and mTORC1 may also be critical during liver development. Heterozygous $apc^{mcr/+}$ zebrafish develop enlarged, hyperplastic livers, which can be rescued by mTORC1 inhibition (Figure 3-4). β-catenin signaling is active in $apc^{mcr/+}$ livers and β-catenin knockdown also rescues liver enlargement. Apoptosis during liver development in $apc^{mcr/mcr}$ zebrafish results in failure to form a morphologically identifiable liver (Goessling et al., 2008). Similarly, Apc deletion in murine hepatoblasts causes liver degeneration and lethality (Decaens et al., 2008). Significant apoptosis is also observed in the livers of Gsk-$3β$ knockout mice, resulting in liver degeneration and lethality around the same time as the hepatoblast-specific Apc mutants (Hoeflich et al., 2000). $Tsc2$ null mice also die around the same time from severe liver hypoplasia (Onda et al., 1999). Taken together, these data suggest repression of mTORC1 and β-catenin by APC is essential for liver development and control of liver size. Immunohistochemical staining for phosphorylated S6 should be performed in the future to see if mTORC1 is aberrantly active in Apc and Gsk-$3β$ mutant livers. These fish and mice can also be treated with Wnt and mTOR inhibitors alone or

in combination to see if they block liver apoptosis and rescue liver development.

APC regulation of GSK-3 and mTORC1 may also be essential for development of other organs and tissues, and for stem cell regulation in adults. As examples, abnormal body curvature in $apc^{mcr/mcr}$ zebrafish is rescued by combined mTORC1 and Wnt/β-catenin inhibition, suggesting suppression of both of these signaling pathways by APC is essential for morphogenesis of the anterior-posterior axis. GSK-3 knockdown in mouse bone marrow causes short term expansion of hematopoietic stem cells (HSCs), which requires β-catenin, and long term depletion which is caused by mTORC1 activation (Huang et al., 2009). Conditional APC deletion in mouse bone marrow similarly causes short term HSC expansion and long term depletion (Qian et al., 2008). These data in combination with our observations suggest widespread roles for APC through regulation of GSK-3 and mTORC1.

4-6. *Apc* mutations promote tumorigenesis through combined Wnt and mTORC1 activation

APC is a tumor suppressor that is mutated in most colorectal cancers. Apc^{mcr} zebrafish contain a premature stop codon within the mutation cluster region of *Apc,* resulting in expression of a truncated protein analogous to those commonly found in human colorectal cancers (Hurlstone et al., 2003). Heterozygous $Apc^{mcr/+}$ zebrafish develop adenomas in multiple tissues, including

the intestine (Haramis et al., 2006). We find mTORC1 is aberrantly active in Apc^{mcr} zebrafish, and mTORC1 contributes to many development defects in these fish. We hypothesize that oncogenic *Apc* mutations reduce GSK-3 activity and activate Wnt and mTORC1 which combine to strongly promote tumorigenesis. In support of this, we find reduced GS phosphorylation and active mTORC1 in adenomas in Apc^{min} mice. Furthermore, we find mTORC1 is constitutively active in *Apc* mutant colorectal cancer cells and can be turned off by reintroduction of wild type APC.

Reduced GS phosphorylation in intestinal adenomas in Apc^{min} mice could be due to additional mutations. Therefore future work should address the effects of truncating *Apc* mutations on GSK-3 activity in more detail. To provide further evidence that truncating *Apc* mutations reduce GSK-3 activity, we can examine phosphorylation of multiple GSK-3 substrates in wild type compared to $apc^{mcr/mcr}$ zebrafish, or in *Apc* mutant intestinal adenomas. GSK-3 or Axin can also be immunoprecipitated followed by in vitro assay for GSK-3 activity based on its ability to phosphorylate added substrates. Interestingly, inducible deletion of *Gsk-3* in mouse gastric epithelium phenocopies *Apc* deletion in gastric epithelium. Within a few days of *Gsk-3* or *Apc* deletion, intestinal-type gastric adenomas develop in the antrum, and fundic gland polyps form in the corpus (Radulescu et al., 2012). These data suggest loss of GSK-3 activity could be responsible for tumorigenesis resulting from APC loss of function.

Our data suggest mTORC1 activation may contribute to tumorigenesis

downstream of *Apc* mutation. In support of this, the mTORC1 inhibitors rapamycin and RAD001 reduce the number of intestinal polyps and extend survival of *Apc^{min}* mice (Koehl et al., 2010; Metcalfe et al., 2010). RAD001 also reduces polyp number and polyp size, and extends survival of *Apc$^{\Delta716}$* mice that also have a truncating *Apc* mutation within the mutation cluster region (Fujishita et al., 2008).

β-catenin is also important downstream of *Apc*. β-catenin knockdown reduces the size of resultant tumors when *Apc* mutant colorectal cancer cells are xenografted to mice (Roh et al., 2001; Scholer-Dahirel et al., 2011). β-catenin knockdown also reduces adenoma cell proliferation and the number of adenomas in *Apc^{min}* mice (Foley et al., 2008). These data suggest both mTORC1 and β-catenin activation contribute to tumorigenesis. Interestingly, constitutive β-catenin activation in the urothelium synergizes with PTEN loss of function, which activates mTORC1, to promote bladder tumorigenesis. This synergism is blocked by rapamycin (Ahmad et al., 2011). Future experiments should combine β-catenin knockdown with mTORC1 inhibition in *Apc* mutant mice to see if combined treatment is more effective than either treatment alone at reducing the number and size of adenomas, as well as extending survival. Sphingadiene treatment reduces Wnt signaling in *Apc^{min}* mice and reduces the number of intestinal adenomas, similar to rapamycin. Combining sphingadiene and rapamycin treatment has an additive effect of reducing adenoma number (Kumar et al., 2012). These data suggest combined Wnt and mTORC1 inhibition is more

effective than inhibition of either pathway alone. However, this result should be confirmed with more specific and well-established methods of inhibiting Wnt signaling (such as β-catenin knockdown).

Interestingly, *Apc* mutation may initiate adenoma formation prior to activation of Wnt/β-catenin signaling. In humans, nuclear β-catenin is detected in less than 10% of sporadic *Apc* mutant polyps and early adenomas in FAP, but is detected much more frequently in later stage carcinomas (Anderson et al., 2002; Kobayashi et al., 2000; Phelps et al., 2009). Nuclear β-catenin is also rarely observed in microadenomas in a rat model of FAP (Amos-Landgraf et al., 2007). Intestinal hyperproliferation in *apc$^{mcr/mcr}$* zebrafish also occurs in the absence of detectable nuclear β-catenin or expression of a Wnt reporter (Phelps et al., 2009). These data raise the possibility that *Apc* mutant intestinal adenomas could develop initially in the absence of active β-catenin signaling. Thus it is interesting to speculate that mTORC1 activation may have a role in early adenoma formation. To test this possibility we could conditionally delete (or truncate) *Apc* in rat intestine and observe the timing of mTORC1 activation compared to nuclear β-catenin localization and activation of Wnt target genes. We could then inhibit mTORC1 or β-catenin signaling at various times after *Apc* deletion to determine when each pathway is required during tumor development and progression. This method would also allow us to distinguish between a requirement for mTORC1 or β-catenin signaling in tumor initiation as opposed to growth or maintenance of established tumors.

4-7. Conclusion

In conclusion, we identify new roles for APC through its ability to directly enhance GSK-3 activity. We find previously unappreciated roles for APC in regulating Glycogen Synthase and mTORC1, and our work provides a mechanism for APC function in numerous other GSK-3 dependent cellular processes and signaling pathways. We also identify new mechanisms for regulation of APC and GSK-3 in the Wnt signaling pathway to answer the longstanding question of how Wnt signaling reduces GSK-3 activity. Finally, we demonstrate that mTORC1 is essential for mediating the effects of oncogenic *Apc* mutation, thus establishing a new model of colorectal cancer pathogenesis.

Future work will focus on defining the breadth of GSK-3 substrates regulated by APC and exploring the mechanisms of Axin-independent APC/GSK-3 interaction. Future work will also test the prediction that combined inhibition of mTORC1 and Wnt/β-catenin signaling will efficiently block development and progression of colorectal tumors.

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