Mapping Function and Cooperativity in the Cancer Genome: RSPO2 and WAC

A DISSERTATION
SUBMITTED TO THE FACULTY OF
UNIVERSITY OF MINNESOTA
BY

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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

Advisor: Dr. David A. Largaespada

September 2014

Acknowledgements

I would like to thank the people who contributed to and supported this work. Many current and former members of the Largaespada lab lent me their time, wisdom, and efforts, including Bobbie Tschida, Sue Rathe, Vincent Keng, Julia Hatler, Branden Moriarty, Adrienne Watson, Eric Rahrmann, Pauli Jackson, Chani Becker, Tim Kuka, Alpay Temiz, and undergraduate students David Hu, Brooke McDonald, and Kelsey Navis. Juan Abrahante, Chris Clark, and Callie Janik from Tim Starr's lab, the "GI working group," including Bob Cormier and Pat Scott, Mike Linden and Khalid Amin from the UMN pathology department, Sekar Seshagiri and Frederic de Sauvage from Genentech, Josep Llovet's lab at Mount Sinai, Michael Burns, and Ruben Harris have also been helpful collaborators.

I also want to thank Yoji Shimizu, Pete Bitterman, Lisa Schimmenti, Tucker LeBien, Susan Shurson, and Nick Berg for their work in administering the MSTP program and providing professional guidance to aspiring physician scientists.

Members of my thesis committee, Scott Dehm, Bob Cormier, Carol Lange, and Kris Hogquist, have been wonderfully supportive and offered insightful suggestions and critiques for this work over the years.

Tim Starr, my unofficial co-advisor, helped me tremendously during my PhD. His post-doc research in the Largaespada lab initially identified *RSPO2* and *WAC* as candidate cancer genes, and he has been a generous collaborator in these projects. I strive to emulate his clarity in scientific writing and presentations.

Lastly, my advisor, David Largaespada has been an inspiring scientist and thoughtful mentor. I want to thank him for giving me permission to explore, fail, and find my own questions during my PhD research. I have learned so much from him about creativity, persistence, and humility in the scientific enterprise.

My research was funded by an F30 fellowship from the NIH (NIH F30 CA171547), training grants through the MSTP program (NIH T32 GM008244) and the Cancer Biology Training Grant (NIH T32 CA009138-36), and NIH Grant R01 CA134759.

To Hilarie,

my wife, partner, and bedrock

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Abstract

Cancer is a leading cause of morbidity and mortality worldwide. Developing molecularly targeted therapies to improve patient outcomes will require comprehensive understanding of the genetic events that give rise to cancer. Large-scale efforts to catalog the genetic aberrations in human tumors are currently underway. Transposon-based insertional mutagenesis screens provide a complementary, comparative genomics approach for cancer gene discovery. *Sleeping Beauty* (*SB*) transposon mutagenesis has been used to identify genes that contribute to intestinal tumor formation. Two of the identified genes, *Rspo2* and *Wac*, were the subjects of this thesis research. *R-spondin 2* (*RSPO2)* belongs to the R-spondin family of secreted Wnt agonists. Activation of *RSPO2* and *3* has been identified in human colorectal and liver cancer, although the functional significance of these lesions has not been proven, and genetic screens in mice suggested that *Rspo2* and *Rspo3* are oncogenic in the mammary gland as well. Here we present an analysis of *RSPO2* and *3* in human colon, breast, and liver cancer. We found that expression of RSPO2 and 3 was increased in subsets of all three tumor types, and correlated with activation of Wnt signaling in these tumors compared to normal tissues. We further investigated the functional significance of increased *RSPO2* in breast and liver cancer models. We showed that *RSPO2* can activate Wnt signaling in non-transformed breast epithelial cells and *RSPO2* overexpression is required for Wnt signaling and proliferation in an *RSPO2*-high breast cancer cell line. We developed an *in vivo* model of *RSPO2* activation in the mouse liver using hydrodynamic tranfection with transposon-based DNA vectors followed by Fah selection. We found that increased

expression of RSPO2 in the liver activated Wnt signaling and promoted hepatomegaly. RSPO2 overexpression cooperated with Trp53 inactivation to initiate tumor formation. Hepatocellular carcinomas that formed in this model exhibited activated Wnt signaling. This model will facilitate further studies of R-spondin signaling and enable development of RSPO-targeted therapy. *WW domain containing adaptor with coiled-coil* (*WAC)* is an adaptor protein required for diverse biological processes, including regulation of gene transcription through histone H2B monoubiquitination. *Wac* was inactivated by transposon insertions in three *SB* screens for genetic drivers of intestinal tumorigenesis in wild type, *Apc*-deficient, and *Trp53*-deficient backgrounds. We found that *WAC* was somatically mutated and downregulated in human colorectal tumors. Further, mutant versions of *WAC* identified in human tumors were unable to transcriptionally activate expression of *cdkn1a* (the gene encoding p21) in a zebrafish embryo model. Depletion of *Wac* cooperated with *Apc* and *Trp53* inactivation to promote anchorage independent growth of mouse colonic epithelial cells. The results of these studies collectively implicate *RSPO2* as an oncogene in multiple wnt-responsive tissues and suggest *WAC* is a tumor suppressor in the colon that cooperates with *APC* and *TP53*. Additional work is warranted to further define the signaling pathways regulated by *RSPO2* and *WAC* in tumorigenesis and to determine if drug targeting of these pathways is a viable strategy for improving the treatment of *RSPO2* and *WAC*-driven cancers.

Table of Contents

List of Tables

List of Figures

Chapter 1. **Page**

Chapter 2. **Page**

Chapter 1: Introduction

Cancer gene discovery as a route to targeted and more effective cancer therapies

Summary

Cancer is the cause of death for one in four Americans. Breast and colon cancer are two of the leading causes of cancer related death, and liver cancer mortality is both common and increasing. Although treatment strategies have improved and record numbers of people are living as cancer survivors, therapeutic options are still non-specific and minimally efficacious for many patients. Molecularly targeted therapy is a strategy in which drugs are designed to target the specific molecular pathways that are dysregulated in cancer. Some molecularly targeted therapies have been successfully integrated into clinical oncology, but this approach is still in its infancy. The Wnt/beta-catenin pathway is an important regulator of organismal development and adult stem cell renewal. Aberrantly activated Wnt/beta-catenin signaling promotes many cancer types, including colon, breast and liver, and Wnt signaling is an attractive therapeutic target in these cancers. Despite challenges, considerable progress has been made recently in developing drugs to target the Wnt pathway, reviewed here with a focus on colon, breast and liver cancer. In addition to targeting known cancer pathways, identification of novel genetic drivers of cancer can provide new avenues for targeted therapies. Forward genetic screens in model systems are a complementary approach to genomic profiling of human tumors for cancer gene discovery. *Sleeping Beauty* (*SB*) transposon mutagenesis is a tool for identifying tissue-specific cancer genes. Two genes of interest, *Rspo2* and *Wac*, were discovered to be candidate cancer genes in intestinal tumors via *SB* transposon screens. The role of *Rspo2* and *Wac* in cancer biology is the subject of research in this thesis.

Molecularly targeted therapy is a major goal of current cancer research

Cancer is a leading cause of morbidity and mortality worldwide (Bray et al., 2013). In the United States, approximately 50% of people born today will be diagnosed with cancer in their lifetime, and one in four people will die due to cancer (AACR Cancer Progress Report Writing Committee et al., 2013; Siegel et al., 2014a). Cancers of the colon, breast, and liver are some of the leading causes of cancer-related mortality, and together will account for 113,740 cancer-related deaths (19% of all cancer-related deaths) in the United States in 2014 (Siegel et al., 2014a).

In the face of this daunting public health problem, there have been substantial advances in cancer prevention and treatment that have yielded a 20% decrease in overall cancer mortality in the United States over the past two decades (Siegel et al., 2014a). As a result, more than 14 million people currently living in the United States are estimated to be survivors of cancer (DeSantis et al., 2014b). Colon and breast cancer are positive examples, with mortality decreasing at a rate of 2-3% per year for colon cancer and ~2% per year for breast cancer in the last two decades (DeSantis et al., 2014a; Siegel et al., 2014b). These gains are attributed to a combination of mitigated lifestyle-associated risk factors, improved screening, and advances in treatment. In contrast, death rates for liver cancer have been increasing in the United States and worldwide (Siegel et al., 2014a). In the U.S., liver cancer mortality increased by ~2% per year in the period from 2000 to 2010 (Altekruse et al., 2014). The increasing burden of liver cancer is attributed to risk factors including cirrhosis, chronic infection with hepatitis viruses, alcoholic liver

disease, and obesity (Siegel et al., 2014b). Simultaneously, there have not been significant improvements in treatment of liver cancer to offset the increased incidence.

For localized liver cancer, surgery may be curative but recurrence occurs in the majority of cases. Additionally, for the ~45% of cases that are diagnosed at an advanced stage chemotherapy does not improve survival (Giuliani and Colucci, 2009; Siegel et al., 2014a). As a result, the overall 5-year survival rate for liver cancer is 18% (Siegel et al., 2014a). Even for colon and breast cancer, where chemotherapeutic options have significantly improved survival, the 5-year survival rate remains 13% for metastatic colon cancer and 24% for metastatic breast cancer (Siegel et al., 2014a).

To improve these outcomes, a major goal in current translational cancer research is the development of molecularly-targeted therapies for personalized cancer medicine **(Figure 1)** (Haber et al., 2011). In this paradigm, the clinical diagnosis of cancer would involve both traditional histopathological assessment and molecular analyses of a patient's tumor, including DNA sequencing or gene expression profiling. These data would be analyzed to identify activation of oncogenic pathways and inhibition of tumor suppressor pathways responsible for the individual tumor's growth. Treatment selection would be based on this information, choosing appropriate targeted therapies to intervene in the dysregulated pathways. Treatment response would then be monitored in real-time by repeat biopsy and imaging to determine if therapies were on target and if the disease responded.

The current state of the art has not yet achieved the ideals of targeted cancer therapy. There are still a relatively small number of targeted drugs that are approved for

clinical use **(Table 1).** Personalized medicine has advanced farthest in the treatment of breast cancer, where standard practice includes molecular subtyping based on expression of hormone and growth factor receptors or microarray-based gene expression analysis, and "targeted" anti-hormone therapies and monoclonal antibodies against HER2 have greatly improved outcomes for selected patients. Yet for the 15-20% of breast cancer patients with triple negative or basal type breast cancer, treatment remains limited to cytotoxic chemotherapy (Shastry and Yardley, 2013). For colon cancer, a few targeted drugs that inhibit angiogenesis or growth factor signaling are in clinical practice **(Table 1).** For liver cancer, only one targeted drug is approved, the multi-kinase inhibitor sorafenib, which was shown in phase III clinical trial to extend survival for a mere 2 months **(Table 1)** (Llovet et al., 2008).

In order to expand and improve the repertoire of targeted cancer therapies, efforts are focused on developing drugs to target known cancer pathways and identifying novel genetic drivers of cancer. Wnt signaling is an important pathway in cancer with significant potential for therapeutic intervention. Despite historical difficulty developing Wnt antagonists, recent progress has moved several Wnt-targeted drugs forward in development pipelines, reviewed here. Ultimately, to reach the goal of personalized cancer medicine we also need a comprehensive understanding of the genetic drivers of cancer. The second part of this chapter will describe strategies for identifying new cancer genes and introduce two genes, *RSPO2* and *WAC*, which are the focus of this thesis research.

Wnt signaling: A developmental pathway hijacked in tumorigenesis

Wnt signaling is a developmental pathway hijacked in tumorigenesis to promote unregulated cell growth. This dual role is exemplified by the initial discovery and naming of the Wnt pathway. In 1976, a novel drosophila mutant was discovered to produce one-winged or wingless offspring (Sharma and Chopra, 1976). The mutated gene was named "*wingless*." Six years later, a mouse mammary tumor virus (MMTV) screen identified a novel site of recurrent viral integration harboring a mammary tumor oncogene (Nusse and Varmus, 1982). This gene was named "*Int-1*." Subsequent cloning revealed *Int-1* and *wingless* to be homologs, leading to the adoption of the hybrid name "*Wnt*." *Wnt1* turned out to be the first secreted ligand in a conserved pathway that would come to have 19 Wnt ligands in mammalian genomes, ten Frizzled receptors, and multiple additional receptors and co-receptors, whose interactions regulate the "canonical," beta-catenin (CTNNB1)-dependent Wnt pathway, as well as the "non-canonical" planar cell polarity (PCP) and calcium-dependent Wnt pathways **(Figure 2)** (Niehrs, 2012).

The first connection of the Wnt/CTNNB1 pathway to human cancer was discovered in the early 1990s, when the gene underlying the hereditary colon cancer syndrome called familial adenomatous polyposis (FAP) was discovered (Kinzler et al., 1991; Nishisho et al., 1991). Individuals with FAP syndrome are predisposed to the formation of hundreds of colonic polyps beginning by their second decade of life (Kinzler and Vogelstein, 1996). Without surgical resection of the colon, progression to invasive colorectal cancer occurs in essentially all cases with average onset around 40 years of age. The gene underlying FAP syndrome was named adenomatous polyposis coli

(*APC*) and it was discovered to interact with core components of the emerging Wnt pathway (Rubinfeld et al., 1993; Su et al., 1993), highlighting the significance of the Wnt pathway in human cancer for the first time.

APC regulates canonical Wnt signaling by binding CTNNB1 in the cytoplasm along with other proteins, including AXIN1, GSK3B, and CK1, in a "destruction complex." CTNNB1 is phosphorylated by GSK3B and CK1 at multiple residues and ubquitinated by beta-TRCP, which targets CTNNB1 for proteasomal degradation. In the absence of nuclear CTNNB1, TCF/LEF-family transcription factors are bound by Groucho proteins in transcriptional repressor complexes, which silence Wnt target gene expression **(Figure 2).** In contrast, when Wnt ligand is present a series of events occur to inhibit the destruction complex and stabilize CTNNB1. Wnt ligands bind to a Frizzled family receptor (FZDs) and a co-receptor of the lipoprotein related protein family (LRP5/6). This precipitates phosphorylation of LRP co-receptors, recruitment of AXIN and GSK3B to the receptor complex, activation of dishevelled (DVL), and inhibition of the destruction complex, allowing newly synthesized CTNNB1 to accumulate in the cytoplasm and translocate to the nucleus. Once in the nucleus, CTNNB1 binds TCF/LEF family transcription factors to activate transcription of a gene expression program regulating cell proliferation, survival, differentiation, polarity, and migration **(Figure 2).**

Activity of the Wnt pathway is regulated by several factors in addition to Wnt ligand. The soluble frizzled related protein (SFRP) and dickkopf WNT signaling pathway inhibitor (DKK) families are secreted inhibitors of Wnt signaling. Recently, a novel module of secreted and cell-surface regulators of the Wnt pathway has been described. R-

spondins (RSPOs) were discovered to be secreted activators of canonical and non-canonical Wnt signaling in a *Xenopus* screen (Kazanskaya et al., 2004). RSPO1-4 were found to dramatically potentiate Wnt signaling in the presence of Wnt ligand (Kim et al., 2008). Since that time, the precise mechanisms of RSPO signaling have been the subject of considerable inquiry and controversy. Initial reports suggested that RSPOs bound directly to FZD receptors and LRP6 (Nam et al., 2006; Wei et al., 2007) or inhibited DKK (Binnerts et al., 2007; Kim et al., 2008) in order to activate Wnt signaling. Another report suggested that RSPOs could interact with Syndecan receptors (SCD4) to promote non-canonical Wnt signaling (Ohkawara et al., 2011). Ultimately, multiple research groups discovered that an orphan family of G-protein coupled receptors, the leucine-rich repeat containing G protein-coupled receptors (LGR4/5/6) function as receptors for the four RSPO ligands (Carmon et al., 2011, 2012a; Glinka et al., 2011a; de Lau et al., 2011). This was confirmed and further specified recently by a series of studies describing the crystallographic structure of RSPOs in complex with LGR receptors (Chen et al., 2013; Peng et al., 2013a; Wang et al., 2013). Despite this clarification, the signaling events downstream of RSPO binding to LGR receptors are still incompletely understood. Recently two groups identified a mechanism by which RSPO/LGR binding destabilizes RNF43 and ZNRF3, E3 ubiquitin ligases that negatively regulate Wnt signaling by promoting clearance of FZD and LRP receptors from the cell surface (Hao et al., 2012; Koo et al., 2012). These interactions have been confirmed by recent structural studies (Peng et al., 2013b; Zebisch et al., 2013). To complicate matters, another group recently proposed a contradictory model in which RSPOs can function as inhibitors of Wnt

signaling through the LGR5 receptor, by stabilizing rather than inhibiting ZNRF3 (Wu et al., 2014). Clarification of signaling events initiated by the RSPO/LGR/ZNRF3/RNF43 module will be important for understanding the role of these factors and Wnt signaling in stem cell and cancer biology.

Wnt signaling in normal stem cells

Wnt signaling promotes stem cell self-renewal in many tissues. The role of Wnt signaling in intestinal, mammary, and liver stem cells will be briefly described here, with an emphasis on the recently revealed role of RSPOs as stem cell growth factors and LGR5 as a marker of adult stem cells in many tissues.

Wnt signaling in intestinal stem cells

The intestinal epithelium is a dynamic tissue, undergoing continual turnover and renewal. Intestinal stem cells reside at the base of invaginations in the intestinal epithelium called crypts (Barker et al., 2008). Intestinal stem cells divide approximately once per day to produce a rapidly dividing progenitor cell compartment of so-called transit-amplifying (TA) cells. As TA cells divide they migrate away from the crypt base toward the surface epithelium. TA cells differentiate into mature lineages of the intestinal epithelium, including absorptive, goblet, enteroendocrine and Paneth cells. Differentiated cells undergo apoptosis and shed into the lumen of the gut as new cells from the stem compartment replace them (Barker et al., 2008).

Numerous genetic studies have defined an essential role for Wnt signaling in intestinal stem cell homeostasis. Deletion of *Tcf7l2*, a transcription factor required for

CTNNB1-dependent transcription, during development or in adult intestinal epithelium results in loss of the intestinal stem cell compartment (van Es et al., 2012; Korinek et al., 1998). A similar phenotype occurs following conditional deletion of *Ctnnb1* (Fevr et al., 2007; Ireland et al., 2004), overexpression of the Wnt inhibitor *Dkk* (Kuhnert et al., 2004; Pinto et al., 2003), or compound deletion of *Lgr4* and *Lgr5* (de Lau et al., 2011). Conversely, conditional deletion of *Apc* in intestinal epithelium leads to activated Wnt signaling, inhibition of differentiation, and maintenance of "crypt progenitor-like" phenotypes in intestinal epithelium (Sansom et al., 2004). Interestingly, transgenic overexpression of human RSPO1 by B cells or injection of recombinant RSPO1 in mice also leads to intestinal hyperplasia and a expansion of the crypt compartment (Kim et al., 2005).

Recently, LGR5 was discovered to be a uniquely specific marker of intestinal crypt stem cells (Barker et al., 2007). Lineage tracing experiments found that LGR5-positive stem cells are capable of differentiating to all the mature cell types of the intestinal epithelium (Barker et al., 2007). Isolated LGR5-positive stem cells can also be propagated *ex vivo* in semi-solid medium where they will proliferate and differentiate to form "mini-guts" (Sato et al., 2009). These "organoid" cultures recapitulate the structure of the intestinal crypt and can be continuously propagated without becoming transformed, emphasizing the capacity of LGR5+ stem cells for both differentiation and self-renewal (Sato and Clevers, 2013; Sato et al., 2009). Consistent with a role in activating Wnt signaling, RSPOs are an essential growth factor in organoid culture (Sato and Clevers, 2013). Combined with the genetic studies implicating *Lgr4/5* and *Rspo1* in intestinal stem

cell maintenance, these data suggest that the RSPO/LGR signaling module is an important regulator of Wnt signaling and intestinal stem cell self renewal.

Wnt signaling in mammary stem cells

In the breast, the mammary gland consists of a branching network of ducts, lined by luminal epithelial cells. A basal layer of myoepithelial cells underlies the luminal layer and contains rare mammary stem cells (Visvader and Stingl, 2014). The identity and function of mammary stem cells have been extensively studied using mouse models of gland reconstitution following transplantation into a cleared mammary fat pad (Deome et al., 1959; Smith et al., 2012). Transplantation studies implicate Wnt signaling in mammary stem cell renewal and gland reconstitution. Analysis of Wnt-activated cells in the mammary gland using an Axin2-LacZ reporter strain found that 5% of cells with a putative stem cell immunophenotype (Lin-, CD24+, CD29hi) expressed Axin2, and the Axin2-positive subset had increased efficiency in gland reconstitution compared to Axin2-negative counterparts (Zeng and Nusse, 2010). Further, culturing mammary stem cells with recombinant Wnt3a *ex vivo* enhanced their ability to reconstitute functional mammary glands upon transplantation (Zeng and Nusse, 2010). Lineage tracing experiments using the same Axin2-LacZ reporter strain found that Axin2-positive progenitors contribute to luminal and basal lineages at different times in development and give rise to alveoli during multiple pregnancies (van Amerongen et al., 2012). Lineage tracing using an Lgr5-GFP-IRES-creERT2 reporter strain found that Lgr5 positive cells are bi-potent progenitors that have developmental stage-specific contributions to different lineages in mammary gland development (Rios et al., 2014; de Visser et al., 2012).

Similar to Axin2 positive cells, Lgr5-positive mammary epithelial cells also reside in the basal compartment and have enhanced regenerative capacity (Plaks et al., 2013). Mice null for the Wnt co-receptor Lrp5 exhibit loss of placodes, mammary gland hypoplasia and a reduced regenerative capacity in transplantation experiments (Lindvall et al., 2006). Mice null for *Wnt4* or *Rspo1* have defects in mammary duct side-branching and alveoli formation (Brisken et al., 2000; Chadi et al., 2009). These genetic studies emphasize the importance of Wnt signaling in mammary gland development and stem cell function.

Wnt signaling in hepatic stem cells

The liver is an organ capable of massive regeneration following injury or partial surgical resection (Michalopoulos, 2007). The role of hepatic stem cells in liver regeneration is incompletely understood and likely to depend on the type of liver injury. In response to some injuries, such as partial hepatectomy, fully differentiated hepatocytes and cholangiocytes (biliary duct epithelial cells) are able to re-enter the cell cycle in order to regenerate liver tissue (Michalopoulos, 2007). However, the adult liver also contains a population of bi-potent progenitor cells that are quiescent in healthy liver tissue but capable of regenerating both hepatocyte and cholangiocyte lineages in the context of chronic or toxin-induced liver injury. In rodent models, bi-potent progenitors are identified as “oval cells.” Several studies have shown that Wnt signaling is activated in oval cells during regeneration following partial hepatectomy (Monga et al., 2001) or toxic injury (Apte et al., 2008; Hu et al., 2007; Itoh et al., 2009; Yang et al., 2008). Additionally, expression of constitutively active CTNNB1 expands the oval cell population in the regenerated liver following toxic injury (Yang et al., 2008). A

significant recent study further clarified the role of Wnt signaling in promoting hepatocyte regeneration. This study identified a novel mechanism in which expression of Wnt3a by macrophages in the liver promotes expression of the Notch inhibitor Numb and promotes differentiation of bi-potent progenitors to mature hepatocytes (Boulter et al., 2012). Importantly, lineage-tracing experiments using the Lgr5-creERT2 reporter allele have shown that while Lgr5 is not expressed in normal adult liver, Lgr5 is upregulated in bi-potent progenitor cells following multiple types of liver injury (Huch et al., 2013a). Further, isolated Lgr5-positive progenitor cells from injured liver can be clonally expanded *ex vivo* in culture medium containing Rspo1 (Huch et al., 2013a). Although questions remain regarding the requirement of Wnt signaling in normal hepatocyte homeostasis and regeneration following injury, these studies indicate that liver stem cells are R-spondin responsive and exhibit Wnt activation during regeneration. Collectively, these studies emphasize an important role for Wnt signaling in self-renewal of tissue stem cells in the intestine, breast, and liver.

Wnt signaling in cancer and cancer stem cells

The cancer stem cell theory posits that there is a subset of cells within a tumor that have enhanced capacity for tumor initiation, including stem cell renewal and differentiation to bulk tumor cells (Vermeulen et al., 2012). Cancer stem cells are believed to be responsible for tumor progression and relapse following treatment, and consequently there is significant interest in developing treatments to specifically target cancer stem cells. Wnt signaling is aberrantly activated in many tumor types, and may be a critical mediator of cancer stem cell proliferation **(Figure 3)** (Curtin and Lorenzi,

2010). The evidence for Wnt activation in colon, breast and liver cancer and cancer stem cells are briefly summarized here.

Wnt signaling in colorectal cancer

Colorectal cancer is the prototypical Wnt-initiated cancer type. Following the discovery that mutations in the *APC* gene underlie FAP syndrome, somatic mutations in *APC* were identified in sporadic cases of colon cancer (Kinzler and Vogelstein, 1996). Inactivation of *APC* occurs in ~80% of colorectal cancers, and is thought to be an early initiating event in colonic polyp formation (Barker and Clevers, 2006; Fodde et al., 2001). *APC* loss of function results in constitutive activation of Wnt signaling and promotes a "progenitor-like" gene expression profile and formation of benign adenomas (van de Wetering et al., 2002). Secondary mutations in additional cancer pathways correspond with progression to invasive carcinoma (Fodde et al., 2001; Vogelstein et al., 1988). In colon tumors that retain wild type *APC*, Wnt signaling is frequently activated by other events, including activating mutation of *CTNNB1* or disruption of *AXIN1* or *2* **(Figure 3)** (Cancer Genome Atlas Network, 2012a). Recently, increased expression of *RSPO2* or *RSPO3* due to recurrent genomic rearrangements was identified in 4-10% of CRCs (Seshagiri et al., 2012; Shinmura et al., 2014). Overall, it is estimated that over 90% of colon tumors exhibit activated Wnt signaling (Cancer Genome Atlas Network, 2012a).

The role of Wnt signaling in colon cancer stem cells specifically has recently been clarified following the identification of LGR5-positive intestinal stem cells (Barker et al., 2007). Following deletion of *Apc* in murine Lgr5-positive intestinal stem cells, Ctnnb1

rapidly accumulates and leads to microadenoma and adenoma formation (Barker et al., 2009). In contrast, deletion of *Apc* in transit amplifying compartment does not lead to adenoma formation in this model (Barker et al., 2009). A second study using a different stem cell marker to express constitutively activated Ctnnb1 in intestinal stem cells similarly initiated abnormal proliferation of stem cells and adenoma formation (Zhu et al., 2009). Significantly, in both models, the adenomas formed as a consequence of Wnt activation in intestinal stem cells continued to have a minority population (~7%) of cells that retained expression of stem cell markers (Barker et al., 2009; Zhu et al., 2009). Further, colon cancer stem cells can be identified by high activity of a Wnt reporter construct, and extrinsic signals from the tumor microenvironment are able to promote stemness in bulk tumor cells, indicating plasticity in cancer stem cells and the importance of Wnt signaling in their regulation (Vermeulen et al., 2010).

Wnt signaling in breast cancer

The oncogenic role of Wnt signaling in breast cancer has been investigated since the initial discovery of *Wnt1* activation in MMTV-induced mammary tumors in mice (Nusse and Varmus, 1982). Since then, multiple mouse models have confirmed that activation of Wnt signaling can induce tumor formation in the mammary gland, including models of Wnt ligand overexpression (*MMTV-Wnt1*), Ctnnb1 activation (MMTV-Ctnnb1^{delN90}), and *Apc* inactivation (*Apc*$^{+/1572}$)(Gaspar et al., 2009; Li et al., 2000; Michaelson and Leder, 2001; Tsukamoto et al., 1988). Overexpression of *Rspo2* or *Rspo3* also promote mammary tumorigenesis in MMTV insertional mutagenesis screens (Lowther et al., 2005; Theodorou et al., 2007).

Mouse models indicate that Wnt signaling has a specific role in promoting proliferation and malignant transformation of mammary stem cells (Incassati et al., 2010). Premalignant lesions in *MMTV-Wnt1* mice contain an expanded population of mammary stem cells (Shackleton et al., 2006). Mammary tumors induced by Wnt activation (*Wnt1, Ctnnb1, or Myc*) contain a higher proportion of progenitor-like cells compared to tumors initiated by other drivers (*Her2*, *Hras*, or polyoma middle T antigen) (Li et al., 2003). Expression of Wnt ligands induced by tumor stromal niche factors has also been shown to maintain mammary cancer stem cells, emphasizing the role of the tumor microenvironment in regulating Wnt signaling (Malanchi et al., 2012).

The role of Wnt activation in human breast cancer has been more enigmatic. Nuclear accumulation of CTNNB1 can be detected by immunohistochemistry and used as a measure of Wnt pathway activation. Several groups have identified increased nuclear CTNNB1 in a subset of breast cancer, particularly associated with basal or triple-negative subtype and decreased patient survival (Geyer et al., 2011; Khramtsov et al., 2010; Ozaki et al., 2005) or metaplastic breast cancer (Hayes et al., 2008). However, the genetic events underlying Wnt activation in human breast cancer are not fully understood. Activating mutation in CTNNB1 are seen in 25% of metaplastic breast cancers, but have not been found in other types (Hayes et al., 2008). *APC* mutations are seen in rare breast cancers (Furuuchi et al., 2000). Silencing of *SFRP1* due to promoter hypermethylation is another mechanism that activates Wnt signaling in some breast cancers **(Figure 3)** (Ugolini et al., 2001). The role of R-spondins in human breast cancer has not yet been described.

Wnt signaling in liver cancer

Hepatocellular carcinoma (HCC) commonly arises in the context of chronic liver disease. Chronic infection with hepatitis B or C virus, alcoholic liver disease, or non-alcoholic fatty liver disease can lead to cirrhosis (Flores and Marrero, 2014). In this context, cycles of liver injury, inflammation and regeneration contribute to tumor formation (Hernandez-Gea et al., 2013). Genetic aberrations leading to activation of Wnt signaling are common in HCC. Activating mutations in *CTNNB1* itself are the most common, occurring in 16-40% of cases depending on etiology, followed by loss of function mutation or deletion of *AXIN1* (5-15%), and rarely *APC* (~2%) **(Figure 3)** (Ahn et al., 2014; Guichard et al., 2012; Kan et al., 2013). Silencing of *SFRP1* is also seen in HCC and can result from epigenetic silencing by the hepatitis C virus core protein (Quan et al., 2014). Significantly, amplification and increased expression of *RSPO2* was recently identified in HCC by two studies, in 3% and 22% of cases, although the functional significance of *RSPO2* amplification in these cases was not assessed (Ahn et al., 2014; Kan et al., 2013).

Molecular subtyping of HCCs based on microarray gene expression analysis identified a subtype of HCCs with activated Wnt signaling and wild type *CTNNB1* in which Wnt signaling is activated by crosstalk with the transforming growth factor-beta (TGFB) pathway (Hoshida et al., 2009). Overall, approximately 50% of HCC cases exhibit a gene expression signature consistent with activated Wnt signaling (Lachenmayer et al., 2012).

Expression of liver progenitor cell markers are seen in 28-50% of HCCs, and the role of Wnt signaling in HCC stem cells is an area of active investigation. (Mishra et al., 2009). Wnt signaling has been shown to be active in isolated HCC stem cells (Hernandez-Gea et al., 2013). Further, studies in cultured normal human hepatocytes and HCC cell lines indicate that Wnt signaling regulates expression of the HCC stem cell marker EpCAM (Yamashita et al., 2007). Additional studies are needed to determine if active Wnt signaling is required to maintain HCC stem cells *in vivo*.

In sum, these investigations implicate Wnt signaling as a regulator of proliferation in both normal and malignant stem cells, and suggest that the Wnt pathway may be a viable drug target in colon, breast, liver and other types of cancer.

Development of Wnt targeted therapies

Given the important role of aberrantly activated Wnt signaling in many types of cancer, there is significant interest in developing drugs to inhibit Wnt signaling for cancer therapy. To date, no specific inhibitors of Wnt signaling have received FDA approval, although some agents are now in early clinical trials **(Table 2).** Several challenges have complicated the development of Wnt-targeted therapy, including redundancy of pathway components, "undruggable" pathway components, and limited drug targets downstream of common cancer-associated mutations (Anastas and Moon, 2013). Specifically, although inhibition of Wnt ligand/receptor interactions could effectively block Wnt signaling in tumors where the pathway is activated by upstream events, such as silencing of SFRP genes, the partial redundancy of 19 Wnt ligands and 10 Frizzled receptors suggests that specific inhibition of one ligand/receptor interaction might not be sufficient

to block tumor cell growth. Targeting the pathway downstream of the receptor has also been a challenge due to the "undruggable" nature of the pathway, which contains few activating kinases. In many tumors, the Wnt pathway is activated at the level of *APC* or *CTNNB1* mutations, and options for inhibiting the pathway downstream of these components may be limited to protein-protein interactions between transcriptional regulators. In addition, the role of Wnt signaling in maintaining stem cells in many tissues, reviewed above, suggests that Wnt targeted therapies could have unwanted side effects on normal stem cell populations.

Despite these obstacles, progress has been made. A successful strategy for circumventing these challenges has been the use of high throughput screens to identify small molecule Wnt inhibitors in cancer cell lines (Barker and Clevers, 2006). These efforts have produced a number of agents that intervene in the Wnt pathway at all levels. C59, IWP and LGK974 are Porcupine inhibitors that prevent lipid modification and secretion of Wnt ligands **(Figure 4)** (Chen et al., 2009; Liu et al., 2013; Proffitt et al., 2013). Both C59 and LGK974 were shown to inhibit *MMTV-Wnt1* mammary tumor formation (Liu et al., 2013; Proffitt et al., 2013). LGK974 is currently in a phase I clinical trial for advanced solid tumors **(Table 2).** XAV939, IWR, and JW55 are Tankyrase (TNKS) inhibitors that stabilize AXIN, thereby increasing CTNNB1 inhibition by the destruction complex **(Figure 4)** (Chen et al., 2009; Huang et al., 2009; Waaler et al., 2012). A number of compounds have also been identified that inhibit protein interactions of CTNNB1 in the nucleus **(Figure 4).** For example, ICG-001 was identified in a small molecule screen for inhibitors of Wnt reporter expression in SW480 CRC cells (Emami

et al., 2004). ICG-001 was found to be a potent inhibitor of Wnt signaling (IC50=3uM) that functions by preventing CTNNB1 recruitment of CREB binding protein (CBP), a histone acetylase that facilitates CTNNB1-dependent transcription (Emami et al., 2004). PRI-724 is a second-generation inhibitor of CTNNB1/CBP, which is currently in phase I/II clinical trials for treatment of several leukemia and solid tumors **(Table 2)** (Lenz and Kahn, 2014).

Rational drug design has also produced some promising Wnt inhibitors. OTSA101-DTPA-90Y is a chimeric humanized monoclonal antibody (mAb) against FZD10 conjugated to Yttrium-90, which is currently in a phase I trial for advanced synovial sarcoma **(Table 2).** OMP-54F28 is a fusion protein that contains the FZD8 extracellular domain conjugated to a human immunoglobulin Fc domain that is capable of binding multiple Wnt ligands. OMP-54F28 is currently in a phase 1 trial for advanced solid tumors **(Table 2).** Further descriptions of additional Wnt inhibitors in preclinical development are available in several excellent review articles (Anastas and Moon, 2013; Baarsma et al., 2013; Barker and Clevers, 2006).

Cancer gene discovery is a prerequisite for targeted therapy development

Despite the significance of the Wnt pathway and other established cancer pathways, there is a need to broaden our knowledge of the genetic drivers of cancer, in order to identify new therapeutic targets and define genetic contexts for their application. Colorectal cancer provides an example of this principle. Although the Wnt pathway is dysregulated in the vast majority of CRCs, Wnt activation alone is not sufficient for tumor formation. Rather, a series of lesions in multiple pathways are required for the

development of invasive carcinoma (Fearon, 2011; Vogelstein et al., 1988). Some lesions are highly penetrant (eg. mutations in *KRAS* and *TP53*) but most occur in a minority of cases (Wood et al., 2007). For this reason, there has been substantial interest and challenge in creating a complete catalog of cancer genes.

Large-scale "cancer genome projects" are underway to comprehensively identify the genes and pathways altered in human cancer, including The Cancer Genome Project (TCGA) and the International Cancer Genome Consortium (ICGC) (Cancer Genome Atlas Research Network et al., 2013a; International Cancer Genome Consortium et al., 2010). These projects seek to profile large numbers of tumors of various types at the DNA, RNA, protein and epigenetic levels, by analyzing somatic mutations, DNA copy number changes, promoter methylation, mRNA expression, microRNA expression, and protein expression. While these projects are ongoing, comprehensive profiles of colorectal and breast cancer have already been published (Cancer Genome Atlas Network, 2012a, 2012b). Importantly, a statistical analysis of somatic mutations in >4,500 tumors of various types found that 600-5,000 samples of each tumor type might be required to reach saturation in the detection of mutated genes that contribute to cancer (Lawrence et al., 2014). This suggests that additional cancer genes remain to be discovered. Additionally, cancer genes that are altered epigenetically or by large genomic amplifications and deletions remain difficult to identify by molecular profiling of human tumors alone.

SB transposon-mediated mutagenesis for cancer gene discovery

Forward genetic screens in mice are a complementary approach to human cancer genome projects for enabling cancer gene discovery (Copeland and Jenkins, 2010). Comparative genomics can highlight the functional significance of cancer genes that are altered at low frequency in human tumors. Transposon-based insertional mutagenesis has been developed as tool for generating tumors in a tissue-specific manner **(Figure 5)** (Copeland and Jenkins, 2010; Starr and Largaespada, 2005). Briefly, *Sleeping Beauty (SB)* is a cut-and-paste DNA transposon system. The *SB* transposase enzyme recognizes specific DNA sequences flanking the transposon element (inverted repeat/direct repeat sequences). *SB* transposase cuts the genomic DNA at the recognition sites and pastes the transposon randomly into another location in the genome at a "TA" dinucleotide **(Figure 5B).** Transposon insertions into the genome can function as a mutagen, and SB transposons have been engineered to cause gain- and loss-of-function mutations **(Figure 5C).** Tissue-specific mobilization of *SB* transposons is achieved by combining two alleles: a conditional *SB* transposase allele, in which a Lox-stop-Lox cassette prevents *SB* expression, and a tissue-specific Cre recombinase allele **(Figure 5A).** Expression of *SB* transposase in the tissue of interest leads to insertional mutagenesis and tumor formation. Transposon insertion sites within the tumor genomic DNA are sequenced and mapped. Analysis of transposon insertion sites from multiple tumors allows identification of common insertion sites (CIS) which harbor putative cancer genes **(Figure 5D)** (Starr and Largaespada, 2005).

SB screens for drivers of intestinal tumorigenesis identified novel candidate cancer genes: Rspo2 and Wac

Prior work in the Largaespada lab used the *Sleeping Beauty* system to perform three insertional mutagenesis screens for genetic drivers of intestinal tumorigenesis (Starr et al., 2009, 2011). These studies were designed to identify novel cancer genes that function in a wild type genetic context or cooperate with known predisposing alleles ($Apc^{Min/+}$ or $Trp53^{R270H/+}$). Over 150 CIS genes were identified, including 77 genes on the wild type background, 33 genes on the $Apc^{Min/+}$ background, and 57 genes on the $Trp53^{R270H/+}$ background. *Apc* was the most frequently altered CIS gene in all three screens **(Figure 6),** and the overall list of CIS genes was enriched for known cancer genes, further underscoring the relevance of genes identified by insertional mutagenesis to human cancer genetics (Starr et al., 2009). The list of CIS genes also included many novel candidate cancer genes. Two genes were selected for further study in my thesis research, *R-spondin 2* (*Rspo2*) and *WW domain containing adaptor with coiled-coil (Wac*). Both *Rspo2* and *Wac* were among the top 10 CIS genes identified by frequency of alteration **(Figure 6).** In the wild type screen, activating insertions in *Rspo2* suggested that *Rspo2* may function as an oncogene. In contrast, *Wac* was disrupted by transposon insertions in all three screens, suggesting that *WAC* may be a tumor suppressor gene.

Thesis Statement

The following dissertation explores the hypothesis that *RSPO2* and *WAC* are cancer genes whose altered expression promotes tumorigenesis. Specifically, I propose that *RSPO2* is an oncogene that activates Wnt signaling in subsets of colon, breast and liver cancer. In tumors with elevated *RSPO2* expression, *RSPO2* may be required to maintain active Wnt signaling and cell growth, providing a rationale for targeted therapy. Further, I hypothesize that *WAC* functions as a tumor suppressor, and that inactivation of *WAC* cooperates with *APC* and *TP53* inactivation to promote colon cancer.

Figure 1: A theoretical framework for personalized cancer medicine using molecularly targeted therapies. In this paradigm, clinical diagnosis of a tumor incorporates both histopathological analysis and genomic profiling, including somatic mutation, DNA copy number, gene expression, and other analyses. Genomic information is used to subtype the tumor and identify specific genetic lesions that contribute to activation of oncogenic pathways and disruption of tumor suppressive pathways. Targeted therapies are used for treatment to intervene in the specific dysregulated pathways identified. For cases in which an appropriate targeted therapy is not available, genetic information can be used to identify appropriate clinical trials to expedite development and evaluation of novel targeted drugs. Monitoring in real-time using repeat biopsies and PET scans or other imaging technologies is used to evaluate pharmacodynamics and tumor responsiveness to treatment. Figure inspired by (Haber et al., 2011).

Figure 1

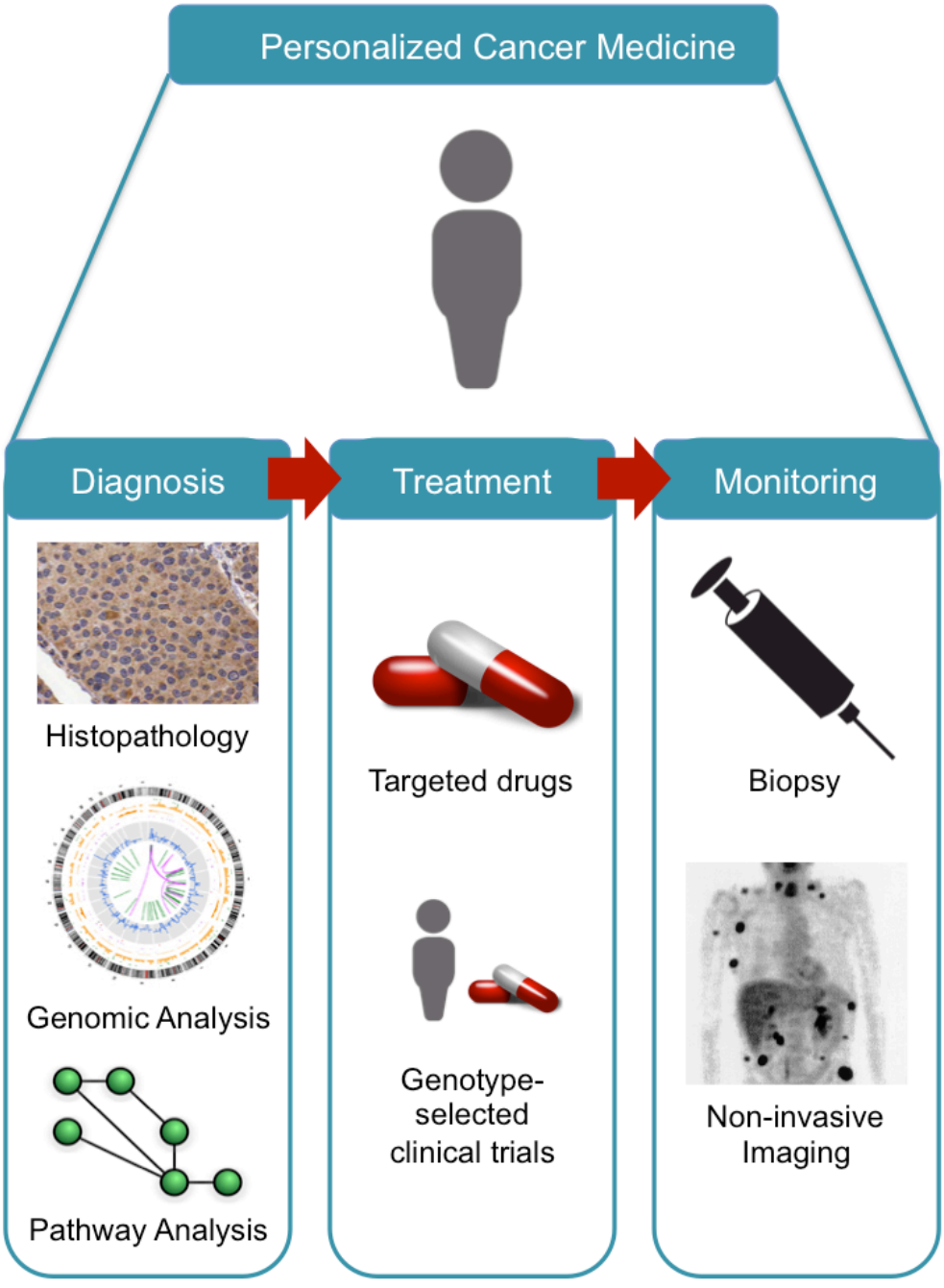

Personalized Cancer Medicine
Diagnosis
Treatment
Monitoring
Histopathology
Genomic Analysis
Pathway Analysis
Targeted drugs
Genotype-selected clinical trials
Biopsy
Non-invasive Imaging

Figure 2: Diagram of the canonical (CTNNB1-dependent) Wnt pathway. In the absence of activation by WNT ligands (left), CTNNB1, the effector of the pathway, is bound in the cytoplasm by a group of proteins called the "destruction complex" which includes APC, AXIN, and other members. CTNNB1 is phosphorylated at multiple residues by GSK3B and CK1 and ubiquitinated by BTCR(also known as beta-TRCP), which targets CTNNB1 for proteasomal degradation. Expression of Wnt target genes is repressed by TLE (also known as Groucho) binding to TCF/LEF family transcription factors and recruitment of HDACs. In contrast, when ligand is present (right), binding of WNT to Frizzled (FZD) family receptors and LRP co-receptors results in phosphorylation of LRP, activation of Disheveled (DVL), recruitment of the destruction complex to the cell membrane, and inhibition of the destruction complex. CTNNB1 is allowed to accumulate in the cytoplasm and translocate to the nucleus where it binds TCF/LEF family transcription factors and activates transcription of Wnt target genes. Wnt signaling is further regulated by secreted factors, including inhibitory DKK and SFRP factors, and activating RSPOs. See main text for additional details.

Figure 2

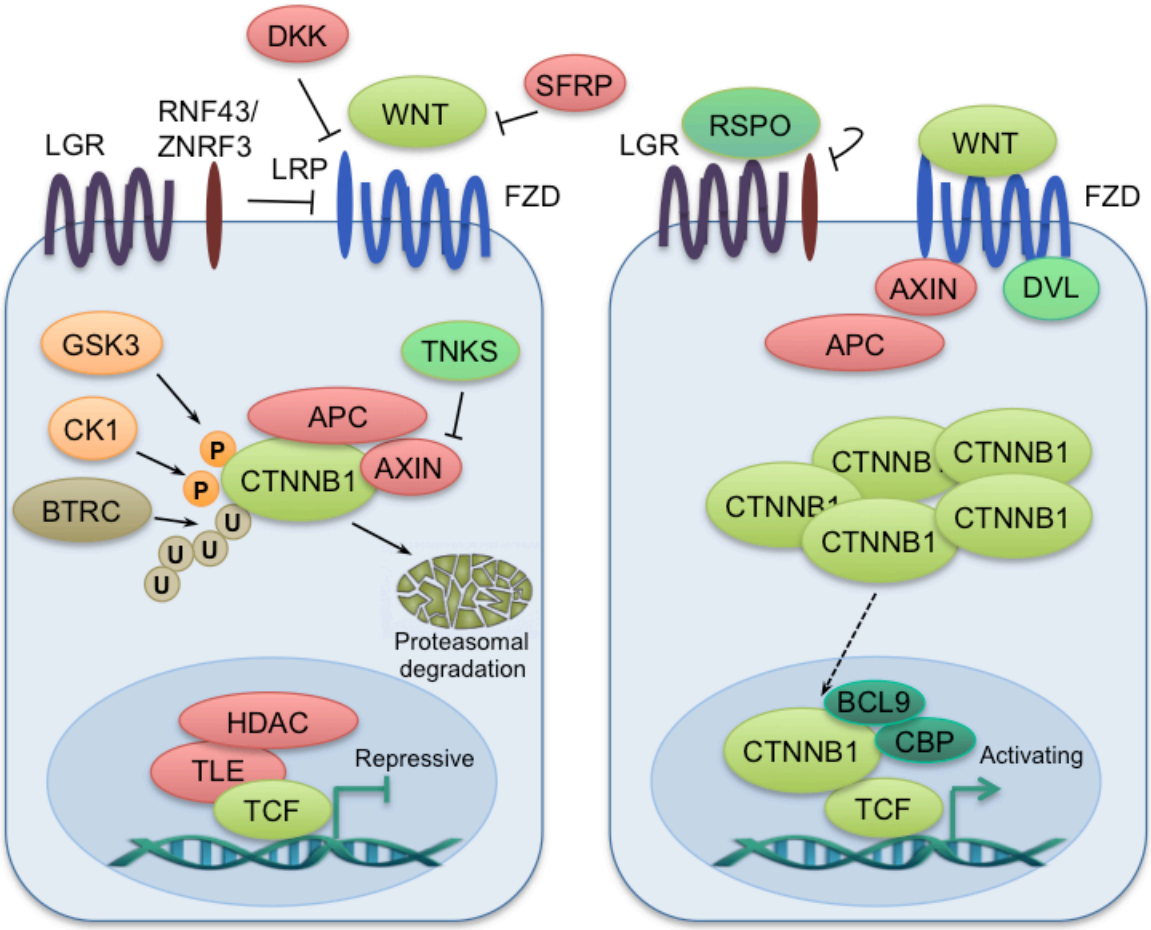

DKK
SFRP
WNT
RNF43/
ZNRF3
LGR
LRP
FZD
GSK3
TNKS
CK1
APC
P
P
CTNNB1
AXIN
BTRC
U
U
U
U
Proteasomal
degradation
HDAC
TLE
TCF
Repressive
RSPO
LGR
WNT
FZD
AXIN
DVL
APC
CTNNB1
CTNNB1
CTNNB1
CTNNB1
BCL9
CTNNB1
CBP
Activating
TCF

Figure 3: Wnt signaling is aberrantly activated in many types of cancer. Diagram of the canonical Wnt pathway indicates components that are recurrently mutated or altered in different cancers. Common mechanisms of Wnt dysregulation include inactivation of *APC* by truncating mutation or deletion, loss of function mutation of *AXIN1, AXIN2* or *RNF43*, and constitutive activation of *CTNNB1* by mutation or deletion of residues in exon 3 which are subject to phosphorylation by the destruction complex. Silencing of *SFRP1* due to promoter hypermethylation and overexpression of *RSPO2* and *RSPO3* due to genomic rearrangements can also activate Wnt signaling in diverse tumor types. Alterations in other components of the Wnt pathway have also been observed, including overexpression of Frizzled receptors and mutation or deletion of *DKK*, *FBXW7*, *ARID1A*, *FAM123B*, and *SOX9*. However, the functional relevance of these events in Wnt signaling and tumorigenesis has not been investigated.

Figure inspired by (Curtin and Lorenzi, 2010).

Figure 3

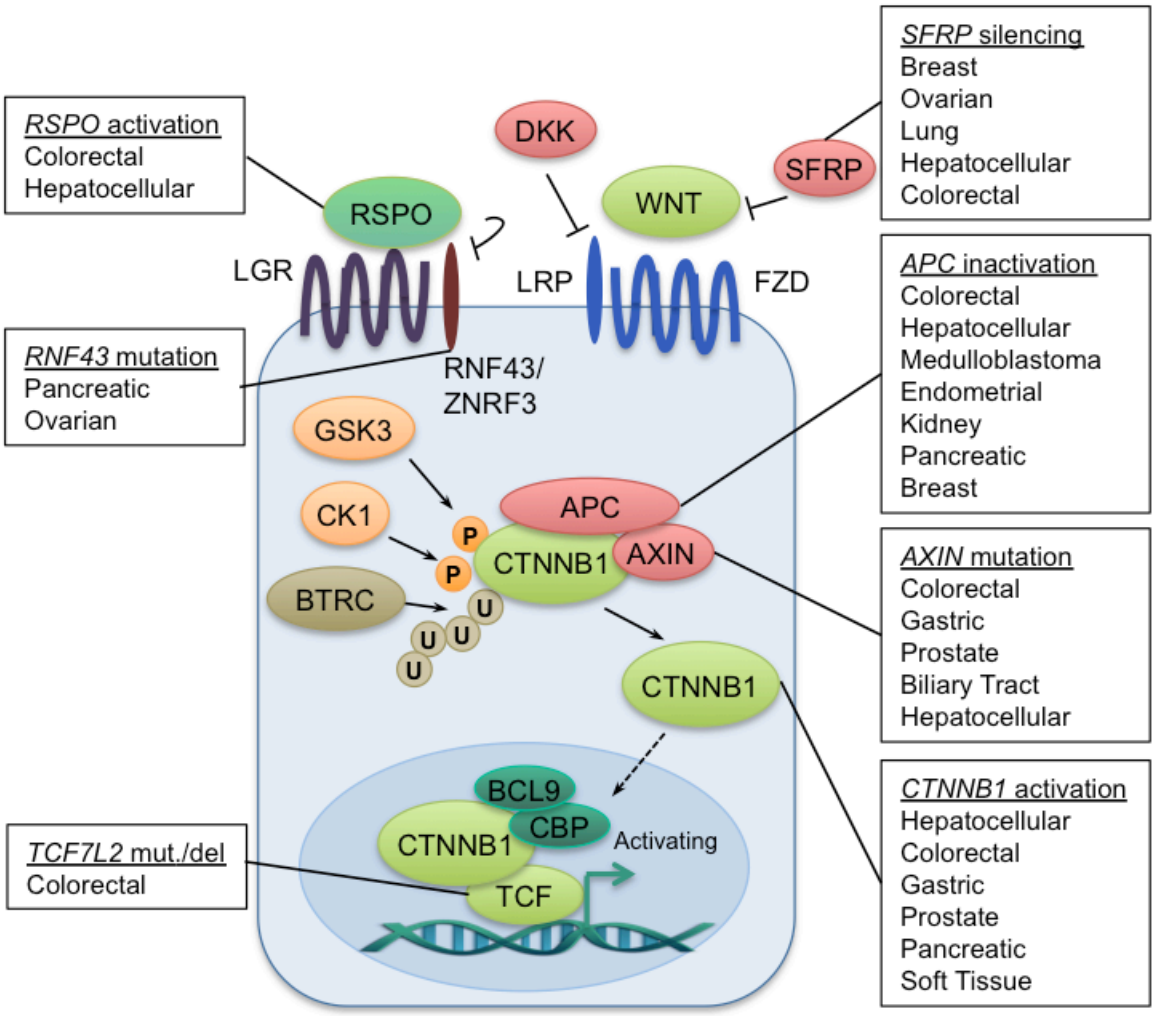

SFRP silencing
Breast
Ovarian
Lung
Hepatocellular
Colorectal
RSPO activation
Colorectal
Hepatocellular
DKK
SFRP
WNT
RSPO
LGR
LRP
FZD
APC inactivation
Colorectal
Hepatocellular
Medulloblastoma
Endometrial
Kidney
Pancreatic
Breast
RNF43 mutation
Pancreatic
Ovarian
RNF43/
ZNRF3
GSK3
CK1
APC
AXIN
P
P
CTNNB1
BTRC
U
U
U
U
AXIN mutation
Colorectal
Gastric
Prostate
Biliary Tract
Hepatocellular
CTNNB1
BCL9
CBP
CTNNB1
Activating
TCF
CTNNB1 activation
Hepatocellular
Colorectal
Gastric
Prostate
Pancreatic
Soft Tissue
TCF7L2 mut./del
Colorectal

Figure 4: Therapeutic agents for inhibition of Wnt signaling. Diagram of the canonical Wnt pathway annotated with small molecule activators and inhibitors of pathway components (red boxes). Wnt inhibitors target all levels of the pathway, including inhibition of WNT ligand secretion with Porcupine inhibitors (LGK974 and IWP), stabilization of the destruction complex with Tankyrase inibitors (IWR, JW55, XAV939), and inhibition of CTNNB1 transcriptional activation with inhibitors of protein-protein interactions (PNU-7465431, PFK115-584, PFK118-310, BC21, CGP049090, IQ-1, and others). Drugs marked with an asterix are non-specific inhibitors of Wnt signaling that have FDA approval for other clinical indications. See main text for details. Figure inspired by (Anastas and Moon, 2013; Baarsma et al., 2013).

Figure 4

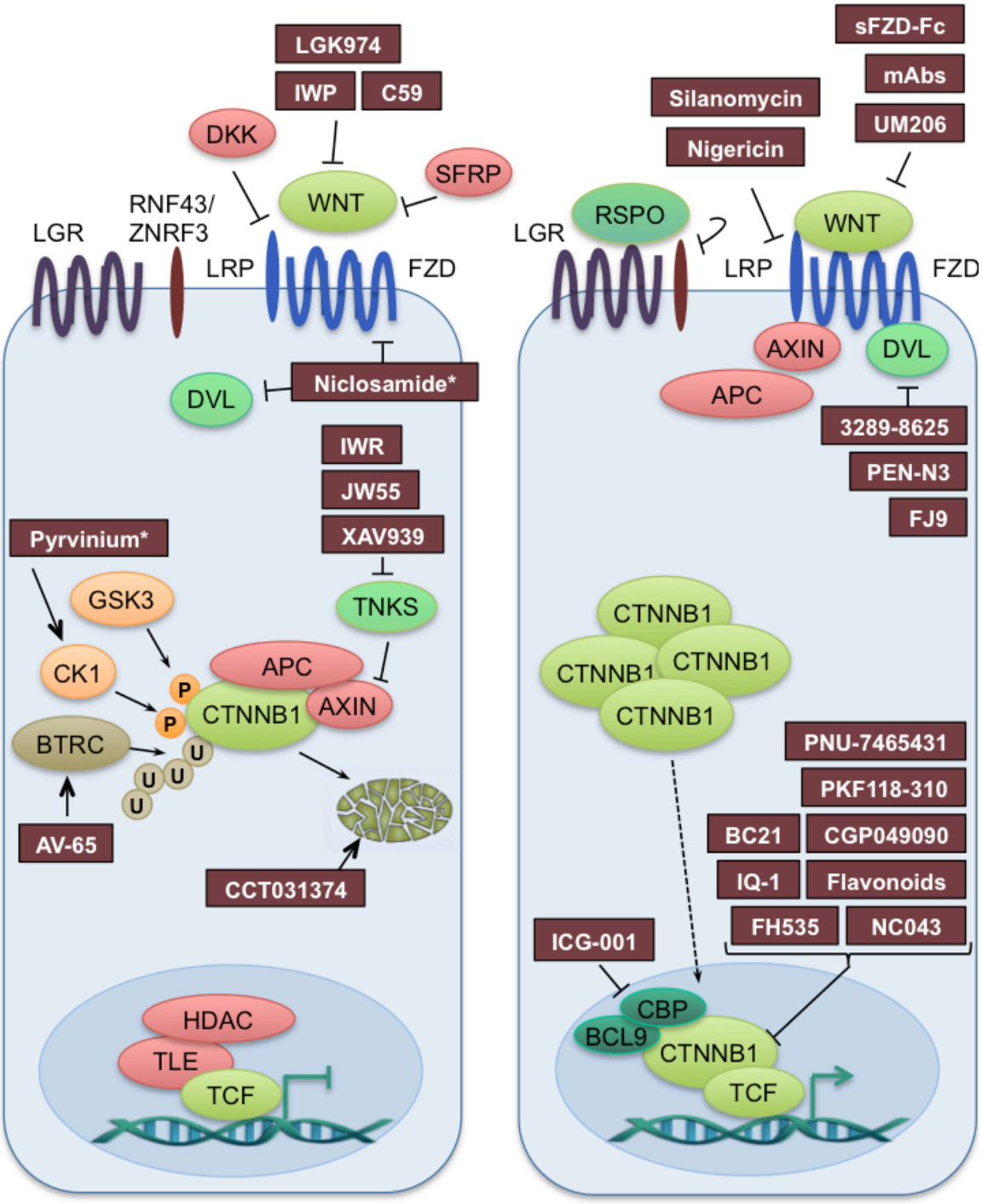

LGK974
IWP
C59
DKK
SFRP
WNT
RNF43/
ZNRF3
LGR
LRP
FZD
DVL
Niclosamide*
IWR
JW55
XAV939
TNKS
Pyrvinium*
GSK3
CK1
APC
AXIN
CTNNB1
P
P
BTRC
U
U
U
U
AV-65
CCT031374
HDAC
TLE
TCF
sFZD-Fc
mAbs
UM206
Silanomycin
Nigericin
RSPO
WNT
LGR
LRP
FZD
AXIN
DVL
APC
3289-8625
PEN-N3
FJ9
CTNNB1
CTNNB1
CTNNB1
CTNNB1
PNU-7465431
PKF118-310
BC21
CGP049090
IQ-1
Flavonoids
FH535
NC043
ICG-001
CBP
BCL9
CTNNB1
TCF

Figure 5: Schematic diagram depicting a Sleeping Beauty transposon insertional mutagenesis screen for cancer gene discovery. (A) Tissue-specific mobilization of *Sleeping Beauty* transposons is achieved by breeding mice to harbor three transgenes: a tissue-specific Cre recombinase allele, a conditional transposase allele, and an SB transposon concatamer allele. For intestinal tumor screens, transposase expression is restricted to the gut by the *Villin-Cre* and *Rosa26-LsL-SB11* alleles. The Lox-stop-Lox cassette (LsL) blocks transcription of the transposase (*SB11*) from the ubiquitous *Rosa26* promoter. Where Cre is expressed in epithelial tissues of the gut, the stop cassette is excised and transposase is expressed. Active transposase mobilizes transposons from the donor transposon allele (T2/Onc.) (B) Transposon insertional mutagenesis occurs in tissues where the transposase (SB11) is expressed. SB is a DNA-based "cut-and-paste" transposon system. Transposase enzyme recognizes specific DNA sequences flanking the transposon element (inverted repeat/direct repeat sequences, indicated by <<>>). The transposase cuts the genomic DNA at the recognition sites and pastes the transposon randomly into another location in the genome at a "TA" dinucleotide. (C) Transposon insertions cause gain and loss-of-function mutations and drive tumorigenesis. The *T2/Onc* transposon contains a promoter sequence from the murine stem cell virus 5' long terminal repeat (LTR) and splice donor (SD) sequence that can activate expression of oncogenes. *T2/Onc* can also cause loss-of-function insertions within tumor suppressor genes (TSG) as it contains bidirectional splice acceptor-polyadenylation sequences (^{SA}pA). Insertional mutagenesis results in tumor formation. (D) Transposon insertion sites are sequenced and mapped to the mouse genome for multiple independent tumors in order to define recurrently mutated common insertion sites (CIS). These loci contain putative cancer genes, dysregulation of which contributes to tumor formation.
Figure modified from (Starr and Largaespada, 2005).

Figure 5

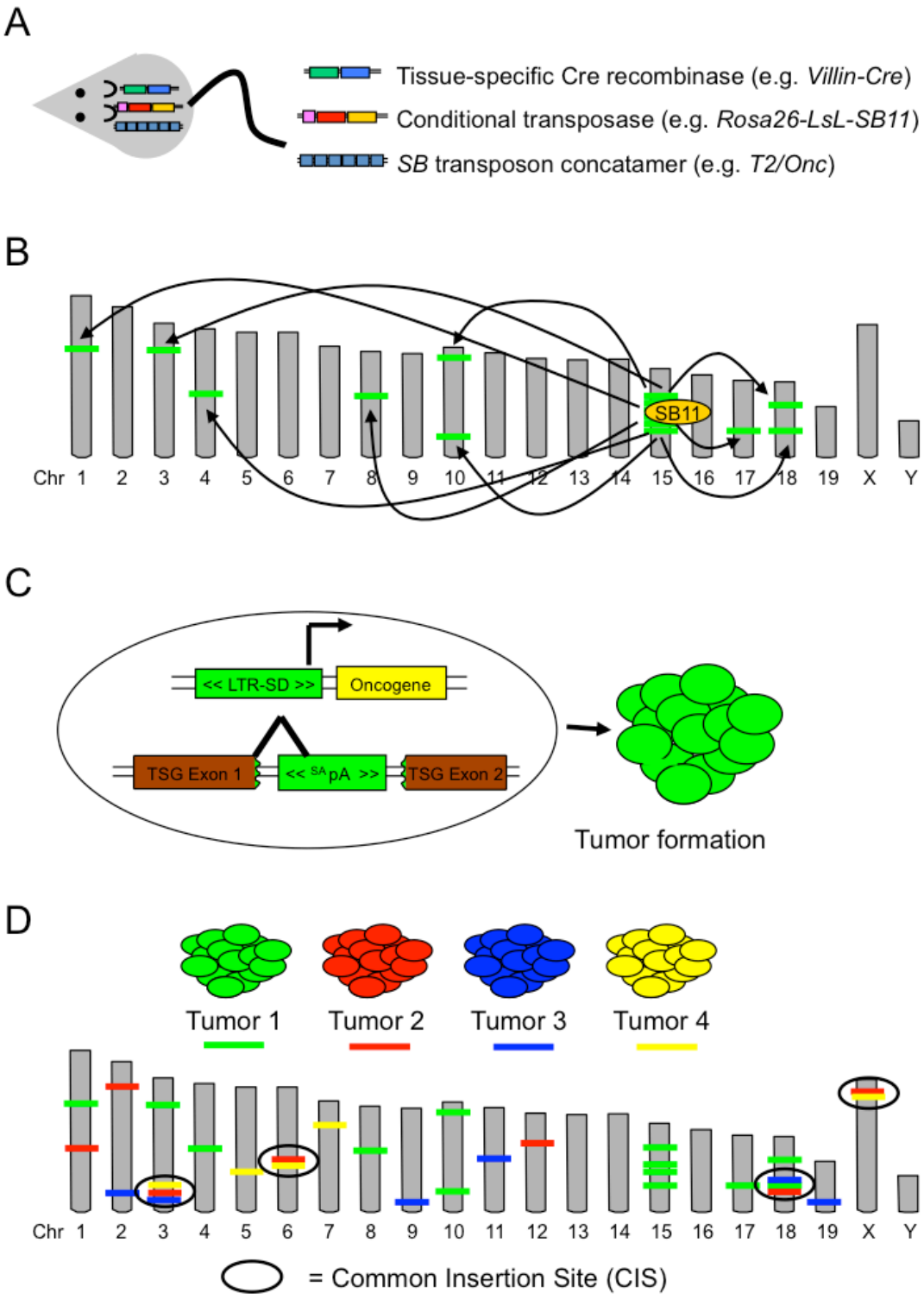

A
Tissue-specific Cre recombinase (e.g. Villin-Cre)
Conditional transposase (e.g. Rosa26-LsL-SB11)
SB transposon concatamer (e.g. T2/Onc)
B
SB11
Chr 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 X Y
C
<< LTR-SD >>
Oncogene
TSG Exon 1
<< SA pA >>
TSG Exon 2
Tumor formation
D
Tumor 1
Tumor 2
Tumor 3
Tumor 4
Chr 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 X Y
= Common Insertion Site (CIS)

Figure 6: Top 10 Common Insertion Site (CIS)-associated genes identified by three SB transposon mutagenesis screens for genetic drivers of intestinal tumor formation. CIS-associated genes were ranked by frequency of transposon insertions identified in unique tumors. Results are shown for screens conducted on wild type (WT), APC-deficient ($Apc^{Min/+}$), and p53-deficient ($TP53^{R270H/+}$) genetic backgrounds. Two CIS genes selected for further study, *Rspo2* and *Wac*, are highlighted (Starr et al., 2009, 2011).

Figure 6

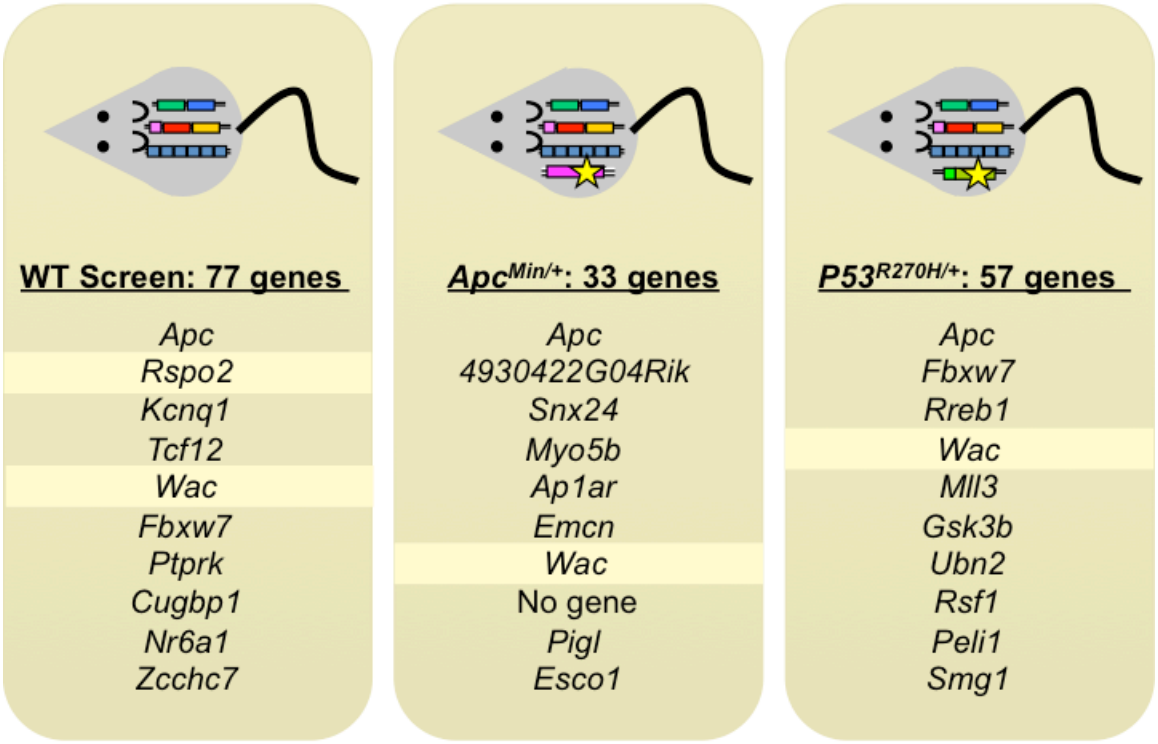

WT Screen: 77 genes
Apc
Rspo2
Kcnq1
Tcf12
Wac
Fbxw7
Ptprk
Cugbp1
Nr6a1
Zcchc7
ApcMin/+: 33 genes
Apc
4930422G04Rik
Snx24
Myo5b
Ap1ar
Emcn
Wac
No gene
Pigl
Esco1
P53R270H/+: 57 genes
Apc
Fbxw7
Rreb1
Wac
Mll3
Gsk3b
Ubn2
Rsf1
Peli1
Smg1

Table 1: Targeted agents with FDA approval for treatment of colon, breast, and liver cancer

Colon Cancer				
Drug (generic name)	**Drug (trade name)**	**Class**	**Mechanism**	**Approved Indications**
bevacizumab	Avastin	angiogeneses inhibitor	Antibody against VEGF	Metastatic CRC, advanced NSCLC, metastatic kidney cancer, glioblastoma multiforme
regorafenib	Stivarga	angiogeneses inhibitor	Multi-kinase inhibitor (VEGFR, PDGFR, KIT, FGFR, RAF, TIE2, and others)	Metastatic CRC, advanced GIST
ziv-aflibercept	Zaltrap	angiogeneses inhibitor	VEGFR-Fc inhibits VEGF and PGF	Metastatic colorectal cancer
panitumumab	Vectibix	cell signaling inhibitor	Antibody against EGFR	KRAS wild type metastatic CRC
cetuximab	Erbitux	cell signaling inhibitor	Antibody against EGFR	KRAS wild type metastatic CRC Head and neck cancer
Breast cancer				
Drug (generic name)	**Drug (trade name)**	**Class**	**Mechanism**	**Approved Indications**
ado-trastuzumab	Kadcyl	cell signaling inhibitor	Antibody against HER2 conjugated to DM1 chemotherapeutic agent	HER2+ metastatic breast cancer
lapatinib	Tykerb	cell signaling inhibitor	Dual kinase inhibitor (HER2 and EGFR)	HER2+ metastatic breast cancer
pertuzumab	Perjeta	cell signaling inhibitor	Antibody against HER2	HER2+ metastatic breast cancer
trastuzumab	Herceptin	cell signaling inhibitor	Antibody against HER2	HER2+ breast cancer and metastatic BCA
everolimus	Afinitor	cell signaling inhibitor	mTOR inhibitor	HER2+ metastatic breast cancer, metastatic kidney cancer, metastatic PNET
exemestane	Aromasin	hormone/anti-hormone	Aromatase inhibitor	ER+ breast cancer and metastatic BCA
letrozole	Femara	hormone/anti-hormone	Aromatase inhibitor	Breast cancer and metastatic BCA
fulvestrant	Faslodex	hormone/anti-hormone	Estrogen receptor inhibitor	ER+ metastatic breast cancer
megestrol acetate	Megace	hormone/anti-hormone	Synthetic progestin	Advanced breast cancer, endometrial cancer
tamoxifen	Nolvadex	hormone/anti-hormone	SERM	ER+ breast cancer and metastatic BCA
Liver cancer				
Drug (generic name)	**Drug (trade name)**	**Class**	**Mechanism**	**Approved Indications**
sorafenib	Nexavar	angiogeneses inhibitor	Multi-kinase inhibitor (VEGFR, PDGFR, RAF, and others)	Unresectable hepatocellular carcinoma Advanced renal cell carcinoma Differentiated thyroid carcinoma

Abbreviations: BCA, breast cancer; CRC, colorectal cancer; EGFR, epidermal growth factor receptor; GIST, gastrointestinal stromal tumors; mTOR, mammalian target of rapamycin; PDGFR, platelet-derived growth factor receptor; PGF, placental growth factor; PNET, pancreatic neuroendocrine tumors; SERM, selective estrogen-receptor modulator; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor.

References: Therapeutic agents were identified from (AACR Cancer Progress Report Writing Committee et al., 2013; Cantley et al., 2012).

Table 3: Wnt inhibitors currently in clinical trials

Drug (Manufacturer)	Mechanism	Conditions	Phase	Clinicaltrials.gov Identifier
PRI-724 (PRISM pharma)	Blocks CTNNB1 interaction with CBP	Relapsed AML,CML Advanced solid tumors	Phase I/II Phase I	NCT01606579 NCT01302405
CWP232291 (JW Pharmaceutical)	Enhances CTNNB1 degradation	Relapsed-Refractory AML	Phase I	NCT01398462
LGK974 (Novartis)	Porcupine inhibitor	Advanced Melanoma, breast cancer, pancreatic cancer	Phase I	NCT01351103
OTSA101-DTPA-90Y (OncoTherapy Science)	Radiolabelled mAb to FZD10	Advanced synovial sarcoma	Phase I	NCT01469975
OMP-54F28 (OncoMed)	Pseudo-receptor (FZD8-Fc)	Advanced solid tumors	Phase I	NCT01608867
Genistein (generic)	Phytoestrogen	Metastatic CRC	Phase I/II	NCT01985763

Abbreviations: CTNNB1, beta-catenin; CBP, CREB binding protein; FZD10, Frizzled 10; FZD8-Fc, Frizzled 8 conjugated to a soluble constant fragment; AML, acute myeloid leukemia; CML, chronic myeloid leukemia; CRC, colorectal cancer. Clinical trials were identified by search for interventional studies at clinicaltrials.gov, updated August 2014.

Chapter 2: R-spondin 2 drives Wnt signaling and tumorigenesis in multiple tissues

Summary

R-spondins are secreted regulators of Wnt signaling that function in development and promote tissue stem cell growth. In murine insertional mutagenesis screens *Rspo2* has been identified as a candidate oncogene in intestinal and breast tumors. Further, oncogenic activation of *RSPO2* and *RSPO3* mediated by recurrent genomic rearrangements has been identified in human colorectal cancer. To determine if R-spondins are oncogenic in other human cancers characterized by Wnt signaling activation, we analyzed *RSPO2/3* expression in primary breast and liver tumors. *RSPO2* was highly expressed in a subset of primary human breast tumors and significantly associated with the basal and Her2 subtypes. Similarly, *RSPO2* was elevated in the subset of primary human liver cancers with an activated Wnt signaling gene expression profile. *RSPO2* overexpression increased Wnt signaling in a non-tumorigenic human breast epithelial cell line, and knockdown of RSPO2 decreased Wnt signaling and proliferation in human breast cancer cells with high basal *RSPO2* expression. An *in vivo* model of somatic *RSPO2* overexpression revealed that *RSPO2* promoted Wnt signaling and hepatomegaly in the mouse liver. Overexpression of *RSPO2* combined with depletion of *Trp53* (RSPO2/shp53) also significantly enhanced liver tumor formation. Liver tumors that formed in mice expressing RSPO2/shp53 were characterized as hepatocellular carcinomas with necrosis and lymphoid infiltrate. These data strongly suggest that RSPO2 overexpression promotes tumorigenesis in multiple Wnt-responsive tissues, including colon, liver and breast and may be a novel drug target. We have developed *in*

vitro and *in vivo* models of RSPO-driven cancer for future preclinical testing of R-spondin and Wnt targeted therapy.

Introduction

Wnt/beta-catenin (CTNNB1) is a critical signaling pathway in normal development and maintenance of adult stem cells in many tissues (Clevers, 2006; Holland et al., 2013). Activation of "canonical" (CTNNB1-dependent) Wnt signaling induces transcription through the CTNNB1–TCF complex, activating a gene expression program that regulates cell proliferation, survival, differentiation, polarity, and migration (Baarsma et al., 2013; Niehrs, 2012). Aberrant activation of Wnt signaling is a feature of many types of cancer, including cancer of the colon, liver, lung, brain, breast, ovary, prostate, and other tissues (Curtin and Lorenzi, 2010; Polakis, 2012). Consequently, there is significant interest in targeting the Wnt pathway therapeutically (Anastas and Moon, 2013; Barker and Clevers, 2006). Multiple genetic lesions or molecular events can activate Wnt signaling. In colon cancer, inactivating mutation or deletion of *APC*, a core negative regulator of CTNNB1, is the predominant mechanism of Wnt activation, occurring in ~80% of cases (Barker and Clevers, 2006; Fodde et al., 2001). Activating mutations in *CTNNB1*, loss of function mutations in *AXIN1/2*, and other pathway components are alternate routes to Wnt activation in colon cancer (Polakis, 2012). Recently, overexpression of R-spondin 2 and 3 (*RSPO2* and *RSPO3*) due to recurrent genomic rearrangements was discovered and proposed to be a novel mechanism of Wnt activation in colorectal cancer (Seshagiri et al., 2012). Specifically, Seshagiri et al. identified recurrent genomic deletions and inversions associated with increased

expression of *RSPO2* or *RSPO3* in 10% of human colorectal cancers (Seshagiri et al., 2012). Studies in mice support the hypothesis that RSPOs are oncogenes that activate Wnt signaling. An insertional mutagenesis screen for intestinal tumors in mice identified *Rspo2* as the second-most commonly altered gene after *Apc* (Starr et al., 2009). Significantly, in both human CRCs and mouse intestinal tumors, activation of *RSPO2/3* was mutually exclusive with inactivation of *APC*, suggesting these lesions operate in the same pathway (Seshagiri et al., 2012; Starr et al., 2009). Mouse mammary tumor insertional mutagenesis screens identified *Rspo2* and *Rspo3* as candidate oncogenes in breast cancer as well (Lowther et al., 2005; Theodorou et al., 2007). Consistent with oncogenic function, R-spondins were overexpressed in mammary tumors with RSPO insertions and ectopic expression of *Rspo2* or *Rspo3* promoted tumor formation of mouse mammary epithelial cells in xenograft experiments (Klauzinska et al., 2012; Theodorou et al., 2007). Recent efforts to comprehensively catalog genomic alterations in hepatocellular carcinoma (HCC) identified amplification and increased expression of *RSPO2* in 3-22% of cases, although the functional significance of *RSPO2* amplification in these cases was not assessed (Ahn et al., 2014; Kan et al., 2013). These studies suggest that RSPOs might function as oncogenes in multiple tissue types, perhaps by activating Wnt signaling.

Indeed, RSPOs were initially discovered and characterized as secreted enhancers of Wnt signaling (Kazanskaya et al., 2004). Subsequently, RSPOs have been shown function in development and promote tissue stem cell growth (Jin and Yoon, 2012; Schuijers and Clevers, 2012). *Rspo2* and *Rspo3* are both are essential in mice. *Rspo2* null

mice die in the immediate postnatal period with developmental defects of the lungs, kidney, limbs, and craniofacial structures, while *Rspo3* null mice die by day E10 due to placental defects (Jin and Yoon, 2012). R-spondins promote *ex vivo* growth of tissue stem cells derived from small intestine, colon, stomach, liver and pancreas and are an essential media component in organoid cultures from these tissues (Barker et al., 2010; Huch et al., 2013a, 2013b; de Lau et al., 2014; Sato and Clevers, 2013). Transgenic expression of RSPO1 or injection of recombinant RSPO1 in mice leads to intestinal hyperplasia, suggesting that RSPO1 is a growth factor promoting proliferation in the intestinal epithelium (Kim et al., 2005).

How RSPOs regulate Wnt signaling has been area of intense study and some controversy in recent years (de Lau et al., 2014). Initial studies established that all four members of the RSPO family (RSPO1-4) are able to enhance Wnt signaling in the presence of Wnt ligand, although these studies proposed contradictory receptors and signaling mechanisms (Binnerts et al., 2007; Kazanskaya et al., 2004; Kim et al., 2008; Nam et al., 2006; Ohkawara et al., 2011; Wei et al., 2007). In 2011, three groups reported nearly simultaneous discoveries, definitively establishing the orphan G-protein coupled receptors LGR4, LGR5, and LGR6 as receptors for RSPOs (Carmon et al., 2011; Glinka et al., 2011a; de Lau et al., 2011). Interestingly, expression of LGR5 identifies stem cells in many tissues including the intestine, colon, liver, and mammary gland (Huch et al., 2013a; Koo and Clevers, 2014; Plaks et al., 2013; de Visser et al., 2012). LGR5-positive intestinal stem cells have been identified as the cell of origin for adenoma formation

(Barker et al., 2009). This suggests that both normal tissue stem cells and cancer stem cells are potentially responsive to RSPO signals.

The signaling events downstream of RSPO binding to LGR receptors remain incompletely understood. It has been proposed that upon RSPO-binding, LGR proteins associate directly with the Wnt receptor complex (FZD/LRP) to enhance Wnt signaling (Carmon et al., 2012b; de Lau et al., 2011). Alternatively, two groups determined that RSPO/LGR enhance Wnt signaling by destabilizing RNF43 and ZNRF3, E3 ubiquitin ligases that negatively regulate Wnt signaling by promoting clearance of FZD and LRP receptors from the cell surface (Hao et al., 2012; Koo et al., 2012). In this model, when RSPOs bind LGR receptors, RNF43 and ZNRF3 are cleared from the membrane and Wnt receptors remain, leading to enhanced signaling response to Wnt ligand (Hao et al., 2012; Koo et al., 2012). Contradicting this view, another group recently proposed that RSPO2 could act as a repressor, rather than enhancer, of Wnt signaling via LGR5-dependent stabilization, rather than inhibition, of ZNRF3/RNF43 (Wu et al., 2014). Wu et al. observed that *RSPO2* expression was suppressed in the majority of CRCs due to promoter hypermethylation, and RSPO2 was able to inhibit Wnt signaling through LGR5 in CRC cell lines with decreased RSPO2 expression (Wu et al., 2014). They proposed an alternate model in which RSPO2 functions as a tumor suppressor and Wnt antagonist in CRC. Supporting this view, LGR5 has been proposed to participate in a stem cell-specific negative feedback loop to limit Wnt signaling (Garcia et al., 2009).

Further complicating the picture, contradictory roles have also been reported for other members of the RSPO/LGR/RNF43/ZNRF3 signaling module in cancer. RNF43

was initially characterized as an oncogene in CRC and HCC (Xing et al., 2013; Yagyu et al., 2004). Subsequently, RNF43 has been shown to function as a tumor suppressor gene in CRC, pancreatic cancer, and mucinous ovarian cancer (Jiang et al., 2013; Koo et al., 2012; Ryland et al., 2013; Wu et al., 2011). LGR5 expression is increased in large subsets of human CRC and HCC (McClanahan et al., 2006; Uchida et al., 2010; Yamamoto et al., 2003). Yet reports conflict as to whether LGR5 is oncogenic (Hsu et al., 2014; Tsuji et al., 2014) or tumor suppressive (Walker et al., 2011) in CRC.

This study addresses the contradictory models of RSPO function in human cancer, and finds support for an oncogenic role of *RSPO2* in subsets of colon, liver and breast cancer. We investigated the expression of *RSPO2* and *RSPO3* in primary human colon, liver and breast cancers compared to normal tissues, and found that while *RSPO2* and *RSPO3* expression was downregulated in the majority of CRC tumors compared to normal colon, expression was upregulated in a subset of tumors corresponding with expression of *RSPO*-fusion genes and activation of Wnt signaling. In breast and liver tumors, *RSPO3* expression was significantly downregulated compared to normal tissues. However, *RSPO2* expression was increased in subsets of human breast and liver cancers with activated Wnt signaling. In breast cancer, elevated *RSPO2* expression was associated with the basal subtype. We found that RSPO2 was required for CTNNB1-dependent transcription and proliferation in a basal breast cancer cell line with high RSPO2-expression. We also developed and *in vivo* model of RSPO2 activation in the mouse liver, and found that elevated expression of RSPO2 in the mouse liver promoted hyperplastic growth and an enlarged liver phenotype. In the context of p53 inhibition,

RSPO2 expression dramatically increased liver tumor formation. These studies support an oncogenic role for R-spondins in multiple Wnt-responsive tissue types.

Results

RSPO2 and RSPO3 are upregulated in fusion-transcript-positive colon cancer

Two prior studies identified recurrent genomic rearrangements associated with *RSPO2* and *RSPO3* overexpression in 4-10% of colorectal cancers (Seshagiri et al., 2012; Shinmura et al., 2014). Although *APC* is mutated in ~80% of non-hypermutated/microsatellite-stable (MSS) CRC overall (Cancer Genome Atlas Network, 2012a), RSPO-high tumors in both studies were MSS and, yet, retained wildtype *APC*, suggesting that *RSPO* overexpression and *APC* loss of function could be mutually exclusive mechanisms for activation of canonical Wnt signaling in colorectal cancer. The initial studies characterizing RSPO activation in CRC examined a modest number of samples (70 and 75) (Seshagiri et al., 2012; Shinmura et al., 2014). In order to determine the frequency of these events in a larger cohort of patients and model their effects on cell signaling, we analyzed RNA-seq data from 434 CRC tumors and 41 normal colon samples obtained through The Cancer Genome Atlas (TCGA 2012, http://cancergenome.nih.gov). In this cohort, *RSPO2* and *RSPO3* expression was decreased in the majority of CRCs compared to normal colon. Specifically, 422 of 434 (97.2%) CRCs have more than four-fold decreased *RSPO2* expression, and 250 of 434 (57.6%) have more than four-fold decreased *RSPO3* expression **(Figure 1A)**. This concurs with previous observations indicating *RSPO2* and *RSPO3* expression are

suppressed in the vast majority of CRCs (Kazanskaya et al., 2004; Seshagiri et al., 2012; Shinmura et al., 2014; Wu et al., 2014).

In contrast, a small subset of CRCs expressed high levels of *RSPO2/3* and RSPO fusion transcripts. "RSPO-high" tumors were defined as having greater than four-fold elevated mRNA levels compared to normal colon. In this cohort, 2 of 434 CRCs (0.5%) were RSPO2-high and 6 of 434 CRCs (1.4%) were RSPO3-high **(Figure 1A)**. Compared to the corresponding RSPO-low tumors, RSPO-high tumors expressed 231-fold higher *RSPO2* levels and 59-fold higher *RSPO3* levels. Next we sought to determine if RSPO-high samples expressed RSPO-fusion genes (Seshagiri et al., 2012; Shinmura et al., 2014). Three of the eight RSPO-high tumors had paired-end RNA-seq data available from TCGA, which allowed us to check for expression of RSPO fusion transcripts using DeFuse (McPherson et al., 2011). All three (100%) expressed *PTPRK-RSPO3* fusions, as described previously **(Figure 1A)** (Seshagiri 2012, Shinmura 2014).

RSPO-high human CRCs have activated Wnt signaling and wild type APC

Many studies have shown that R-spondins enhance Wnt pathway activity *in vitro* (Binnerts et al., 2007; Carmon et al., 2011; Glinka et al., 2011a; Kazanskaya et al., 2004; Kim et al., 2008; de Lau et al., 2011; Nam et al., 2006; Ohkawara et al., 2011; Wei et al., 2007). Accordingly, the initial identification of RSPO gene fusions in CRC found that RSPO fusion-positive tumors had activated Wnt signaling in the absence of *APC* or *CTNNB1* mutations, consistent with a model where RSPOs function as oncogenes by activating the Wnt pathway. However, a contradictory model proposes that R-spondins are capable of inhibiting Wnt signaling through LGR5/ZNRF3 stabilization and there is

selective pressure to lose this negative feedback by downregulating RSPO2 in CRC (Wu et al., 2014). In the set of CRCs analyzed by TCGA, expression of Wnt target genes was increased in RSPO-high tumors compared to normal colon **(Figure 1B).** Interestingly clustering CRC and normal samples based on expression of 123 Wnt target genes failed to separate RSPO-high CRCs from tumors with other pathway alterations, suggesting equivalent Wnt activation **(Supplementary Figure S1).** Consistent with prior reports, RSPO-high status was mutually exclusive with *APC* mutation (Seshagiri et al., 2012; Shinmura et al., 2014). Somatic mutation data was available for four of the eight RSPO-high CRCs. Of these, 4 of 4 (100%) retained wild type *APC*. These data are consistent with a model wherein R-spondins are an alternate route to activation of Wnt signaling.

Additionally, although Wu et al. found that an RSPO-high CRC expressing the *EIF3E-RSPO2* fusion transcript had downregulated expression of *LGR5*, presumably to prevent Wnt inhibition through the RSPO/LGR5 axis, in our analysis of TCGA CRC patients we found that *LGR5* mRNA was downregulated in only 1 of 8 RSPO-high tumors **(Supplementary Figure S2).** No coding mutations in LGR5 were identified (data not shown). These data suggest that LGR5 inactivation is not an absolute requirement in RSPO-high tumors.

These findings support a model in which *RSPO2/3* are upregulated in a minority of *ACP* wild type CRCs due to gene fusions or other events, correlating with activation of Wnt signaling in those tumors. Because Wnt activation can promote tumorigenesis in other tissues, we next examined expression of *RSPO2* and *RSPO3* in primary human

breast and liver cancer, using both TCGA datasets and independently obtained primary samples.

RSPO2 is upregulated in human breast cancer

Previous studies identified activation of Wnt signaling in a subset of breast cancers, correlating with the basal subtype (Geyer et al., 2011; Khramtsov et al., 2010). Silencing of *SFRP1*, a secreted negative regulator of the Wnt pathway, is one mechanism that promotes Wnt signaling in breast cancer (Curtin and Lorenzi, 2010; Ugolini et al., 2001). However, events leading to Wnt activation in breast cancer are incompletely understood. *Rspo2* and *Rspo3* were identified as potential breast cancer oncogenes by MMTV insertional mutagenesis screens (Lowther et al., 2005; Theodorou et al., 2007) and overexpression of *Rspo2* was shown to promote invasiveness and tumor formation in mouse mammary epithelial cells (Klauzinska et al., 2012). Interestingly, *LGR5* expression marks stem cells in the mammary epithelium (Plaks et al., 2013; de Visser et al., 2012), suggesting that the stem cell compartment may be R-spondin-responsive in the breast.

To analyze R-spondin expression in breast cancer, we obtained pairs of matched normal and tumor tissues from 41 patients. *RSPO2* and *RSPO3* mRNA expression levels were quantified by qRT-PCR. Six tumors (14.6%) expressed *RSPO2* mRNA levels >4-fold higher than their matched normal controls and were defined as “RSPO-high” (Range: 6.7 to 28.2-fold elevation) **(Figure 2).** In the TCGA dataset, we compared 1,048 breast tumors to 111 normal breast tissues (TCGA 2012b, http://cancergenome.nih.gov). *RSPO2* mRNA was expressed at a very low level in normal breast samples (RPKM <1 in

100 of 111, 90.1%, of samples). The majority of breast tumors also maintained low *RSPO2* expression (RPKM <1 in 842 of 1,048, 80.3%, of samples.) However, 122 of 1,048 (10.6%) of breast tumors had >4-fold increased RSPO2 expression compared to the normal average (range, 4.0-300.7-fold elevated) **(Supplementary Figure S3A).**

Similar to our observations in CRC, *RSPO3* levels were decreased in the majority of breast tumors, both in the set of 41 matched tumor/normal samples and in the TCGA dataset (data not shown and **Supplementary Figure S3A)**. In the TCGA dataset, 881 of 1,048 (83.5%) of breast tumors were RSPO3-low, while rare tumors had elevated *RSPO3* levels (3 of 1,048, 0.3%, **Supplementary Figure S3).** DeFuse analysis of RSPO-high breast tumors in the TCGA set did not identify expression of *RSPO* fusion transcripts.

RSPO2-high breast cancers are associated with basal/HER2 subtypes and active Wnt signaling.

To determine the clinical relevance of *RSPO2* overexpression, we analyzed PAM50 clinical subtype information for 521 breast tumors in the TCGA dataset. RSPO2-high status was significantly associated with basal (p=2.07E-5) and HER2 (p=0.0259) subtypes, and anti-correlated with luminal A (p=0.0052) and luminal B (p=0.0093) subtypes **(Supplementary Figure S3B).** For example, while basal type tumors account for only 19% of tumors overall, 41% of RSPO-high breast tumors were basal type.

While active Wnt signaling, as measured by presence of nuclear CTNNB1, is associated with basal subtype tumors (Geyer et al., 2011; Khramtsov et al., 2010), expression of Wnt target genes in RSPO-high breast tumors was not consistently elevated compared to normal tissue **(Supplementary Figure S3C).** Specifically, while expression

of the Wnt target gene *LEF1* was significantly elevated in RSPO-high tumors, *AXIN2* and *TCF7* were significantly downregulated **(Supplementary Figure S3C).** Examination of Wnt target gene expression in breast tumors with *APC* loss of function mutations or reduced expression of *SFRP1* revealed the same pattern, suggesting Wnt signaling is equivalently activated in these subsets **(Supplementary figure S3C).**

RSPO2 regulates Wnt signaling and proliferation in breast cancer cells

To determine the functional significance of elevated RSPO2 expression in breast cancer we overexpressed RSPO2 in a non-transformed breast epithelial cell line with basal origin (MCF10A, (Debnath et al., 2003)) and we knocked down RSPO2 in an RSPO2-high basal-type breast cancer cell line (BT-549 (Barretina et al., 2012)). MCF10A cells overexpressing RSPO2 showed transcriptional upregulation of Wnt/CTNNB1 target genes **(Figure 3A)**, while there was no effect on proliferation rate in MCF10A cells (data not shown.). BT-549 cells express extremely high levels of RSPO2 compared to the 60 breast cancer cell lines profiled in the Cancer Cell Line Encyclopedia (**Figure 3B** (Barretina et al., 2012)). Knockdown of RSPO2 in BT-549 cells resulted in decreased expression of Wnt target genes, as well as reduced cell proliferation **(Figure 3C and D).** These results suggest that RSPO2-high breast cancers may require RSPO2 expression for activation of Wnt signaling and enhanced growth.

RSPO2 is upregulated in human liver cancer and is associated with activated Wnt signaling

Aberrant activation of Wnt/CTNNB1 signaling is a common feature of hepatocellular carcinoma (HCC) (Ahn et al., 2014; Guichard et al., 2012; Kan et al., 2013). A study of 642 HCCs showed that activation of the Wnt pathway, defined by gene expression profiling, occurs in approximately 50% of cases due to activating mutations in *CTNNB1* or other events (Lachenmayer et al., 2012). Activating mutations in *CTNNB1* occur in approximately one third of cases (Ahn et al., 2014; Guichard et al., 2012; Kan et al., 2013). Importantly, recent whole genome sequencing of 81 hepatitis B virus-associated HCCs identified genomic amplification and increased mRNA expression of *RSPO2* in 22% of cases, although the functional effect of these lesions was not assessed (Kan et al., 2013). To further characterize the role of R-spondins in HCC, we analyzed *RSPO2* and *RSPO3* mRNA levels by microarray analysis in two sample cohorts including 319 HCCs, 199 chirrhotic or premalignant samples, and 23 normal liver samples (Lachenmayer et al., 2012). *RSPO2* expression was uniformly low in normal liver and cirrhotic/pre-malignant samples, but was increased in HCC ($p<0.05$ vs. cirrhotic liver, n/s vs. normal) **(Figure 4A).** Analogous to colon and breast tumors, HCC samples express significantly lower *RSPO3* levels compared to normal, cirrhotic, and pre-malignant samples **(Supplementary Figure S5B).**

Gene expression-based molecular subclass analysis was performed to further characterize RSPO-high liver cancers (Chiang et al., 2008; Lachenmayer et al., 2012). *RSPO2* expression was significantly elevated in the subclass of HCC characterized by

activated Wnt signaling (CTNNB1-class), while *RSPO3* expression was suppressed in all classes **(Figure 4B** and data not shown). *RSPO2* levels were higher in HCC samples with mutated *CTNNB1* ($p<0.001$), indicating that these events tended to co-occur. However, 50% of RSPO2-high HCC samples belonged to the CTNNB1-class based on gene expression profiling yet retained wildtype *CTNNB1*.

RSPO2 and *RSPO3* expression was also assessed in 200 liver tumors and 50 normal liver samples from TCGA. In this cohort, RSPO3 was suppressed in 178 of 200 (89%) liver tumors, while 24 of 200 samples (12%) were RSPO2-high and 1 of 200 samples (0.5%) was RSPO3-high **(Supplementary Figure S5A).** RSPO expression was increased 29.3-fold on average in RSPO-high tumors compared to normal liver. RSPO-high tumors had significantly elevated expression of Wnt target genes (*AXIN2*, *TCF7*, and *LEF1*) compared to normal liver **(Supplementary Figure S5D)**. Expression of LGR5 was also significantly elevated in RSPO-high tumors compared to normal liver **(Supplementary Figure S5C)**. Again, we found significant co-occurrence of RSPO-high status and *CTNNB1* mutation in this cohort ($p=1.72E-09$). DeFuse analysis of RSPO-high liver tumors in the TCGA set did not identify expression of RSPO fusion transcripts.

R-spondin overexpression is not induced by activated CTNNB1

The co-occurrence of high *RSPO2* expression and *CTNNB1* mutation in liver cancer suggests a model in which CTNNB1 causes transcriptional upregulation of R-spondins. Indeed, RSPOs were shown to be Wnt target genes in Xenopus (Kazanskaya et al., 2004). In this model, R-spondin upregulation may provide positive feedback to enhance Wnt signaling, rather than being an initiating event.

To test this hypothesis we used an *in vivo* model of constitutive CTNNB1 activation in the mouse liver. We generated mice that somatically expressed *CTNNB1-S33Y* in the liver by hydrodynamic tail vein injection of DNA transposon vectors in Fumarylacetoacetate hydrolase (Fah) deficient mice (Wangensteen et al., 2008). Liver cells in Fah deficient mice will die unless the mice are maintained on the drug NTBC. By delivering *CTNNB1-S33Y* along with a functional copy of *Fah* on the same transposon and then withdrawing NTBC, the liver becomes repopulated only with cells that have incorporated both *CTNNB1-S33Y* and *Fah* in their genome. In this experiment, transposon vectors expressing *Fah*, *luciferase*, *GFP,* and either *CTNNB1-S33Y* or *GFP* control were injected into *Fah* null mice with ubiquitous transposase expression (*Fah-/-;Rosa26-SB11,* **Figure 5A**)(Keng et al., 2013). Tumors formed in the livers of all four (100%) mice injected with *CTNNB1-S33Y* by 90 days post-hydrodynamic injection (PHI), and were classified as hepatocellular carcinomas by histopathological analysis **(Supplementary Figure S4A).** No tumors formed in the *GFP*-injected control mice (zero of six) by 90 days PHI. RNA was isolated from samples of grossly normal liver from *CTNNB1-S33Y*- and *GFP*-injected mice, and from tumors of *CTNNB1-S33Y*-injected mice and used for gene expression analysis. *CTNNB1-S33Y* was well expressed in experimental tissues (data not shown). As expected, grossly normal liver and tumors from *CTNNB1-S33Y*-injected mice showed increased expression of the Wnt target gene *Axin2* compared to *GFP*-injected livers, confirming activation of Wnt signaling in these tissues **(Supplementary Figure S4B)**. However, neither *Rspo2* nor *Rspo3* was upregulated in *CTNNB1-S33Y*-injected livers. Indeed, analogous to human HCC, *Rspo3* expression was

significantly suppressed in tumors from *CTNNB1-S33Y*-injected mice **(Supplementary Figure S4B)**. Despite prior literature suggesting R-spondins constitute a positive feedback loop in Wnt regulation, *Rspo2/3* were not transcriptionally activated by CTNNB1 in the mouse liver, rather *Rspo3* was significantly downregulated in tumors initiated by activated CTNNB1. Similarly, treating MCF10A breast cells with recombinant Wnt ligand did not induce expression of *RSPO2* or *RSPO3* **(Supplementary figure S4C).** These results suggest that although high RSPO2 expression co-occurs with other lesions in the Wnt pathway in liver cancer, elevated RSPO2 expression is not merely a consequence of activated Wnt signaling and may, rather, be an independently selected event.

RSPO2 promotes Wnt signaling and tumorigenesis in the mouse liver

To study the *in vivo* effects of elevated *RSPO2* expression we next generated mice that somatically expressed *RSPO2*, alone or with knockdown of *Trp53*, by hydrodynamic injection and *Fah* selection, as described above (Wangensteen et al., 2008). Control mice were injected with transposon vectors expressing *Fah*, *GFP*, and *luciferase* to track liver repopulation. Experimental mice were injected with vectors expressing *Fah*, *GFP*, *luciferase*, and *RSPO2* and/or *Trp53* shRNA (**Figure 5A).** Necropsies were performed 15 and 25 days PHI, and at 30 day intervals between day 60 and 150 PHI to assess liver mass and tumor formation. At early time points (day 15-25 PHI) mice injected with *RSPO2* had slightly enlarged livers compared to *GFP* controls (1.2-1.5-fold, $p>0.05$, data not shown). At later time points there was a prominent phenotype of enlarged liver (hepatomegaly) in mice injected with *RSPO2* with or without *Trp53* shRNA compared to

GFP controls (1.6-2.2-fold enlarged, p<0.01, **Figure 5B and C**). Mice injected with *Trp53* shRNA did not develop enlarged livers **(Figure 5B and C)**. There was no difference in liver size between male and female mice injected with RSPO2 **(Figure 5D).**

To characterize the enlarged liver phenotype, sections of grossly normal liver were stained with DAPI to quantify nuclear density. Mice injected with *RSPO2* or *RSPO2* and *Trp53* shRNA had 30-40% increased nuclear density compared to control mice receiving *GFP* or *GFP* and *Trp53* shRNA, suggesting that the enlarged livers in these mice arose due to hyperplastic growth **(Figure 5D and E)**.

At 150 days PHI, mice injected with *GFP* or *GFP* and *Trp53* shRNA had low tumor penetrance (4-5%), consistent with the background level of tumor formation in this model **(Figure 6A).** Mice injected with *RSPO2* alone had a non-significant increase in tumor penetrance (18%). However, expression of *RSPO2* combined with *Trp53* shRNA dramatically increased tumor penetrance (63% of *RSPO2/Trp53* shRNA mice vs. 5.2% of *GFP/Trp53* shRNA mice, p=3.37E-5, **Figure 6A**). *RSPO2/Trp53* shRNA-injected mice formed 1.8 tumors/mouse on average (range 1- 5). Histologic examination indicated the tumors were adenocarcinomas with areas of necrosis and lymphoid infiltrate **(Figure 6B and C).**

Expression of *RSPO2* and its effect on Wnt signaling were assessed by qRT-PCR and IHC. Nuclear localization of CTNNB1 and expression of Wnt target genes (*Axin2* and *Tcf7*) was significantly increased in livers and tumors from *RSPO2/Trp53* shRNA-injected mice compared to *GFP/Trp53* shRNA-injected controls **(Figure 7A-C).** At early time points (days 15-25 PHI) expression of Wnt target genes was also significantly

increased in the livers of mice injected with *RSPO2* compared to *GFP*-injected controls **(Supplementary Figure S6).** These data confirm that RSPO2 expression enhances Wnt signaling and promotes tumor formation in the mouse liver.

Discussion

Activation of Wnt/CTNNB1 signaling is an important oncogenic driver and potential therapeutic target in many types of cancer (Barker and Clevers, 2006; Curtin and Lorenzi, 2010; Polakis, 2012). The molecular events underlying Wnt pathway activation are heterogeneous. R-spondins are recently discovered regulators of Wnt signaling that exert their effect through LGR/RNF43/ZNRF3-dependent regulation of Wnt receptor stability (de Lau et al., 2014). RSPO1 has been shown to be a potent growth factor for intestinal epithelium *in vivo* (Kim et al., 2005). Recent work identified a potentially oncogenic role for RSPO2 and RSPO3 in CRC and other cancers (Kan et al., 2013; Seshagiri et al., 2012; Watson et al., 2013). In contrast, another study suggested that RSPO2 functions as a tumor suppressor gene in CRC (Wu et al., 2014). To address this controversy, the current study examined evidence for oncogenic activation of *RSPO2* and *RSPO3* in human colon cancer, and showed that subsets of human breast and liver cancer also overexpressed *RSPO2*. We further demonstrated that elevated *RSPO2* expression was functionally significant for Wnt signaling activation, cell proliferation, and tumorigenesis using cell line and mouse models.

We analyzed mRNA expression of *RSPO2*, *RSPO3,* and Wnt pathway target genes in primary human tumors of the colon (N=434), breast (N=1089), and liver (N=519) compared to their respective normal tissues (N=41 colon, 152 breast, and 73

liver samples) using publically available RNAseq data from the TCGA and independently acquired primary tumor samples. We found that the majority of tumors of all three types expressed significantly lower *RSPO2* and *RSPO3* levels compared to normal tissues or, in the case of *RSPO2* expression in the breast, maintained a low basal level **(Figure 1A, 2, and 4A, and Supplementary Figure S3A and S5A).** This concurs with the recent observation that *RSPO2* expression is suppressed in the majority of CRCs reported in (Wu et al., 2014) and further suggests that downregulation of *RSPO2* and *RSPO3* may be selected for during tumorigenesis in multiple Wnt-responsive tissues. Further studies are needed to address if *RSPO2/3* suppression in breast and liver cancers is due to promoter hypermethylation, as seen at the *RSPO2* promoter in CRC (Wu et al., 2014). Functional studies will also be required to determine how *RSPO2/3* suppression affects Wnt signaling in breast and liver and whether restoration of *RSPO2/3* expression is growth suppressive in RSPO-low tumors.

In contrast to the suppression of *RSPO2/3* seen in the majority of cases, we confirmed that a subset of CRC expressed highly elevated levels of *RSPO2* or *RSPO3*, associated in some cases with detection of RSPO fusion transcripts **(Figure 1A)** (Seshagiri et al., 2012; Shinmura et al., 2014). In this cohort, RSPO-high CRCs occurred at a lower frequency (2%) than in previous reports. Overexpression of *RSPO2* was also observed in 11% of breast and 12% of liver cancers, while *RSPO3* overexpression was less common in these tissues **(Figure 2A and 4A, Supplementary Figure S3A and S5A).** We defined tumors as "RSPO-high" if they expressed >4-fold elevated mRNA levels compared to normal tissues. As a consequence, the absolute level of *RSPO*

expression defined as "RSPO-high" varied by tissue type. For example, the average expression of *RSPO2* in RSPO2-high colon tumors was 139-fold higher than in RSPO2-high breast tumors. This difference in *RSPO2* expression level may reflect tissue-specific differences in responsiveness to R-spondins, or differences in the optimal level of Wnt activation required to promote tumorigenesis in different tissues. Consistent with this idea, analysis of the tumor spectrum initiated by different mutations of *Apc* in mice has show that *Apc* mutations that activate the Wnt pathway to a lesser degree promote mammary tumors, while mutations associated with greater Wnt pathway activation promote intestinal tumors (Bakker et al., 2013; Gaspar et al., 2009).

RSPO fusion transcripts were not detected in breast or liver tumors, raising the question of how R-spondins are upregulated in these tissues. We investigated the possibility that *RSPO2/3* were overexpressed as a consequence of activated Wnt signaling initiated by other events, constituting a positive feedback regulatory loop (Kazanskaya et al., 2004). We found that *RSPO2* and *RSPO3* were not transcriptionally upregulated in breast epithelial cells treated with recombinant Wnt3a ligand or in murine liver tissues expressing a constitutively active *CTNNB1* transgene **(Supplementary Figure S4B and C).** Rather, *Rspo2* and *Rspo3* expression were unchanged in grossly normal liver expressing activated *CTNNB1*. In *CTNNB1*-initiated tumors, *Rspo3* expression was significantly decreased, reminiscent of the pattern seen in human liver tumors.

Wnt target gene expression was increased in RSPO-high tumors compared to normal tissues **(Figure 1B and 4B; Supplementary Figure S1, S3C, and S5)**. Although

RSPO-high CRC samples retained wild type *APC* as previously reported (Seshagiri et al., 2012; Shinmura et al., 2014) in HCC RSPO-high status significantly co-occurred with mutations in *CTNNB1*. In these cases, multiple genetic lesions in the Wnt pathway within one tumor may represent selection for the "just right" level of pathway activation (Leedham et al., 2013; Segditsas and Tomlinson, 2006) or may represent the presence of multiple, potentially cooperating clones within a tumor (Halberg and Dove, 2007; Merritt et al., 1997). An alternate explanation for the lack of mutual exclusivity could be that R-spondin overexpression is selected based on regulation of other pathways in addition to Wnt/CTNNB1 signaling. Indeed, R-spondins have been shown to regulate the "non-canonical" Wnt/planar cell polarity (PCP) pathway (Glinka et al., 2011a; Ohkawara et al., 2011). Dysregulation of Wnt/PCP has been associated with cancer progression and metastasis (Wang, 2009). Further research will be required to determine if regulation of Wnt/PCP or other pathways is a function of RSPO overexpression in cancer.

Wu et al. proposed that downregulation of LGR5 is required in RSPO-high tumors, in order to prevent negative feedback inhibition of Wnt signaling through LGR5 (Wu et al., 2014). Our observations suggest LGR5 downregulation is not a requirement in RSPO-high tumors. LGR5 was highly expressed in 7 of 8 RSPO-high CRCs, consistent with its role as a Wnt target gene **(Supplementary Figure S2).** Similarly, LGR5 levels were significantly elevated in RSPO-high breast and liver tumors compared to their respective normal tissues (data not shown and **Supplementary Figure S5C).** High LGR5 expression in these tissues is a marker of activated Wnt signaling (Van der Flier et al., 2007). These data do not support the negative feedback loop model proposed by Wu et al.

However, further investigation may reveal other mechanisms by which such a feedback loop is subverted in RSPO-high tumors.

We investigated the functional significance of elevated RSPO2 expression in the breast and liver using cell line and mouse models. Prior literature established that overexpression of RSPO2 promoted tumor formation of transplanted mouse mammary epithelial cells (Klauzinska et al., 2012). We took the reverse approach of identifying an RSPO2-high human breast cancer cell line for loss of function experiments. The BT-549 cell line was derived from a basal subtype tumor and expresses 33-fold elevated RSPO2 levels compared to the average expression of 60 breast cancer cell lines **(Figure 3B).** BT-549 is an appropriate cell line model, as ~40% of RSPO-high breast cancers are basal subtype **(Supplementary Figure S3B).** Depletion of RSPO2 in BT-549 cells decreased expression of Wnt target genes and reduced cell proliferation **(Figure 3C and D).** This suggests that RSPO-high basal breast cancers rely on RSPO2 expression for growth and oncogenic signaling, and that RSPO-targeted therapy may be effective in these tumors. BT-549 may represent a useful cell line model for the 11% of breast cancers and 25% of basal subtype breast cancers that express elevated RSPO2 levels and may respond to drugs inhibiting RSPO or Wnt signaling.

We developed a model of *RSPO2* overexpression in the mouse liver. High *RSPO2* levels in the mouse liver promoted a hyperplastic, enlarged liver phenotype **(Figure 5B-E).** This phenocopies a similar degree of hepatomegaly reported in multiple models of *CTNNB1* activation in the liver (Harada et al., 2002; Stein et al., 2011). Indeed, we found Wnt signaling was activated by *RSPO2* expression in liver, indicated by increased

nuclear CTNNB1 and increased expression of Wnt target genes **(Figure 7B and C).** RSPO2-driven liver enlargement is also consistent with the mitogenic effect of *RSPO1* expression seen in intestinal epithelium (Kim et al., 2005).

Expression of *RSPO2* alone in the mouse liver did not significantly increase tumor formation in this model **(Figure 6A).** However, *RSPO2* overexpression combined with depletion of *Trp53* gave rise to tumors in 63% of mice by day 150 PHI, compared to only 5% of mice with *Trp53* depletion alone **(Figure 6A).** RSPO2/shp53 tumors were classified as adenocarcinomas with features of lymphoid infiltration and necrosis. These data strongly suggest that RSPO2 is oncogenic in the liver, but that additional mutations or alterations are required for tumor formation. This *in vivo* model of RSPO2-driven liver tumor formation will be a valuable tool for future studies and preclinical testing of RSPO and Wnt targeted therapies.

In sum, these data indicate that R-spondin overexpression can activate canonical Wnt signaling and promote tumor formation in the breast and liver as well as the colon. This raises the enticing possibility that R-spondins could be used as biomarkers or therapeutic targets in the diagnosis and treatment of multiple types of cancer. However, many important questions remain. Future studies should be designed to determine if RSPO-high tumors require RSPO expression for tumor maintenance and whether therapeutic inhibition of R-spondins themselves or downstream components of the Wnt pathway can an effective treatment strategy for R-spondin driven tumors. Future studies should also investigate whether activation of other pathways, including non-canonical Wnt pathways, contribute to RSPO-driven tumorigenesis.

Methods

Acquisition of RNAseq and somatic mutation data from TCGA

RNAseq and somatic mutation data for colon, breast, and liver tumors were extracted from The Cancer Genome Atlas (TCGA) data matrix (https://tcga-data.nci.nih.gov/tcga/dataAccessMatrix.htm), and updated as of July 2014. These data were generated by the TCGA Research Network (http://cancergenome.nih.gov/).

Primary breast tissue RNA isolation, cDNA synthesis, and qRT–PCR

Normal/tumor matched pairs were obtained from the University of Minnesota Tissue Procurement facility. Briefly, cDNA synthesis and qPCR were performed as described (Burns et al., 2013; Refsland et al., 2010). Tissue RNA was from 100 mg flash-frozen tissue disrupted by a 2-h water bath sonication in 1 ml of Qiazol Lysis Reagent (RNeasy, Qiagen). Cell RNA was made using Qiashredder (RNeasy, Qiagen). qPCR was performed on a Roche Lightcycler 480 instrument. The *RSPO2* and *RSPO3* primer sets were designed using the ProbeFinder version 2.48 for the Human Universal ProbeLibrary (UPL) from Roche Applied Science. The housekeeping gene TBP was used for normalization (Burns et al., 2013; Refsland et al., 2010). Primer and probe sequences are listed in **Supplementary Table S1**.

Primary liver tissues and microarray gene expression analysis

Gene expression profiling of a total of 319 primary HCCs, 199 chirrhotic or premalignant samples, and 23 normal liver samples were analyzed for this study as reported in (Lachenmayer et al., 2012). HCC samples were classified according to subtypes defined

in (Chiang et al., 2008). Briefly, five gene expression classes were defined by hierarchical clustering for a training set of HCCs. Marker genes were identified that were differentially expressed in each class (CTNNB1, IFN, proliferation, Poly7, and unannotated). Expression of marker genes was used to classify subsequent HCC samples (Chiang et al., 2008; Lachenmayer et al., 2012).

Tissue culture reagents and cell lines

BT549, MCF10A, and MCF7 cells were obtained from the American Type Culture Collection (ATCC). BT549 and MCF7 cells were cultured in RPMI 1640 medium supplemented with 10% FBS and 0.023IU/mL bovine insulin. MCF10A cells were cultured as in (Debnath et al., 2003) in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 5% horse serum, 20 ng/ml EGF, 0.5 ug/ml hydrocortisone, 100ng/ml cholera toxin, 10 ug/ml insulin and 1% penicillin/streptomycin. All cells were grown on tissue culture-treated plates under standard conditions of 37°C and 5% CO2.

In vitro gene knockdown and overexpression and proliferation assays

For RSPO2 knockdown experiments, plasmids encoding lentiviral shRNAmirs against RSPO2 or a non-silencing control shRNAmir were purchased from OpenBiosystems. Lentiviral particles were produced in 293T cells using the Trans-Lentiviral Packaging Kit (Thermo Scientific). For overexpression experiments, lentiviral expression vectors were cloned with RSPO2 or dsRed regulated by a CAGGS promoter and followed by an IRES-GFP to monitor transduction efficiency. Lentiviral particles were produced in 293T cells by co-transfection with helper plasmids. For both knockdown and overexpression

experiments, viral supernatant was collected after 24 hours of virus production, cleared, and applied to transduce experimental cells with 12 ug/mL polybrene overnight. Transduced cells were selected with 1 ug/mL puromycin. Knockdown efficiency and overexpression levels were assayed by qRT-PCR.

Mouse strains, hydrodynamic injection, and liver analysis

All animal work was conducted according to an institutionally approved animal welfare protocol. Mouse strains and hydrodynamic injection protocols were as described (Bell et al., 2007; Keng et al., 2011). Briefly, doubly transgenic mice (*Fah*$^{-/-}$*; Rosa26-SB11*) received 20 ug of plasmid DNA by hydrodynamic tail vein injection. Prior to injection mice were maintained on 2-(2-nitro-4-trifluoromethylbenzoyl)- 1,3-cyclohexanedione (NTBC) drinking water, but NTBC was withdrawn immediately post-injection. At the indicated time points, mice were euthanized with CO2 and whole livers were removed, weighed and analyzed for gross tumor formation. Macroscopic hyperplastic nodules were counted. Nodules at least 1mm in size were isolated for RNA extraction. Histological sections were also taken from larger nodules for hematoxylin & eosin (H&E) staining or immunohistochemistry (IHC) analysis. H&E stained sections were reviewed independently by two pathologists. IHC for CTNNB1 was performed as in (Watson et al., 2013) using β-Catenin (6B3) Rabbit mAb #9582 (Cell Signaling) at 1:100 as a primary antibody.

Quantitative reverse transcriptase PCR (qRT-PCR)

RNA was isolated from cell lines and mouse liver tissues using the PureLink RNA Mini Kit according to manufacturer protocol (Ambion). RNA samples were analyzed by gel electrophoresis to assess quality and treated with DNase to remove contaminating genomic DNA (Turbo DNA-free Kit, Ambion). Complementary DNA was synthesized from 1 ug template RNA per sample using random hexamer primers (SuperScript III First-Strand Synthesis System, Invitrogen). qRT-PCR reactions were conducted with FastStart Universal SYBR Green Master mix (Roche), using 0.5 ul of cDNA template per 25 ul reaction. Primer sequences for qRT-PCR reactions are listed in **Supplementary Table S1**. Data were analyzed by normalization to ACTB using the following equation: relative expression = ((2^(CT_ACTB))/((2^(CT_GOI)).

Authors' Contributions

Caitlin B. Conboy – Conceived and designed this study, performed all experiments and analyses except as otherwise noted, and wrote this manuscript.

Michael Burns, PhD – Performed qRT-PCR analysis of primary human breast cancer and normal breast samples.

Sara Toffanin, PhD / Josep Llovet, PhD – Performed microarray analysis of primary human liver cancer, premalignant, and normal liver samples.

Barbra R. Tschida – Performed hydrodynamic injections in Fah-null mice.

David Hu – Assisted with necropsies and tissue processing for liver analyses. Assisted with cell culture experiments with BT-549 and MCF10A.

German Velez Reyes – Assisted with qRT-PCR analysis of tissues from Fah-null mice

Juan E. Abrahante, PhD – Analyzed RNAseq data for expression of RSPO fusion transcripts

Mike Linden, MD, PhD/ Khalid Amin, MD = Pathological review of H&E-stained tumor sections

Tim K. Starr, PhD – Contributed to conception and design of this study and review of this manuscript

David A. Largaespada - Contributed to conception, design, and supervision of this study and review of this manuscript

Grant Support

This work was supported by grants to DAL (R01 CA134759, R01 CA113636) and CBC (T32 GM008244, F30 CA171547).

Acknowledgments

The results published here are in part based upon data generated by the TCGA Research Network.

Figure 1: RSPO2 and RSPO3 are highly expressed in rare colorectal tumors and associated with expression of RSPO fusion transcripts and activated Wnt signaling. (A) RSPO2/3 mRNA levels in 41 normal colon tissues and 434 CRC tissues from TCGA RNA-Seq. RSPO fusion status is indicated by symbol shape and color: Black circle = not assessed, grey triangle = data not collected, green triangle = RSPO2 fusion detected, red triangle = RSPO3 fusion detected, open triangle = no RSPO2 or RSPO3 fusion detected. (B) mRNA expression of Wnt target genes (AXIN2, TCF7, and LEF1) in 41 normal colon tissues and 8 "RSPO-high" CRC tissues with R-spondin expression elevated >4-fold compared to normal colon. Symbol colors: orange = RSPO2-high tumor, blue = RSPO3-high tumor.

Figure 1

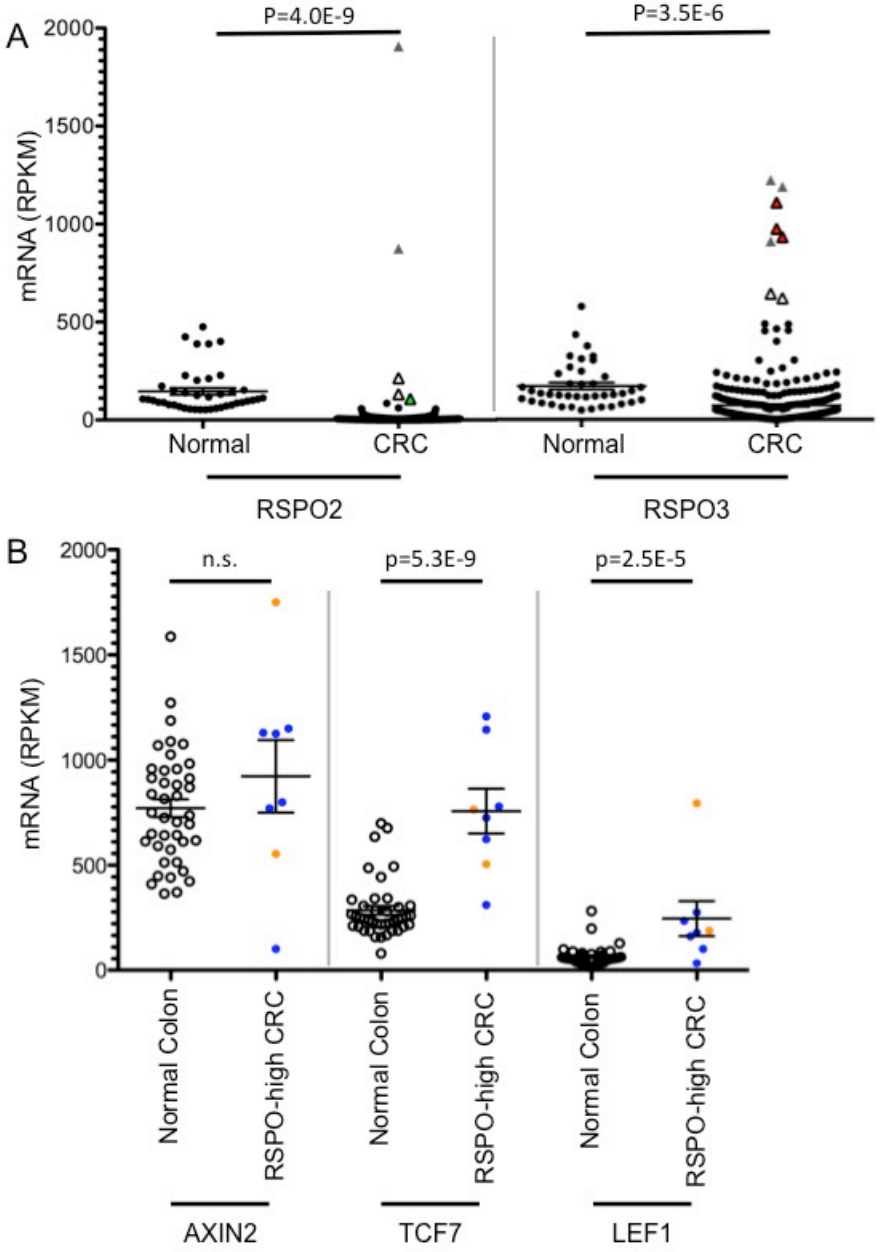

A
P=4.0E-9
P=3.5E-6
mRNA (RPKM)
2000
1500
1000
500
0
Normal
CRC
Normal
CRC
RSPO2
RSPO3
B
n.s.
p=5.3E-9
p=2.5E-5
mRNA (RPKM)
2000
1500
1000
500
0
Normal Colon
RSPO-high CRC
Normal Colon
RSPO-high CRC
Normal Colon
RSPO-high CRC
AXIN2
TCF7
LEF1

Figure 2: RSPO2 is highly expressed in a subset of breast tumors. RSPO2 mRNA levels in tumor (red circles) and matched normal tissue (blue circles) from 41 breast cancer patients. mRNA levels were measured using qRT-PCR and normalized using TBP mRNA levels. Yellow line indicates mRNA expression level 4-fold higher than the normal sample average.

Figure 2

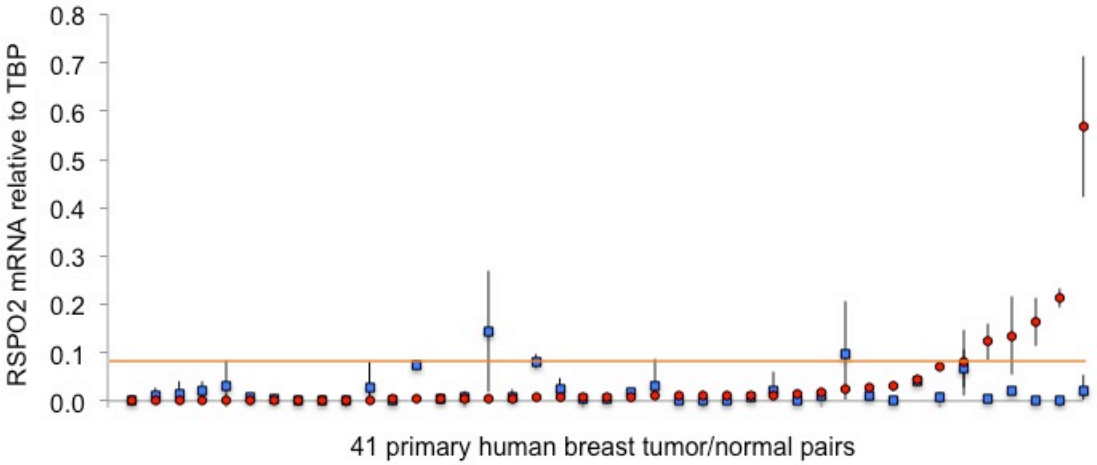

RSPO2 mRNA relative to TBP
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
41 primary human breast tumor/normal pairs

Figure 3: RSPO2 regulates Wnt signaling and proliferation in breast cancer cell lines. (A) Wnt target gene mRNA levels in MCF10A cells transduced with lentivirus expressing RSPO2 or GFP. mRNA levels were measured by qRT-PCR and normalized using ACTB levels (B) RSPO2 mRNA expression in 58 breast cancer cell lines. The BT549 cell line is indicated by data label. Gene-centric RMA-normalized mRNA expression data were extracted from the Cancer Cell Line Encyclopedia (Barretina J, et al. Nature, 2012). (C) Knockdown of RSPO2 in BT549 cells decreases expression of Wnt/beta-catenin target genes. BT-549 cells were transduced with lentivirus encoding shRNA to RSPO2 or a nonsilencing (Nons) control. Knockdown efficiency and expression of Wnt target genes were quantified by qRT-PCR and normalized with beta-actin to the Nonsilencing control. (D) Knockdown of RSPO2 expression decreases proliferation of BT-549 cells. Proliferation was measured by MTS absorbance for three biological replicates per sample.

Figure 3

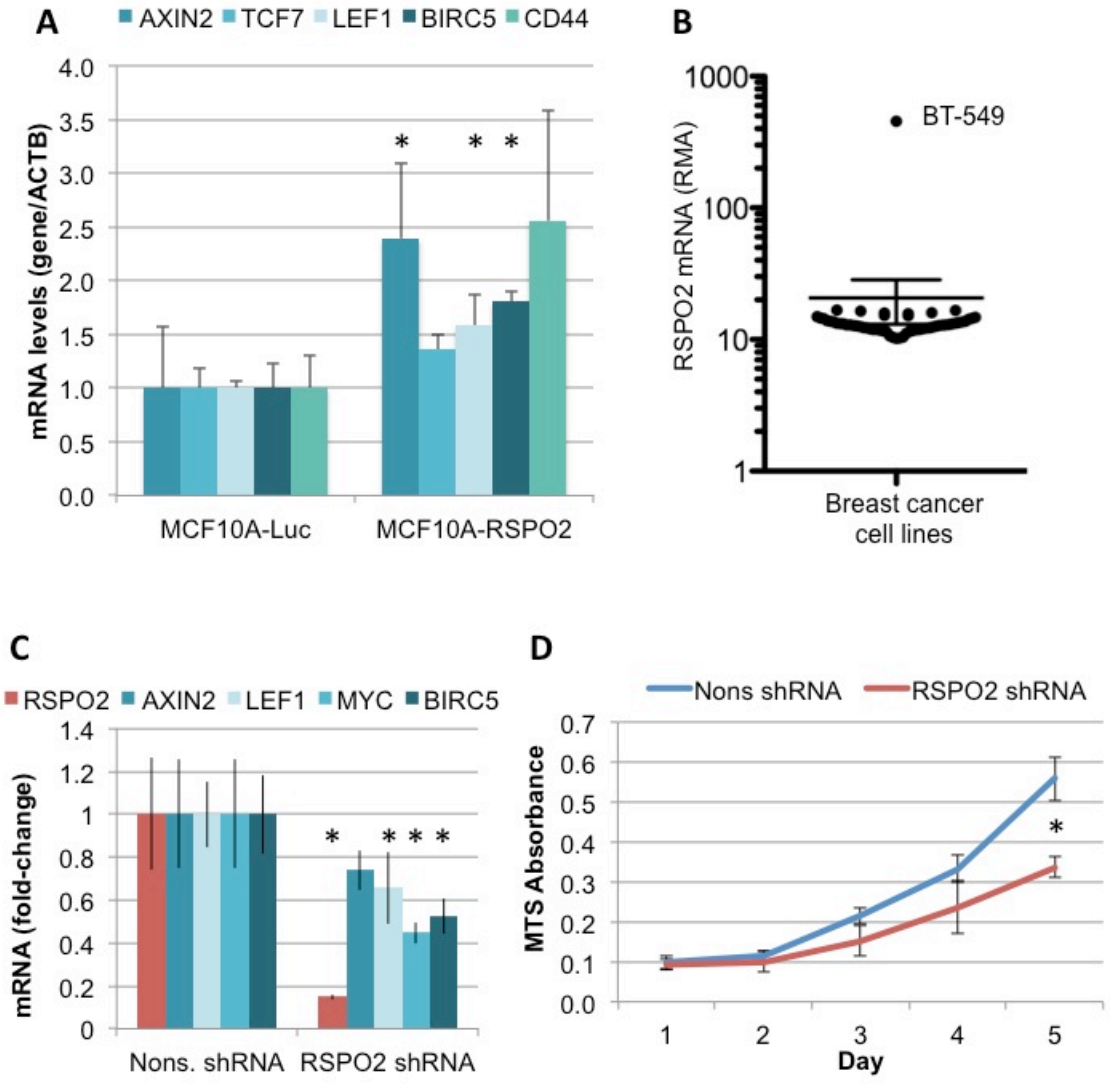

A
AXIN2
TCF7
LEF1
BIRC5
CD44
mRNA levels (gene/ACTB)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
* *
MCF10A-Luc
MCF10A-RSPO2
B
1000
100
10
1
RSPO2 mRNA (RMA)
BT-549
Breast cancer cell lines
C
RSPO2
AXIN2
LEF1
MYC
BIRC5
mRNA (fold-change)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
* * *
Nons. shRNA
RSPO2 shRNA
D
Nons shRNA
RSPO2 shRNA
MTS Absorbance
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
*
1
2
3
4
5
Day

Figure 4: RSPO2 is highly expressed in the subset of HCC with activated Wnt signaling. (A) RSPO2 mRNA expression was determined by microarray analysis (left, Affymetrix U133 2.0) in normal liver (n=10), cirrhosis (n=13), Low-grade dysplastic nodules (LGDN, n=10), high grade dysplastic nodules (HGDN, n=8), and liver cancer (HCC, n=91). Analysis of RSPO2 expression by molecular subclass (right) showed that RSPO2 expression was significantly elevated in tumors with a Wnt-active gene expression signature (CTNNB1, n=25) compared to other classes (IFN, n=18; Proliferation, n=23; poly 7, n=9; other/unannotated, n=17).

Figure 4

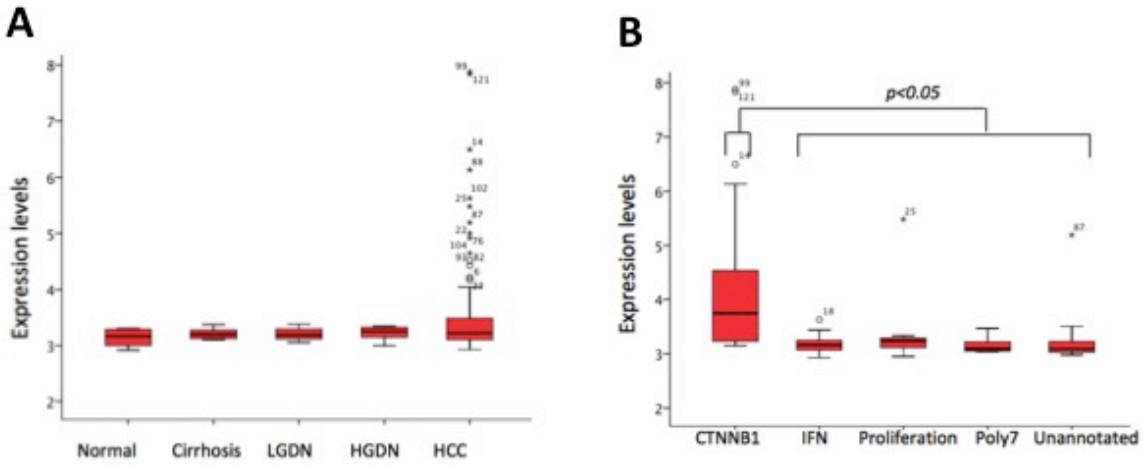
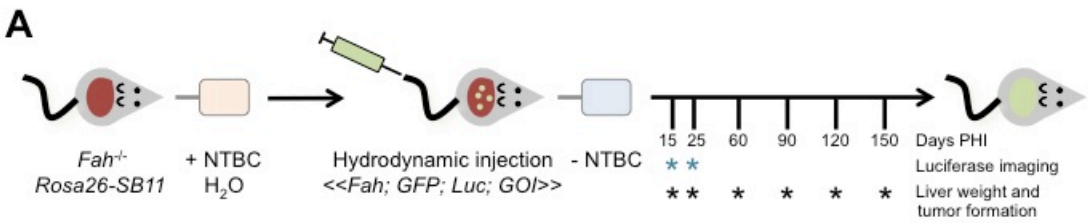

A
Expression levels
Normal
Cirrhosis
LGDN
HGDN
HCC
B
p<0.05
Expression levels
CTNNB1
IFN
Proliferation
Poly7
Unannotated

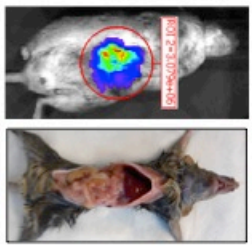
Figure 5: RSPO2 expression drives hyperplastic growth in the mouse liver.

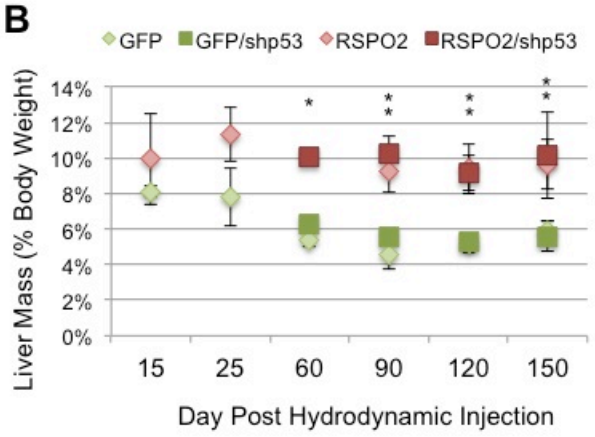
(A) Experimental diagram: Fah-/- mice maintained on NTBC drinking water were hydrodynamically injected with plasmid DNA encoding Fah (selectable marker), luciferase (reporter), and the indicated combination of transgenes from an *SB* transposon-based expression construct. Following hydrodynamic injection, NTBC was withdrawn and modified hepatocytes selectively repopulated the liver. *In vivo* luciferase imaging was used to confirm expression of transgenes in repopulated livers. Mice were necropsied and liver mass and tumor formation were assessed on days 15 and 25 post-hydrodynamic injection (PHI) and then at 30 day intervals from 60 – 150 days PHI. Table indicates number of mice injected with each set of transgenes (B) Average liver mass was significantly increased in mice injected with RSPO2, alone or in combination with Trp53 shRNA (shp53) compared to GFP or GFP/shp53 control mice at all time points 60-150 days PHI. The number of mice included in each time point are listed in part A. *$p<0.05$. (C) At 150 days post-hydrodynamic injection, RSPO2 expression significantly increased average liver mass in both male and female mice, while shp53 had no effect on liver size. **$p<0.005$. (D) Sections of grossly normal liver from mice at 150 days post hydrodynamic injection were stained with DAPI to visualize nuclear density. (E) Nuclei per 40x field were quantified for 6 livers per group. Nuclear density increased 30-40% with RSPO2 expression, consistent with hyperplastic growth. *$p<0.05$.

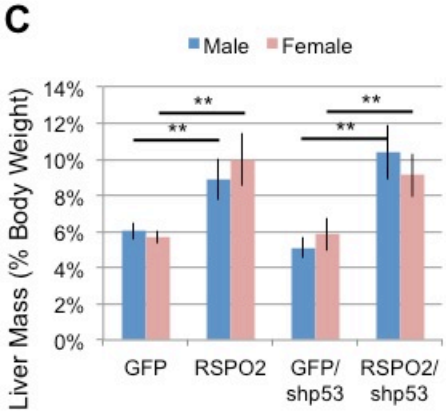
Figure 5

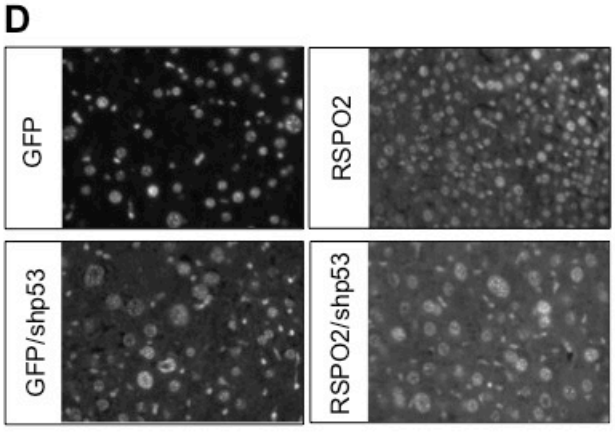


Transgenes encoded by *SB* transposon	# of mice evaluated per time point (days PHI)					
	15	25	60	90	120	150
Fah, Luc, GFP	2	2	5	6	12	20
Fah, Luc, GFP, shp53	--	--	3	4	6	23
Fah, Luc, GFP, RSPO2	2	2	--	2	4	22
Fah, Luc, GFP, RSPO2, shp53	--	--	2	3	2	20
Fah, Luc, CTNNB1-S33Y	--	--	--	4	1	--









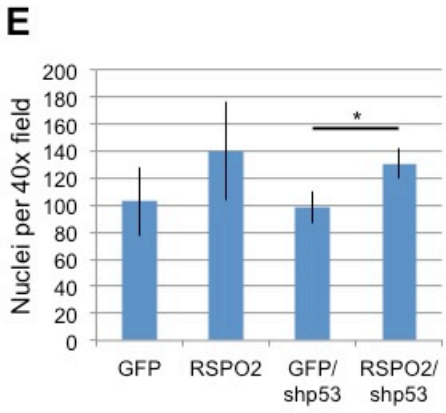


Figure 6: RSPO2 promotes tumor formation in the mouse liver. (A) Tumor penetrance in *Fah*-null mice injected with *GFP*, *RSPO2*, *GFP* plus shRNA against *Trp53* (*GFP*/*shp53*), or *RSPO2* plus shRNA against *Trp53* (*RSPO2*/*shp53*) at 150 days PHI. (B) Gross images of representative livers from *RSPO2*/*shp53*-injected mice at 150 days PHI. Tumors are indicated by blue arrowheads. (C) Representative hematoxylin & eosin-stained section of a liver tumor from an *RSPO2*/*shp53*-injected mouse. Histopathological analysis determined that tumors in RSPO2/shp53-injected mice were hepatocellular carcinomas with features of lymphoid infiltration and necrosis. T, tumor; N, necrosis. 10X magnification.

Figure 6

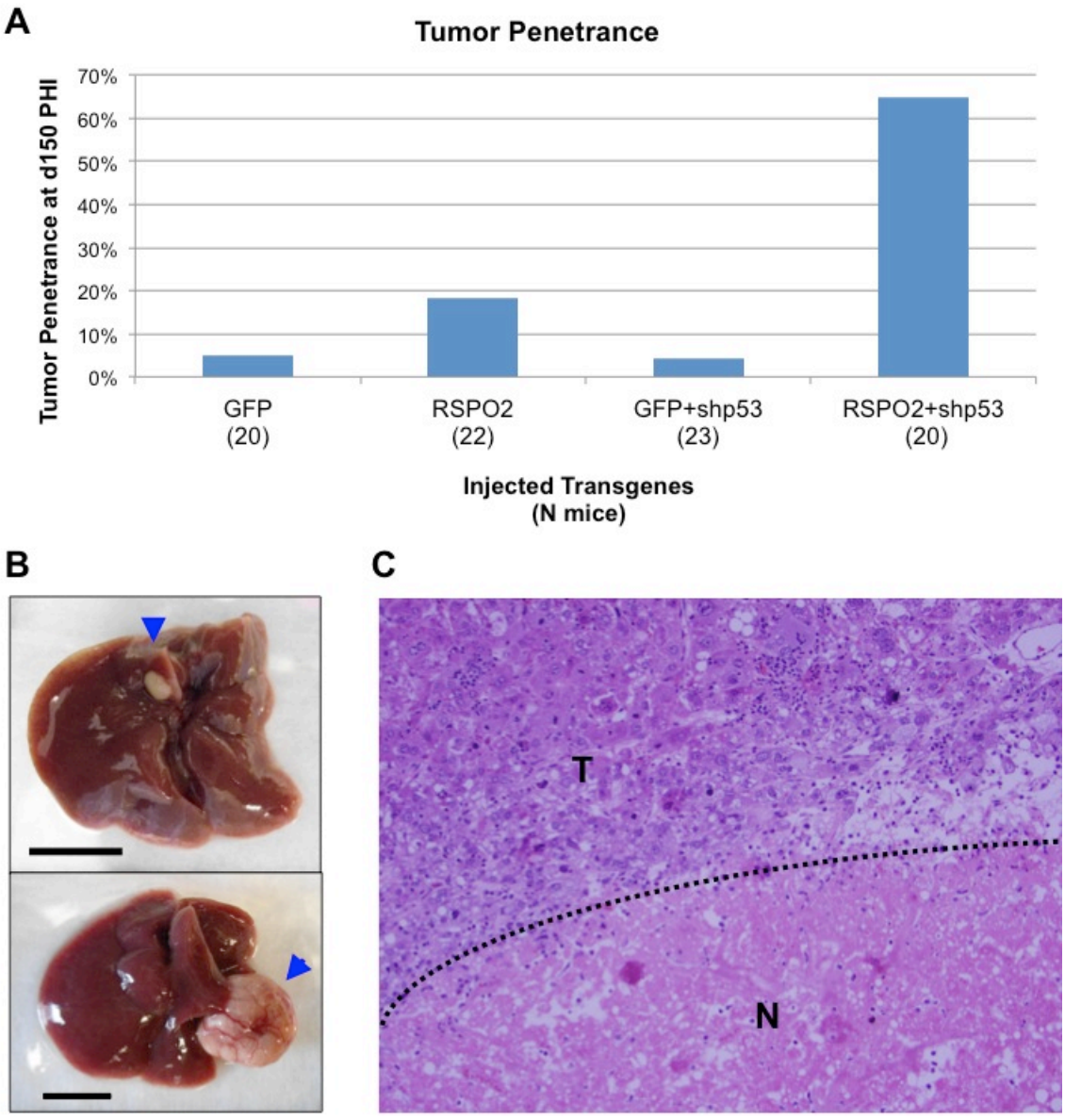
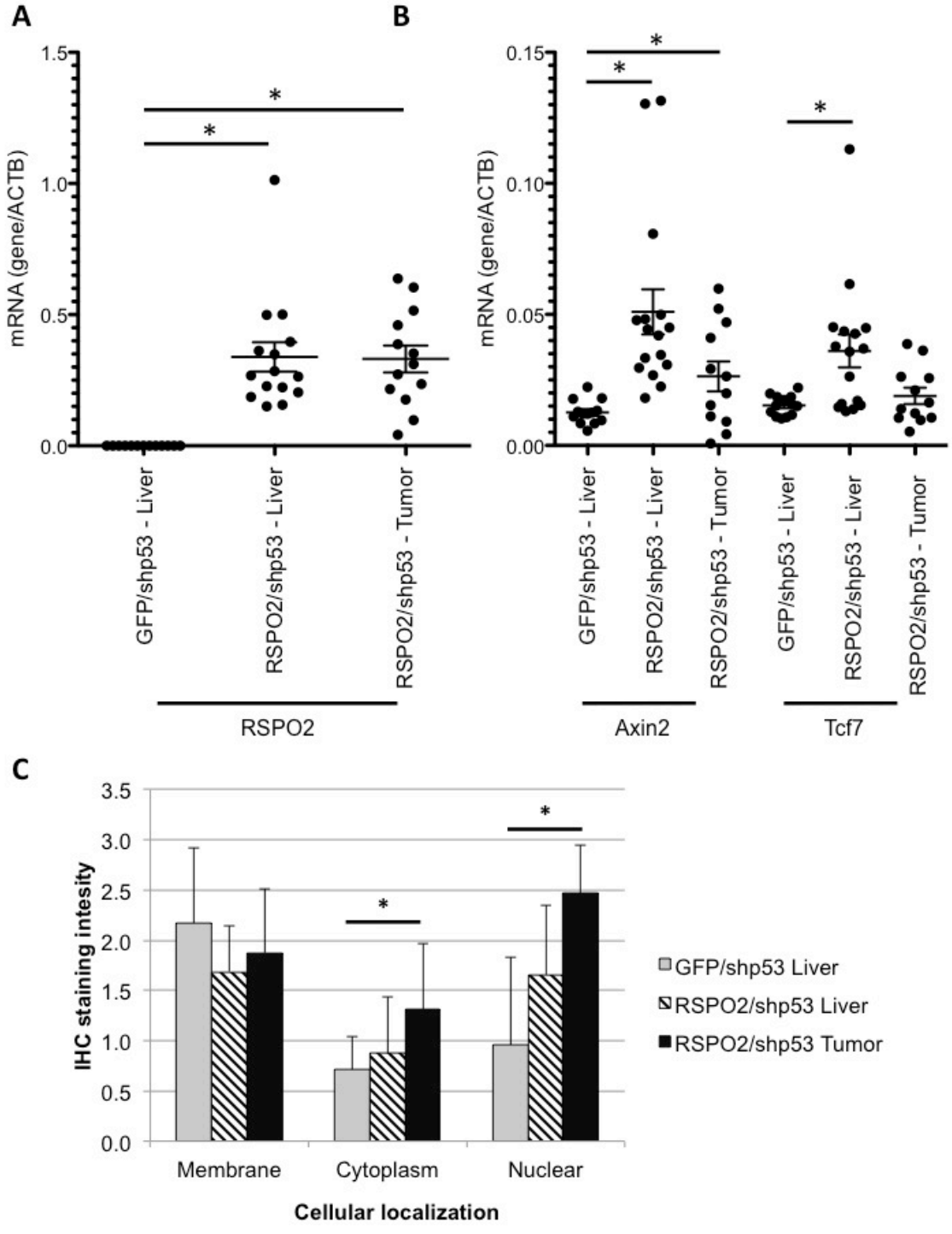

A
Tumor Penetrance
Tumor Penetrance at d150 PHI
70%
60%
50%
40%
30%
20%
10%
0%
GFP
(20)
RSPO2
(22)
GFP+shp53
(23)
RSPO2+shp53
(20)
Injected Transgenes
(N mice)
B
C
T
N

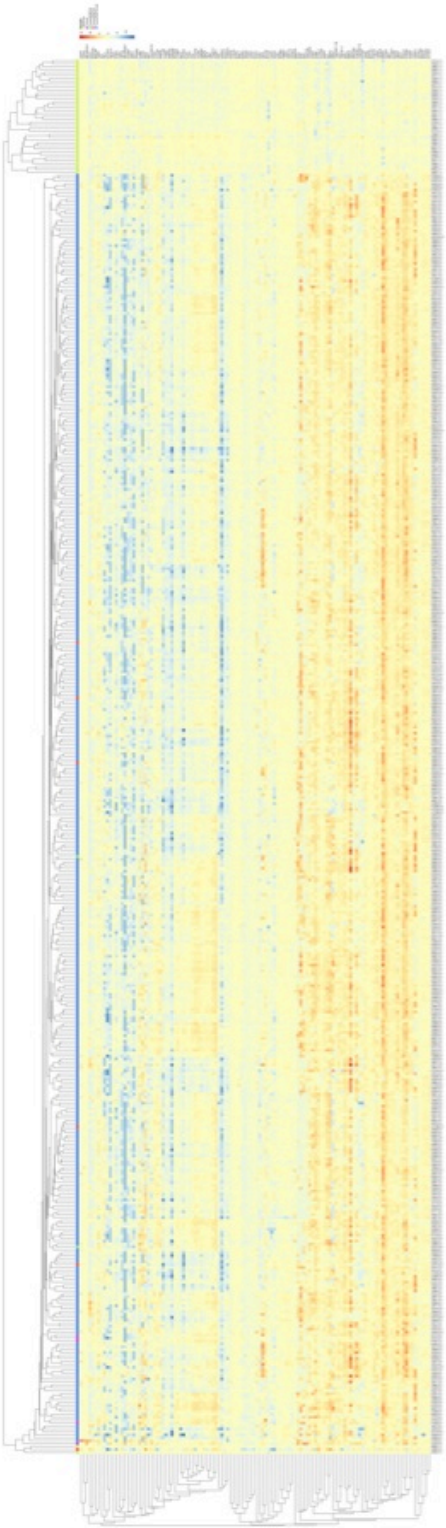
Figure 7: RSPO2 increases Wnt signaling in the mouse liver. (A,B) Expression of RSPO2 (A) and endogenous Wnt target genes (B) were assessed by qRT-PCR in grossly normal liver and tumor samples from mice injected with GFP/Trp53 shRNA or RSPO2/Trp53 shRNA. Wnt target genes were significantly elevated in murine liver and tumors expressing RSPO2. (C) Beta-catenin protein expression and localization were examined by immunohistochemical staining in normal liver and tumor samples from mice injected with GFP/Trp53 shRNA or RSPO2/Trp53 shRNA. Cytoplasmic and nuclear expression of CTNNB1 were elevated in tumors from RSPO2/shp53-injected mice. *$p<0.05$.

Figure 7


A
B
C
mRNA (gene/ACTB)
1.5
1.0
0.5
0.0
0.15
0.10
0.05
0.00
*
GFP/shp53 - Liver
RSPO2/shp53 - Liver
RSPO2/shp53 - Tumor
RSPO2
Axin2
Tcf7
IHC staining intesity
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Membrane
Cytoplasm
Nuclear
Cellular localization
GFP/shp53 Liver
RSPO2/shp53 Liver
RSPO2/shp53 Tumor

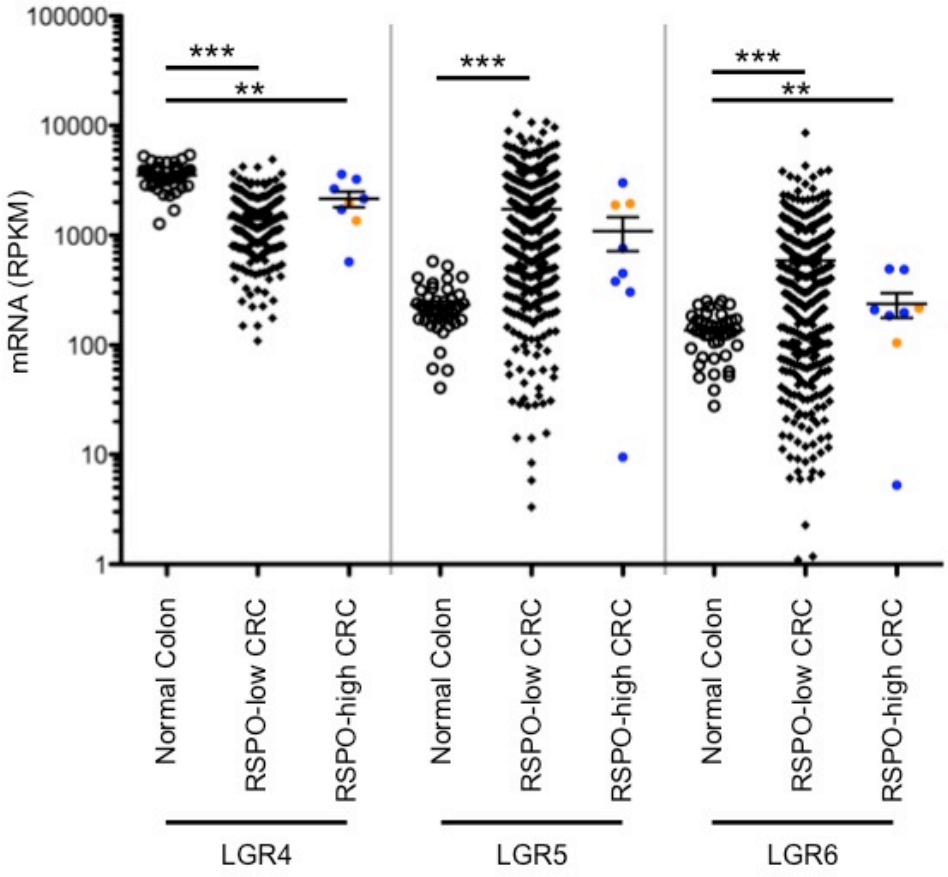
Supplementary Figure S1: Wnt target gene expression in CRC and normal colon samples.

Supplementary Figure S1



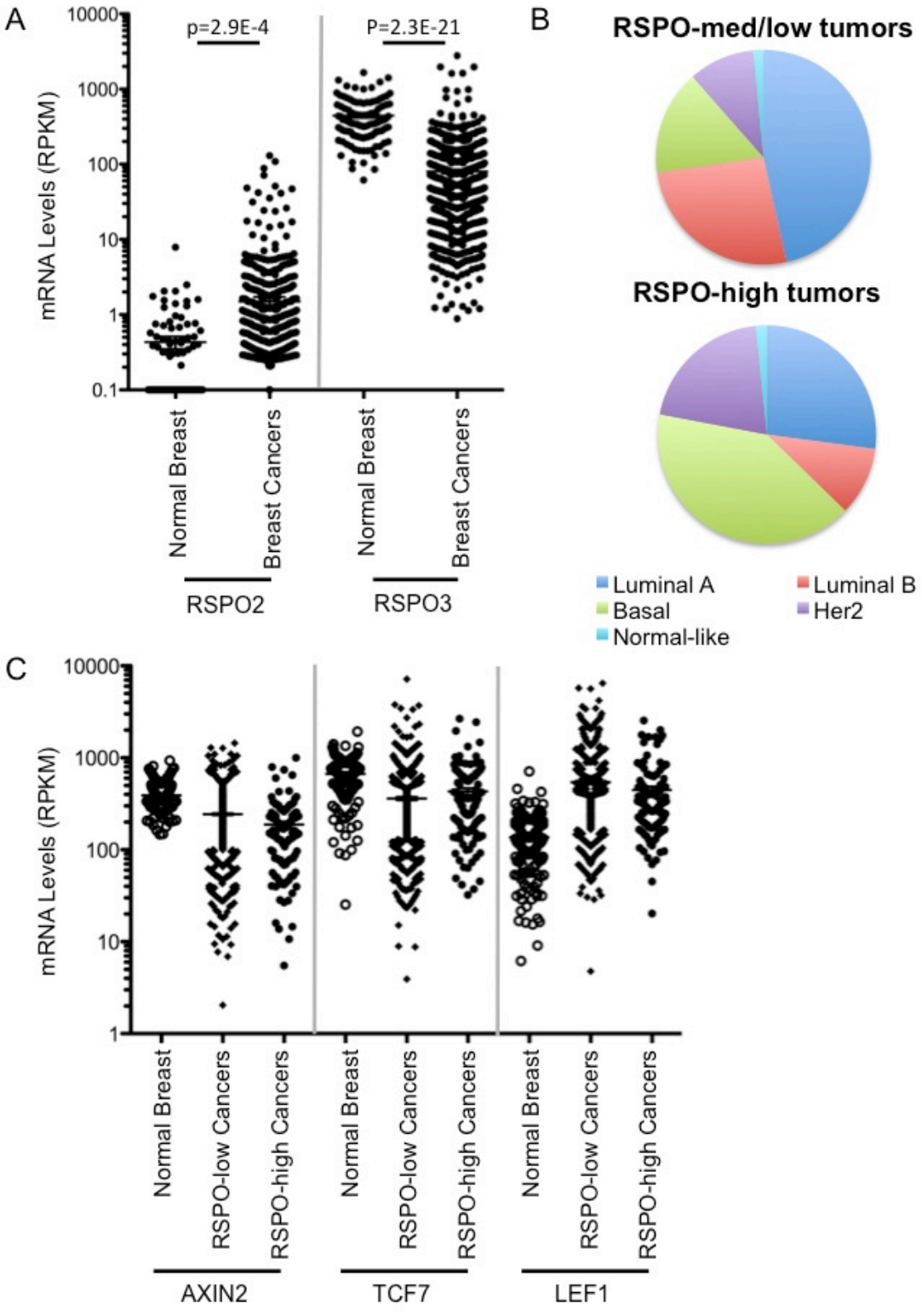
Supplementary Figure S2: Expression of LGR-family receptors in normal colon and CRC samples. LGR4, LGR5 and LGR6 mRNA levels were analyzed for 41 normal colon tissues, 417 RSPO-Low CRCs, and 8 RSPO-High CRCs. Data from TCGA RNA-Seq. Symbol color: orange = RSPO2-high, blue = RSPO3 high. ***, $p<0.0005$; **, $p<0.005$.

Supplementary Figure S2



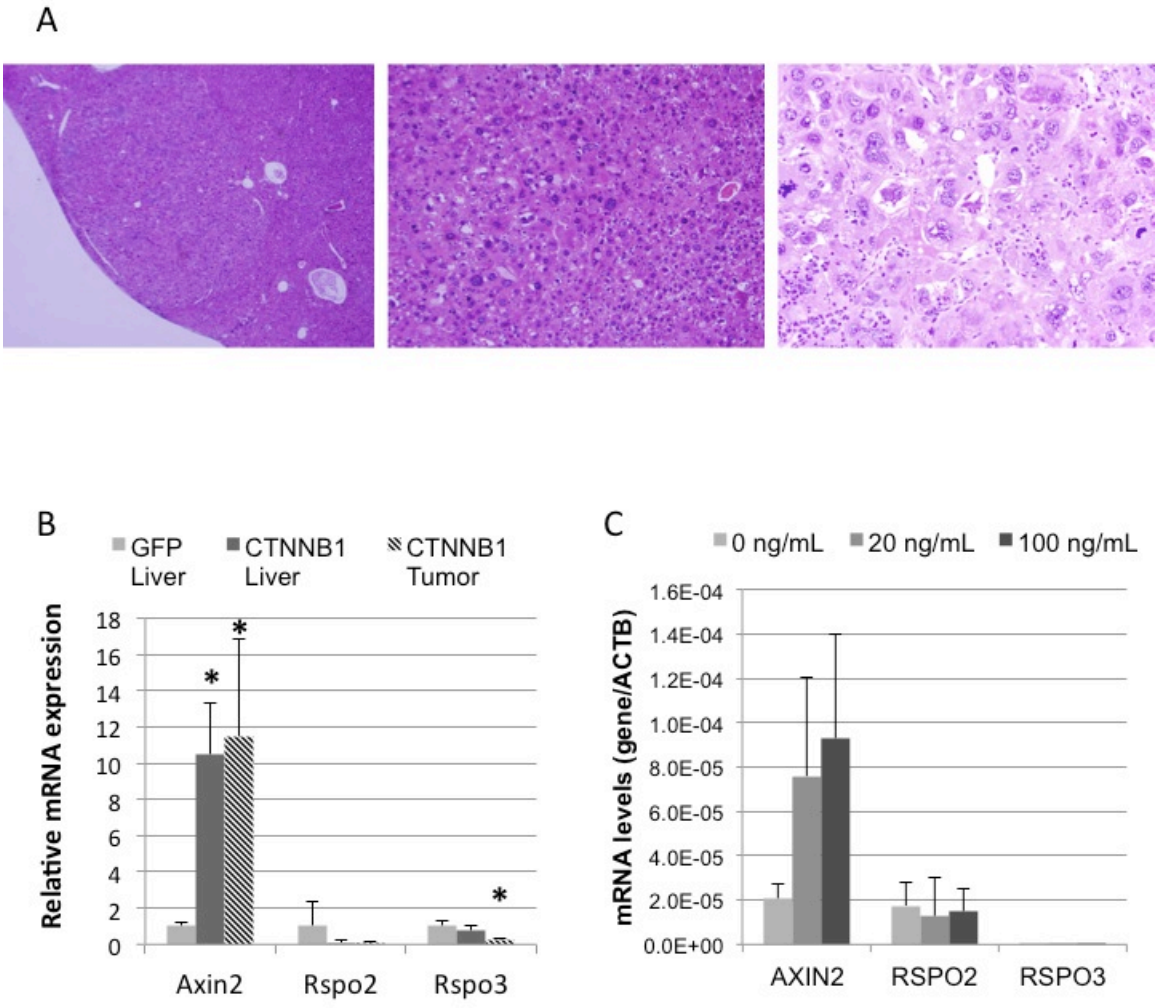
Supplementary Figure S3: RSPO2 is elevated in a subset of breast cancers associated with the basal and HER2 subtypes. (A) RSPO2 and RSPO3 mRNA levels in 1048 breast cancer and 111 normal breast tissue samples from TCGA RNA-Seq. (B) Molecular subtype distribution of 462 tumors with medium or low RSPO expression and 59 breast tumors with elevated RSPO expression. Elevated expression of RSPO2 is correlated with basal subtype (p=2.07E-5) and Her2 subtype (p=0.0259) and negatively correlated with luminal A subtype (p=0.0052) and luminal B subtype (p=0.0093). (C) mRNA expression of Wnt target genes (AXIN2, TCF7, and LEF1) in 111 normal breast tissues 842 RSPO-low breast tumors, and 122 RSPO-high breast tumors, defined by RSPO mRNA expression elevated >4-fold compared to normal breast.

Supplementary Figure S3



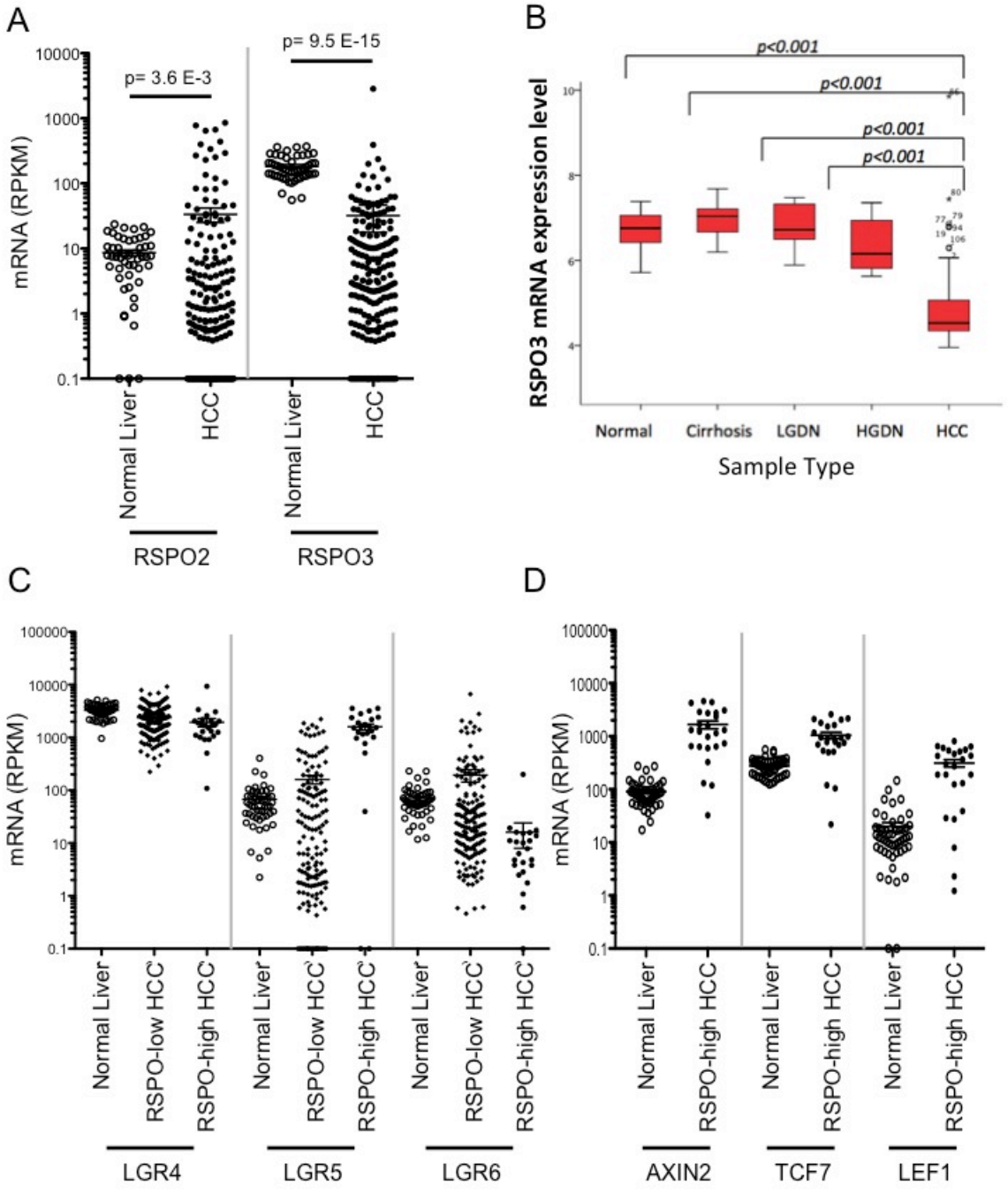
Supplemental Figure S4: RSPO2 and RSPO3 are not transcriptionally activated by Wnt/CTNNB1 in normal liver, liver tumors, or MCF10A breast epithelial cells. (A) Representative hematoxylin & eosin-stained sections of a liver tumor from an *Fah*$^{-/-}$ mouse injected with activated beta-catenin (*CTNNB1-S33Y).* (Left) 2X low power view of a tumor nodule in a background of liver dysplasia. (Middle) 10X view of background hepatocytes with dysplasia. (Right) 20X high power view of a tumor nodule with atypical nuclei and lymphoid infiltrate. (B) Expression of *Axin2*, *Rspo2*, and *Rspo3* in grossly normal liver and tumor tissue from *Fah*$^{-/-}$ mice injected with *GFP* or *CTNNB1-S33Y*. Gene expression was analyzed for eight samples of normal liver per group and seven tumors induced by *CTNNB1-S33Y*. *Axin2* was used as a positive control for induction of CTNNB1-dependent transcription. *Rspo2* expression was not altered by activated Wnt signaling, while *Rspo3* expression was significantly suppressed in tumors with active CTNNB1. (C) MCF10A breast epithelial cells were treated with recombinant mouse Wnt3a (rmWnt3a) ligand for 24 hours. *AXIN2*, *RSPO2*, and *RSPO3* mRNA expression levels were measured by qRT-PCR. *AXIN2* served as a positive control for induction of CTNNB1-dependent transcription. Neither *RSPO2* or *RSPO3* was induced by rmWnt3a treatment.

Supplemental Figure S4



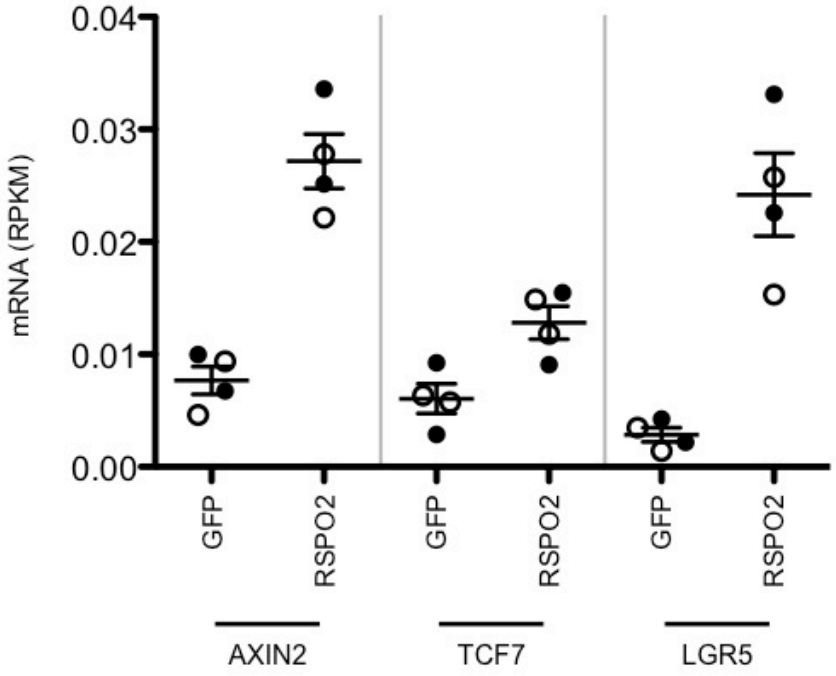
Supplementary Figure S5: RSPO3 is suppressed in HCC and RSPO2-high liver tumors express high levels of Wnt target genes and LGR5. (A) RSPO2 and RSPO3 mRNA levels in 50 normal liver tissues and 200 liver cancers. Data from TCGA RNA-Seq. (B) RSPO3 mRNA levels in an additional cohort of normal liver (n=10), cirrhosis (n=13), Low-grade dysplastic nodule (LGDN, n=10), high grade dysplastic nodule (HGDN, n=8), and liver cancer (HCC, n=91). mRNA expression was determined by microarray analysis (Affymetrix U133 2.0). (C) LGR4, LGR5 and LGR6 mRNA levels in 50 normal liver tissues, 176 RSPO-Low HCCs, and 24 RSPO-High HCCs from TCGA RNA-Seq. (D) mRNA expression of Wnt target genes (AXIN2, TCF7, and LEF1) in 50 normal liver tissues and 24 RSPO-high liver tissues. Data are from TCGA RNA-Seq.

Supplementary Figure S5



Supplementary Figure S6: RSPO2 increases expression of Wnt target genes including LGR5 in the mouse liver. mRNA expression of Wnt target genes (Axin2, Tcf7, and Lef1) in livers from mice hydrodynamically injected with vectors expressing RSPO2 or GFP at early time points (days 15 and 25 PHI). Open circles, day 15; closed circles, day 25.

Supplementary Figure S6



Supplementary Table S1: qRT-PCR primer sequences.

Chapter 3: WAC is inactivated in colorectal cancer and suppresses cellular transformation

Summary

Colorectal Cancer (CRC) is a genetic disease in which a stepwise accumulation of somatic mutations in oncogenes and tumor suppressor genes ("cancer genes") drives tumor initiation and progression. The majority of recurrently mutated genes are affected in <5% of cases and functional validation of these putative cancer genes is a substantial challenge. To identify genes that contribute to the initiation or progression of colorectal cancer (CRC), we recently conducted a set of forward genetic screens using transposon insertional mutagenesis to drive intestinal tumor formation in mice. These studies were designed to identify novel cancer genes that function in a wildtype genetic context or cooperate with known predisposing alleles ($Apc^{Min/+}$ or $Trp53^{R270H/+}$). *WW domain containing adaptor with coiled-coil* (*WAC)*, is a gene not previously associated with cancer that was identified in all three screens. While the function of *WAC* is incompletely understood, previous reports indicate that the WAC protein participates in multiple cellular processes, including golgi biogenesis, autophagy, modulation of gene expression, and induction of p21 expression and cell cycle arrest in response to DNA damage. In human CRC samples, resequencing the *WAC* gene identified non-silent mutations in 3% of samples. Further, we found CRC-associated *WAC* mutants were functionally deficient in induction of p21 in a zebrafish embryo model. We evaluated the role of *WAC* in cellular transformation by depleting *WAC* in mouse and human colonic epithelial cell lines. In these model, loss of *WAC* expression increased anchorage-independent growth

of colonic epithelial cells in cooperation with inactivated *APC* and *TP53*. These data suggest that WAC functions as a tumor suppressor in the colonic epithelium.

Introduction

Colorectal cancer (CRC) is the third leading cause of cancer-related death in both men and women in the United States, and a devastating public health problem (Siegel et al., 2014b). Current treatment relies primarily on surgical resection and traditional cytotoxic chemotherapy. Developing molecularly targeted therapies that selectively kill cancer cells on the basis of their aberrant biology is an important goal of current cancer research, which relies on understanding the genetic mechanisms that drive tumor formation and progression (Haber et al., 2011).

Decades of prior research have established a multistep model of CRC initiation and progression, driven by the stepwise accumulation of somatic mutations in oncogenes and tumor suppressor genes (collectively, "cancer genes") (Fearon, 2011; Vogelstein et al., 1988). Mutation of *Adenomatous Polyposis Coli (APC*) is thought to be a rate-limiting step in the initiation of the majority (70-80%) of human CRCs (Fearon, 2011). *APC* functions as a tumor suppressor at least in part by restraining Wnt signaling (Reya and Clevers, 2005). Canonical Wnt signaling is a critical pathway in normal gut development and homeostasis, which signals through nuclear accumulation of beta-catenin and transcriptional activation of target genes (Reya and Clevers, 2005; Verzi and Shivdasani, 2008). APC antagonizes Wnt signaling by promoting proteosomal degradation of beta-catenin. However, dysregulated beta-catenin following *APC* mutation leads to inappropriate expression of oncogenic target genes including Cyclin D1 and

Myc, and alters cellular differentiation, migration, and survival (de Lau et al., 2007; Sansom et al., 2004; van de Wetering et al., 2002). Thus cells with APC loss of function escape the normal turnover of the GI epithelium, and persist to accumulate additional genetic defects which drive tumorigenesis.

These additional genetic lesions are heterogenous. For example, although mutations in *APC*, *TP53* and *KRAS* are common lesions in CRC and are thought to cooperate in CRC progression, few (<10%) of tumors acquire all three mutations (Smith et al., 2002). Rather, a variety of routes exist to development of invasive disease. Recent whole-exome sequencing of human colorectal tumors revealed an average of ~80 somatic mutations per tumor, less than 15 of which were predicted to be drivers of tumor formation (Wood et al., 2007). Moreover, the majority of recurrently mutated genes were affected in less than 5% of cases (Wood et al., 2007). Determining which of these low-frequency events significantly contribute to tumorigenesis and understanding how they cooperate with predominant cancer genes is an important area of ongoing investigation.

Forward genetic screens in mice can complement efforts to understand genomic changes in human tumors by highlighting genes that are drivers of cancer initiation or progression (Copeland and Jenkins, 2010). We have identified *WW domain containing adaptor with coiled-coil (WAC*) as a candidate tumor suppressor gene in colorectal cancer, based on its predicted loss of function in forward genetic screens for intestinal tumors in mice (Starr et al., 2009, 2011).

WAC is a WW domain-containing adaptor protein with multiple reported functions. The WW domain is a protein-protein interaction motif, similar to the SH3

domain, which binds proline-containing peptides. As a class, WW-domain proteins function in transcription co-activation, ubiquitin-dependent protein degradation, pre-mRNA splicing, and signal transduction (Sudol et al., 2001). WAC also contains a C-terminal coiled-coil domain able to mediate additional protein-protein interactions.

Although the function of WAC has not been fully elucidated, several studies have linked WAC to disparate biological processes. An initial report suggested WAC functions in RNA processing due to co-localization with a member of the pre-mRNA splicing machinery (Xu and Arnaout, 2002). Another group reported WAC complexes with the cytoplasmic deubiquitinase VCIP135 and p97 and is required for golgi biogenesis (Totsukawa et al., 2011). WAC also is required for starvation-induced autophagy (McKnight et al., 2012). In humans, germline deletions on chromosome 10 encompassing *WAC* are associated with multiple congenital abnormalities and mental retardation (Okamoto et al., 2012; Wentzel et al., 2011). Finally, it was recently discovered that WAC functions as an adaptor protein in transcription-coupled histone modification and is required for transcriptional activation of target genes (Zhang and Yu, 2011). Specifically, WAC binds the C-terminal domain of RNA polymerase II through its WW domain and recruits RNF20/40 through its coiled-coil domain. Histone H2B monoubiquitination (H2Bub1) by the RNF20/40 complex is known to regulate transcription at targeted loci (Pavri et al., 2006; Weake and Workman, 2008). Importantly, it was shown that WAC is required for recruitment of the RNF20/40 complex to loci of active transcription, and that loss of WAC expression prevents induction of p21 expression and cell-cycle arrest in response to DNA damage (Zhang and Yu, 2011). RNF20 and H2Bub1 have been shown

to regulate expression of other proto-oncogenes and tumor suppressors in addition to p21 (Shema et al., 2008). Several members of the H2Bub1 pathway, including RNF20, are mutated or dysregulated cancer, suggesting that H2Bub1 may be a novel cancer pathway (Johnsen, 2012).

Results

Murine insertional mutagenesis screens identified Wac is a candidate tumor suppressor gene

Wac has been identified as a CIS-associated gene in ten published transposon insertional mutagenesis screens **(Supplementary Table S1)** (Bard-Chapeau et al., 2014; Berquam-Vrieze et al., 2011; Genovesi et al., 2013; March et al., 2011; Pérez-Mancera et al., 2012; Quintana et al., 2013; Rahrmann et al., 2013; Starr et al., 2009, 2011; Wu et al., 2012). These screens implicated *Wac* dysregulation in tumor formation of 7 unique tissues (colon, liver, pancreas, brain, peripheral nervous system, skin, and blood). In particular, multiple studies identified *Wac* as a candidate tumor suppressor gene in intestinal tumors **(Supplementary Table S1)** (March et al., 2011; Starr et al., 2009, 2011). These studies were conducted in wild type and *Apc*-deficient genetic backgrounds, and showed a pattern of transposon insertions in *Wac* predicted to result in gene loss of function **(Figure 1A).** As a result, these studies suggest that Wac deficiency promotes Apc-driven intestinal tumor formation.

Because p53 mutations are a frequent event late in adenoma formation and are thought to promote the transition to invasive carcinoma, we designed an SB screen to identify cancer genes that cooperate with p53 loss of function. The full results of this

screen will be described in a separate publication. Briefly, a cohort of experimental mice was generated to express a dominant negative Trp53 and *SB* transposase (*SB11)* specifically in the intestinal tract, leading to tissue-specific mobilization of an oncogenic transposon (*T2/Onc*). Quadruple transgenic experimental mice ($Trp53^{LsL\text{-}R270H/+}$; *Rosa26-LsL-SB11; T2/Onc; Villin-Cre*;) had decreased survival and formed intestinal tumors with decreased latency compared to control mice with dominant negative *Trp53* alone (data not shown.) Transposon insertion sites were identified in 30 tumors from experimental mice and 57 CIS-associated genes were identified. *Wac* was the eighth most frequently altered gene in this screen, with insertions in 11 of 30 tumors **(Figure 1A).** Transposon insertions in *Wac* showed a lack of orientation bias and were distributed throughout the gene, suggesting selection for *Wac* loss of function as in prior studies **(Figure 1A).**

To further investigate the status of *Wac* in $Trp53^{R270H}$*/SB*-induced intestinal tumors, we isolated mRNA from six tumors with transposon insertions in Wac and from adjacent normal intestinal tissue. We measured mRNA levels of Wac and its putative target gene p21 by qRT-PCR **(Supplementary Figure S1).** Primers for Wac were designed to probe a region distal to the transposon insertions in all six tumors (exons 5-6). Wac mRNA levels were variable in tumors, and only one of six tumors had significantly decreased Wac expression compared to matched normal tissue **(Supplementary Figure S1A).** Expression of p21 was also variable, but not significantly altered in tumors overall **(Supplementary Figure S1B).** Our ability to detect changes in Wac gene expression caused by transposon insertions may be limited by clonal heterogeneity within tumors or contamination with normal cell types. Despite this

limitation, transposon insertional mutagenesis screens strongly suggest that Wac alterations promote intestinal tumorigenesis on multiple genetic backgrounds.

WAC is somatically mutated and down-regulated in human CRC

To determine if *WAC* loss of function occurs in human colorectal cancer, we analyzed publically available mutation data from The Catalog of Somatic Mutations in Cancer (COSMIC) database (Forbes et al., 2011). Non-silent, somatic mutations in *WAC* occurred in 21 of 690 (3.0%) of samples profiled **(Figure 1B).** Several of these mutations were likely to result in WAC loss of function, including nonsense mutations (E175X, S475X, and Q618X) and point mutations within the coiled-coil domain (L627P and K640N), which is known to mediate binding to RNF20/40 and required for histone monoubiquitination. Interestingly, recurrent mutation of serine 475 was seen in three samples, although that residue is not within a known functional domain.

We analyzed mRNA expression of WAC in 409 human colorectal tumors and 21 normal colon tissues profiled by The Cancer Genome Atlas (Cancer Genome Atlas Network, 2012a). *WAC* mRNA levels were significantly decreased in colorectal tumors compared to normal colon tissues (p=0.00016), with 5.4% of CRCs having more than 2-fold decreased *WAC* mRNA levels compared to normal colon **(Figure 1C).**

Overexpression of wildtype but not mutant WAC induces p21 expression in zebrafish embryo

Because WAC plays a known role in induction of p21 expression (Zhang and Yu, 2011), we chose to use induction of p21 expression as an assay for the functionality of

cancer-associated WAC mutants using an *in vivo* zebrafish embryo model. Injection of wild type WAC mRNA into zebrafish embryos induced a consistent two-fold induction of p21 expression **(Figure 2A).** We also tested whether three cancer-associated Wac mutants were capable of inducing p21 expression. WAC-E172X is a nonsense mutation lacking both the WW and coiled-coil functional domains of the protein. WAC-S475L and WAC-K479N are point mutants of unknown significance, although serine 475 is evolutionarily conserved and may be regulated by phosphorylation **(Figure 2B).** In contrast to wild type WAC, none of the three cancer-associated WAC mutants induced p21 mRNA expression **(Figure 2A).** These results support the hypothesis that cancer-associated *WAC* mutants cause protein loss of function.

WAC regulates p21 expression in colonic epithelial cells

To understand the consequences of WAC loss of function in colonic epithelial cells we chose to use a well-established cellular system. The YAMC and IMCE cell lines are conditionally immortalized colonic epithelial cell lines derived from "Immorto-mouse" tissues, which differ in their *Apc* status (Jat et al., 1991; Whitehead and Joseph, 1994; Whitehead et al., 1993). Conditional immortalization is mediated by the presence of a transgenic allele that expresses a temperature-sensitive SV40 large T antigen from an interferon-responsive promoter (*H-2Kb-tsA58*). Thus under "permissive conditions" (33°C, interferon-gamma-containing media [+INF-γ]), the heat-labile SV40 large tumor antigen (SV40 LTag) is expressed and stable. In contrast, in "non-permissive conditions" (39°C, -INF-γ), the SV40 large T antigen is unstable and cells senesce and die within days. While the YAMC cell line is *Apc* wild type, the IMCE cell line harbors a mutant

Apc allele (*ApcMin/+*). Both YAMC and IMCE cells are non-tumorigenic in nude mice and fail to form colonies in soft agar. These cells represent a valuable tool for investigating the function of a genetic lesion of interest in a genetically-relevant model of "normal" and pre-neoplastic colonic epithelium.

We depleted *Wac* in IMCE cells either transiently or stably, by transfection with siRNA or transduction with a lentiviral vector expressing anti-Wac shRNA respectively. In both cases, knockdown of Wac in IMCE cells led to significantly decreased expression of p21 mRNA **(Figure 2C** and data not shown).

Wac regulates transcription of target genes through recruitment of RNF20/40 and monoubiquitination of histone 2B (H2Bub1) (Zhang and Yu, 2011). A list of genes positively and negatively regulated by RNF20 were identified by gene expression profiling of HeLa cells depleted of RNF20 (Shema et al., 2008). In that study, several pro-apoptosis genes were found to require RNF20 and H2Bub1 for expression. We tested whether Wac was similarly required for expression of the identified apoptosis genes in colonic epithelial cells. However, knockdown of Wac in IMCE cells did not significantly alter mRNA levels of Bad, Bax, Bid, or Bcl2l2 **(Figure 2C** and data not shown).

WAC inhibits colony formation of non-tumorigenic cells

As a candidate tumor suppressor gene, we hypothesized that decreased expression of WAC might contribute to cellular transformation of pre-malignant colonic epithelial cells. Under permissive conditions, in which SV40 LTag is expressed, Knockdown of WAC expression in IMCE cells resulted in increased colony formation in soft agar **(Figure 3A-C).** WAC depletion did not induce colony formation of YAMC cells

harboring wild type *Apc*, or in non-permissive conditions, in which SV40 LTag is destabilized **(Figure 3A and C** and data not shown). This suggests that WAC may function as a tumor suppressor by restraining anchorage-independent growth in the context of mutant APC and inhibited p53/RB.

To further investigate this relationship in a human cell context, we depleted WAC expression in AA/C1 cells. AA/C1 is a cell line derived from an intestinal polyp of a patient with familial adenomatous polyposis (FAP), a syndrome caused by germline mutation of *APC* (Williams et al., 1990*)*. AA/C1 cells have a truncating mutation in *APC* but retain wild type p53. AA/C1 cells are anchorage-dependent and non-tumorigenic in nude mice (Williams et al., 1990, 1994). Previous studies showed that mutation of p53 alone was insufficient to transform AA/C1 cells, despite p53 loss of function being a common late stage event in intestinal adenocarcinoma formation (Williams et al., 1994). Knockdown of WAC alone in AA/C1 cells did not significantly alter colony formation in soft agar **(Figure 3D).** However, simultaneous knockdown of WAC and p53 increased colony formation compared to knockdown of p53 alone **(Figure 3D).**

Discussion

We have presented several lines of evidence suggesting *WAC* functions as a tumor suppressor gene. Insertional mutagenesis screens in mice found *Wac* to be frequently inactivated in intestinal tumors on multiple genetic backgrounds (Starr et al., 2009, 2011; unpublished). In human colorectal cancer, *WAC* was mutated or down-regulated in 8% of cases. Using induction of *Cdkn2a* (p21) gene expression in zebrafish embryos as an assay of WAC function, we showed that mutations in *WAC* identified in human CRC cause loss

of function. Finally, depletion of WAC increased anchorage-independent growth of mouse and human colonic epithelial cell lines when APC and p53 were also mutated or inhibited. Collectively, these data suggest that WAC loss of function is a low penetrance event in CRC that promotes tumorigenesis in the context of APC and p53 depletion. Interestingly, WAC may also be relevant in other tumor types, as *Wac* was identified as a CIS in transposon-induced cancers of the liver, pancreas, brain, peripheral nervous system, skin, and blood in mice, and found to be somatically mutated in >2% of cases of pancreatic, endometrial, and small cell lung cancer in humans (Bard-Chapeau et al., 2014; Berquam-Vrieze et al., 2011; Cancer Genome Atlas Network, 2012a; Cancer Genome Atlas Research Network et al., 2013b; Genovesi et al., 2013; Pérez-Mancera et al., 2012; Quintana et al., 2013; Rahrmann et al., 2013; Rudin et al., 2012; Wu et al., 2012).

The mechanism by which *WAC* functions as a tumor suppressor gene remains to be identified. We showed that cancer-associated mutations prevented the ability of WAC to induce p21 gene expression. This may be relevant to WAC's tumor suppressive function. The p21 cyclin-dependent kinase inhibitor is an important negative regulator of the cell cycle. p21 promotes G1/S phase cell cycle arrest by p53-dependent and independent mechanisms in response to multiple cell stress stimuli (Abbas and Dutta, 2009). As such, p21 is well-positioned to function as a tumor suppressor by restraining cellular proliferation. However, p21 itself is rarely mutated in cancer, and expression of p21 has been documented to have both tumor suppressive and paradoxical oncogenic effects in different contexts (Abbas and Dutta, 2009). Nonetheless, there is evidence that

p21 is tumor suppressive in the colonic epithelium. First, in the normal colonic epithelium, p21 expression is induced (independent of p53) when proliferating progenitor cells migrate away from the crypt base, cease proliferation, and terminally differentiate; a process that is dysregulated in early tumor formation (El-Deiry et al., 1995). Second, although p21 null mice do not develop GI-tract tumors independently, deletion of *Cdkn1a* (encoding p21) collaborates with *Apc* mutation to increase the penetrance, number, and size of intestinal tumors in a mouse model (Yang et al., 2001). Third, immunohistochemical studies of human CRCs have shown that decreased expression of p21, irrespective of p53 expression, correlates with increased metastasis and decreased overall survival (Bukholm and Nesland, 2000; Zirbes et al., 2000). WAC loss may similarly promote tumorigenesis by preventing expression of p21. However, it remains to be tested if p21 is the critical effector of WAC-mediated tumor suppression.

In addition to regulating transcription of multiple cancer genes, H2Bub1 may be a tumor suppressor pathway due to its participation in several other processes (Johnsen, 2012). H2Bub1 functions in DNA double strand break repair and homologous recombination (Moyal et al., 2011; Nakamura et al., 2011; Shema et al., 2008). H2Bub1 is also required for differentiation of embryonic and mesenchymal stem cells (Fuchs et al., 2012; Karpiuk et al., 2012) and WAC was directly shown to be required for some of those phenotypes (Karpiuk et al., 2012). Regulation of cellular differentiation is an intriguing potential mechanism of tumor suppression for WAC. It is known that Wnt signaling an important regulator of stem cell renewal and differentiation in the gut (de Lau et al., 2007). If WAC is required for differentiation of the gut epithelium through

H2Bub1, loss of WAC could cooperate with APC inactivation by preventing terminal differentiation of enterocytes.

Additional studies are needed to specify the mechanism of cooperation between WAC, APC, and p53 in suppressing tumorigenesis. Better understanding of secondary mutations that cooperate with APC inactivation in the progression of CRC will have a significant impact on the field by allowing us to develop better pre-clinical models for testing therapeutic regimens, identify new targets for drug development, and generate hypotheses for rational combination therapy directed against interacting pathways in cancer.

Methods

Somatic mutation and gene expression analysis in human tumors

Human colorectal cancer mutation data for 690 tumors were acquired from the Catalog of Somatic Mutations in Cancer database (COSMIC, http://cancer.sanger.ac.uk/cancergenome/projects/cosmic) (Forbes et al., 2011). WAC mRNA levels were obtained for 409 colorectal tumors and 21 normal colon tissues using data generated by The Cancer Genome Atlas (TCGA) Research Network (http://cancergenome.nih.gov) (Cancer Genome Atlas Network, 2012a).

Zebrafish embryo experiments

In vitro transcribed RNA encoding human wildtype or mutant WAC was micro-injected into one-cell zebrafish embryos obtained from natural matings of wildtype fish. Embryos were raised under standard conditions at 28.5°C in embryo water until 24 hours post

fertilization. Batches of 10 embryos per condition were dechorionated and RNA was extracted for analysis by quantitative RT-PCR. Fold-change in p21 mRNA expression was determined by normalization with S6K to uninjected control embryos. Data represent the average of >3 biological replicates +/- S.E.M. Primers used for qRT-PCR are listed in **Supplementary table S2.**

Cell lines

Immortomouse cell lines (IMCE and YAMC) were acquired from Robert H. Whitehead. Immortomouse cells were cultured in RPMI-1640 medium containing 5% fetal calf serum, 1 mg/ml insulin, and 10E-5 M alpha-thioglycerol. In "non-permissive" conditions, cells were cultured at 37°C and 5% CO2. Under "permissive conditions" culture medium additionally contained 5 units per ml of mouse gamma interferon to upregulate expression of SV40 LTag, and cells were cultured at 33°C, as described (Whitehead and Joseph, 1994; Whitehead et al., 1993).

The AA/C1 cell line was acquired from Christos Paraskeva. AA/C1 cells were cultured in DMEM supplemented with 20% FBS, 2mM Glutamine, 1 ug/ml hydrocortisone, 0.2 units/ml insulin, and penicillin/streptomycin under standard conditions of 37°C and 5% CO2.

RNAi vectors and gene knockdown experiments, and RNA isolation

For transient knockdown experiments, IMCE cells were transfected with a pool of siRNAs against Wac (siGENOME Mouse Wac siRNA SMARTpool, #225131) or a non-targeting control pool (siGENOME Non-Targeting siRNA Pool #1, Thermo

Scientific/Dharmacon) using Lipofectamine RNAiMAX transfection reagent (Invitrogen). siRNA was used at a final concentration of 25 pmol/well with 7.5 ul of lipofectamine and 1E6 cells in a standard 6-well plate. Gene expression was analyzed 72 hrs after transfection as below.

Stable knockdown strains were generated by transduction of YAMC, IMCE, or AA/C1 cells with lentivirus encoding shRNA targeted to WAC, TP53, or a non-silencing control (mouse Wac shRNA V2LMM_20397, human WAC shRNA V2LHS_135342, human p53 shRNA V2LHS_93613, Openbiosystems). Lentiviral particles were produced in 293T cells using the Trans-Lentiviral Packaging Kit (Thermo Scientific). Viral supernatant was collected at 24 hours, cleared, applied to cells with 12 ug/mL polybrene, and incubated overnight. Transduced cells were selected with 1 ug/mL of puromycin (Invitrogen). Knockdown efficiency was evaluated by qRT-PCR.

RNA isolation and quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

RNA was isolated from cell lines and mouse intestinal tissues using the PureLink RNA Mini Kit according to manufacturer protocol (Ambion). RNA samples were analyzed by gel electrophoresis to assess quality and treated with DNase to remove contaminating genomic DNA (Turbo DNA-free Kit, Ambion). Complementary DNA was synthesized from 1 ug template RNA per sample using random hexamer primers (SuperScript III First-Strand Synthesis System, Invitrogen). qRT-PCR reactions were conducted with FastStart Universal SYBR Green Master mix (Roche), using 0.5 ul of cDNA template per 25 ul reaction. Primer sequences for qRT-PCR reactions are listed in **Supplementary**

Table S2. Data were analyzed by normalization to ACTB using the following equation: relative expression = ((2^(CT_ACTB))/((2^(CT_GOI)).

Colony formation in soft agar

Cells were plated in 0.48% low-melt agar (SeaPlaque, Lonza) in complete, permissive media at a density of 10,000 cells per well in 6-well plates, on a base layer of 3.2% agar. Assays were incubated under permissive conditions (33°C and 5% CO2) for three weeks, then fixed and stained with 10% formalin containing 0.005% crystal violet for 1 hour at room temperature to visualize colony formation. Colonies were imaged on a Leica S8 AP0 microscope. Colony counts per well were quantified from digital photographs using ImageJ software (Schneider et al., 2012). Results shown are representative of at least 2 independent experiments.

Authors' Contributions

Caitlin B. Conboy - Conceived and designed the study, performed experiments and analyses unless otherwise noted and wrote this manuscript.

Julia Hatler – Performed experiments in zebrafish embryos (injections, RNA isolations, qRT-PCR) using WAC mutant expression constructs cloned by CBC.

Kelsey Navis – Assisted with tissue culture experiments.

Brooke McDonald – Assisted with RNA isolation and qRT-PCR from mouse tissues.

David Largaespada - Contributed to conception, design, and supervision of this study.

Tim Starr - Contributed to conception, design, and supervision of this study and review of this manuscript. Conducted the *SB* forward genetic screens.

Figure 1: WAC disruption occurs in mouse and human intestinal tumors. (A) *Wac* was identified as a common insertion site (CIS) in three SB screens for GI tract cancer genes. Diagram depicting transposon insertion sites in the *Wac* gene. Triangles depict the location and orientation of transposon insertions. Orange triangles represent transposon insertions with the MSCV promoter within the transposon in the same orientation as *Wac* transcription while purple triangles represent transposon insertions in the opposite orientation (Starr et al., 2009, 2011) and unpublished. (B) Somatic mutations in WAC have been identified in 3.0% of human colorectal cancers. Diagram of the WAC protein, its functional domains, and somatic mutations in WAC identified in human colorectal tumors. Two non-coding mutations predicted to alter WAC splicing (c.610+1G>A and c.1437+1G>A) are not pictured. W, WW domain (aa 129-162). C, coiled-coil domain (aa 617-647) (Forbes et al., 2011). (C) WAC mRNA levels in 21 normal colon tissues and 409 CRC tumors profiled by RNAseq. Data obtained from TCGA (Cancer Genome Atlas Network, 2012a).

Figure 1

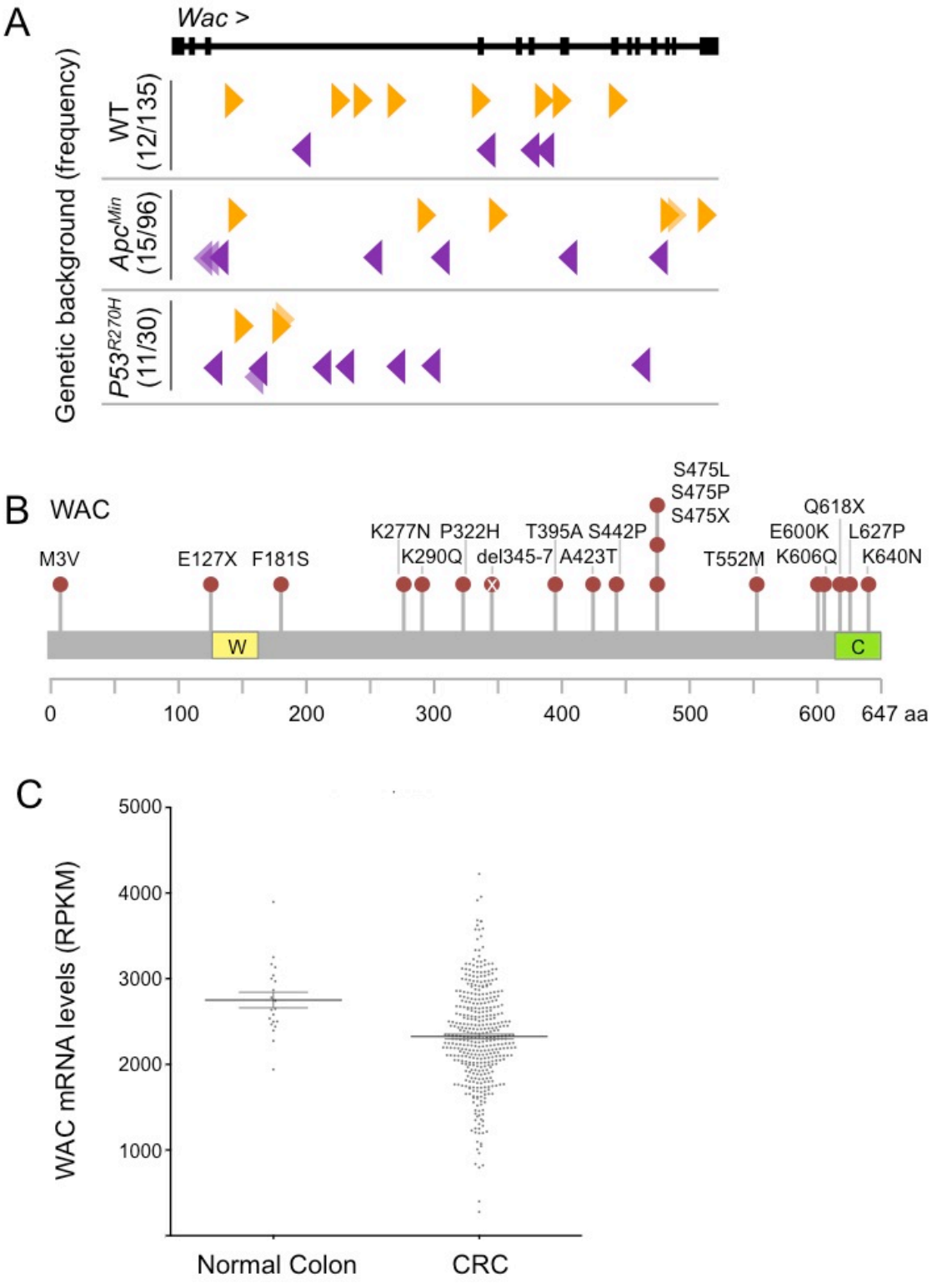

A
Wac >
Genetic background (frequency)
WT (12/135)
Apc^Min (15/96)
P53^R270H (11/30)
B
WAC
M3V
E127X
F181S
K277N
K290Q
P322H
del345-7
T395A
A423T
S442P
S475L
S475P
S475X
T552M
Q618X
E600K
K606Q
L627P
K640N
W
C
0
100
200
300
400
500
600
647 aa
C
WAC mRNA levels (RPKM)
5000
4000
3000
2000
1000
Normal Colon
CRC

Figure 2: Wild type WAC induces expression of p21 but cancer-associated WAC mutants are non-functional. (A) Wild type WAC and cancer-associated WAC mutants were overexpressed in zebrafish embryos by injection with *in vitro* transcribed mRNA. Cdkn1a (p21) mRNA expression levels were measured by qRT-PCR. Fold-change in p21 mRNA expression was determined by normalization with S6K to uninjected control embryos. Data represent the average of >3 biological replicates +/- S.E.M. (B) Wac-S475 is conserved and is a potential phosphorylation site. Diagram indicates evolutionary conservation of Wac serine 475 in human, mouse, zebrafish, xenopus, and drosophila. *In silico* analysis of potential phosphorylation sites indicated the peptide "LISTPPVSSQPKVST" could be a target of several kinases (GSK, STKR, NEK, CDC2, CDK4, Dyrk1, GSK3A, and MAPK) (GPS 2.1, (Xue et al., 2011)). (C) Knockdown of Wac expression by siRNA in mouse colonic epithelial cells (IMCE). Analysis was performed at 72 hours following siRNA transfection. Knockdown efficiency and effect on mRNA expression of p21 and Bax were assessed by qRT-PCR. mRNA levels represent fold-change, normalized with Actb to non-silencing (Nons) control samples. Average data for three biological replicates are shown. *$p<0.05$.

Figure 2

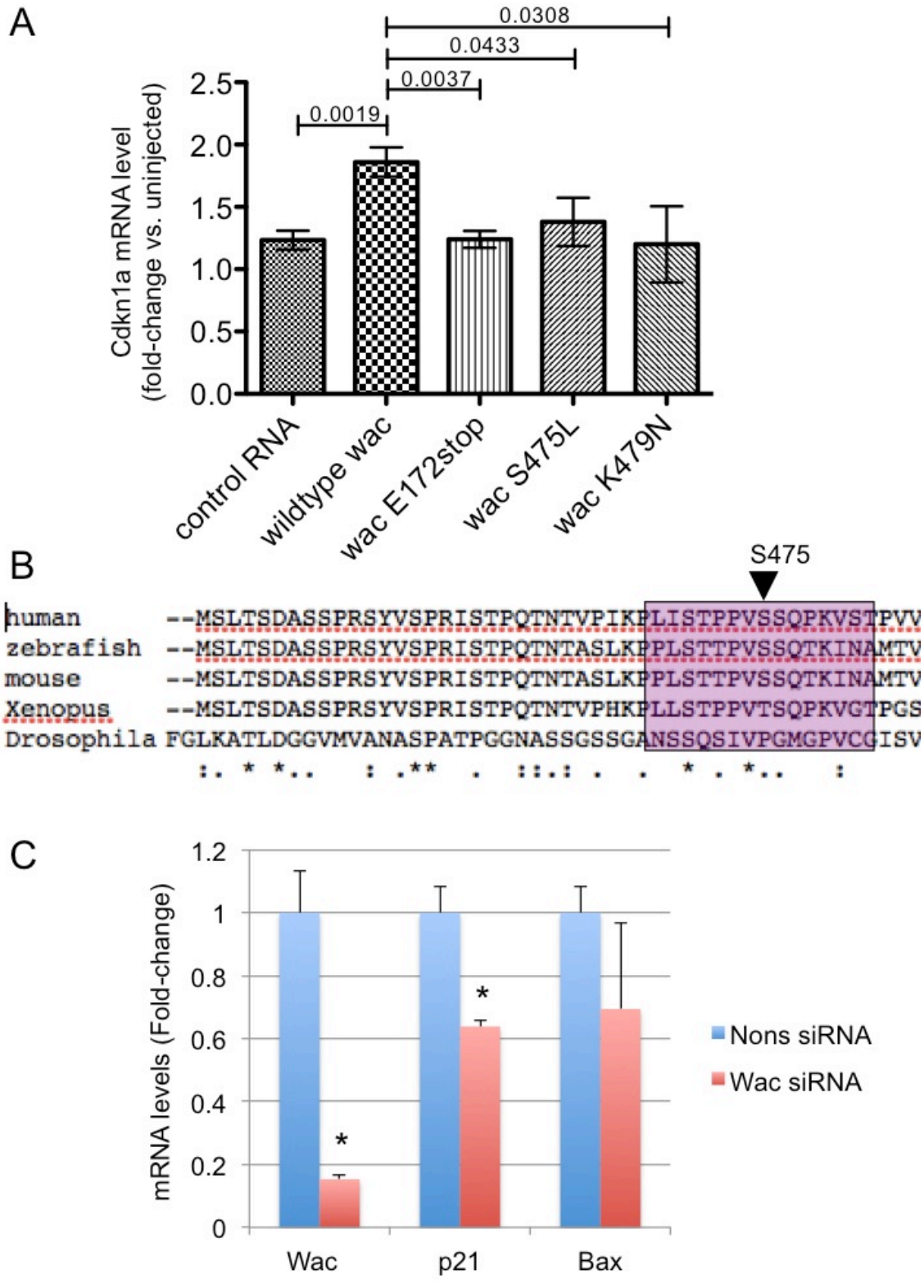
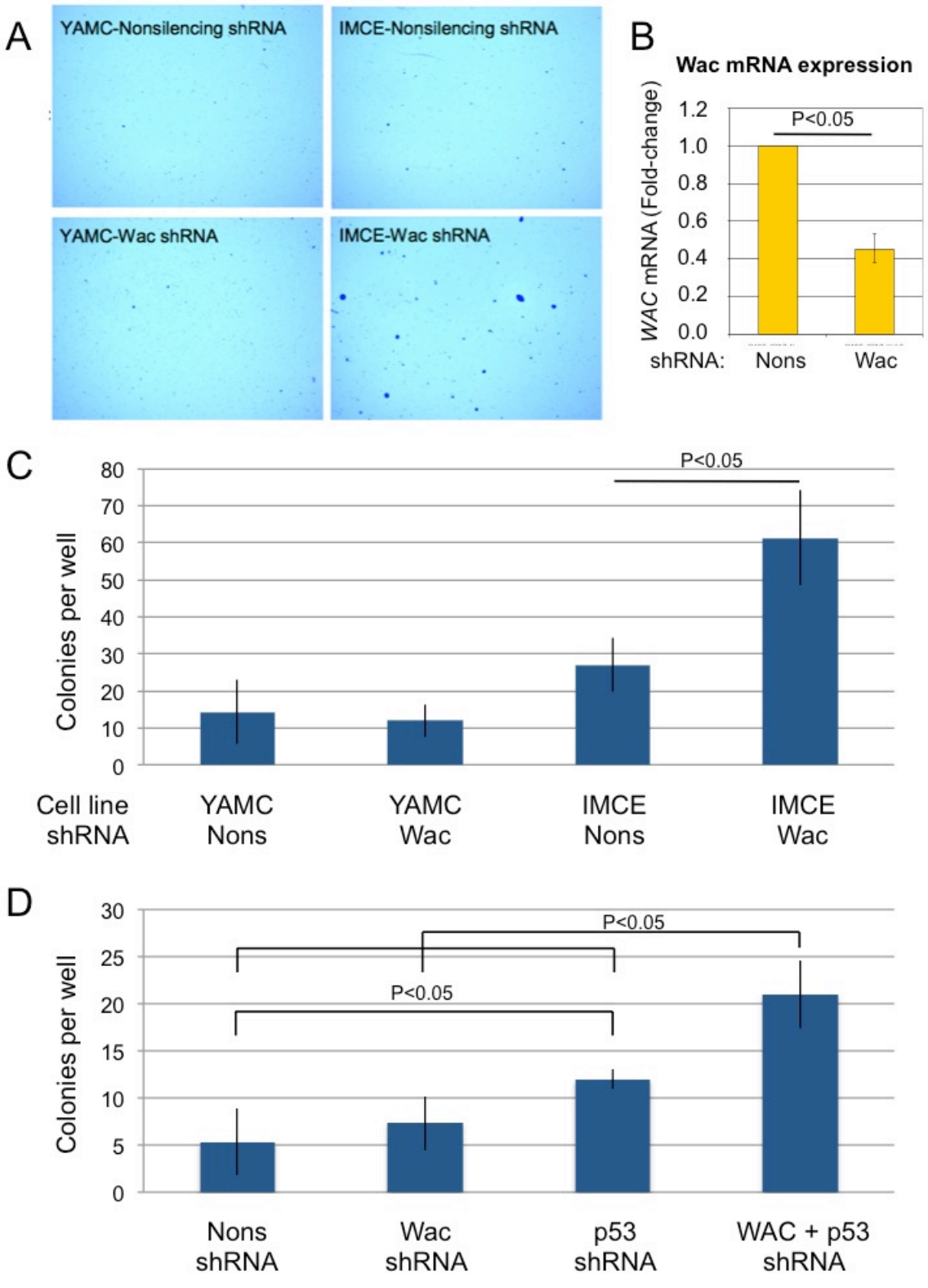

A
0.0308
0.0433
0.0037
0.0019
Cdkn1a mRNA level
(fold-change vs. uninjected)
2.5
2.0
1.5
1.0
0.5
0.0
control RNA
wildtype wac
wac E172stop
wac S475L
wac K479N
B
S475
human --MSLTSDASSPRSYVSPRISTPQTNTVPIKPLISTPPVSSQPKVSTPVV
zebrafish --MSLTSDASSPRSYVSPRISTPQTNTASLKPPLSTTPVSSQTKINAMTV
mouse --MSLTSDASSPRSYVSPRISTPQTNTASLKPPLSTTPVSSQTKINAMTV
Xenopus --MSLTSDASSPRSYVSPRISTPQTNTVPHKPLLSTPPVTSQPKVGTPGS
Drosophila FGLKATLDGGVMVANASPATPGGNASSGSSGANSSQSIVPGMGPVCGISV
:. * *.. : .** . ::.: . . * . *.. :
C
mRNA levels (Fold-change)
1.2
1
0.8
0.6
0.4
0.2
0
*
*
Wac
p21
Bax
Nons siRNA
Wac siRNA

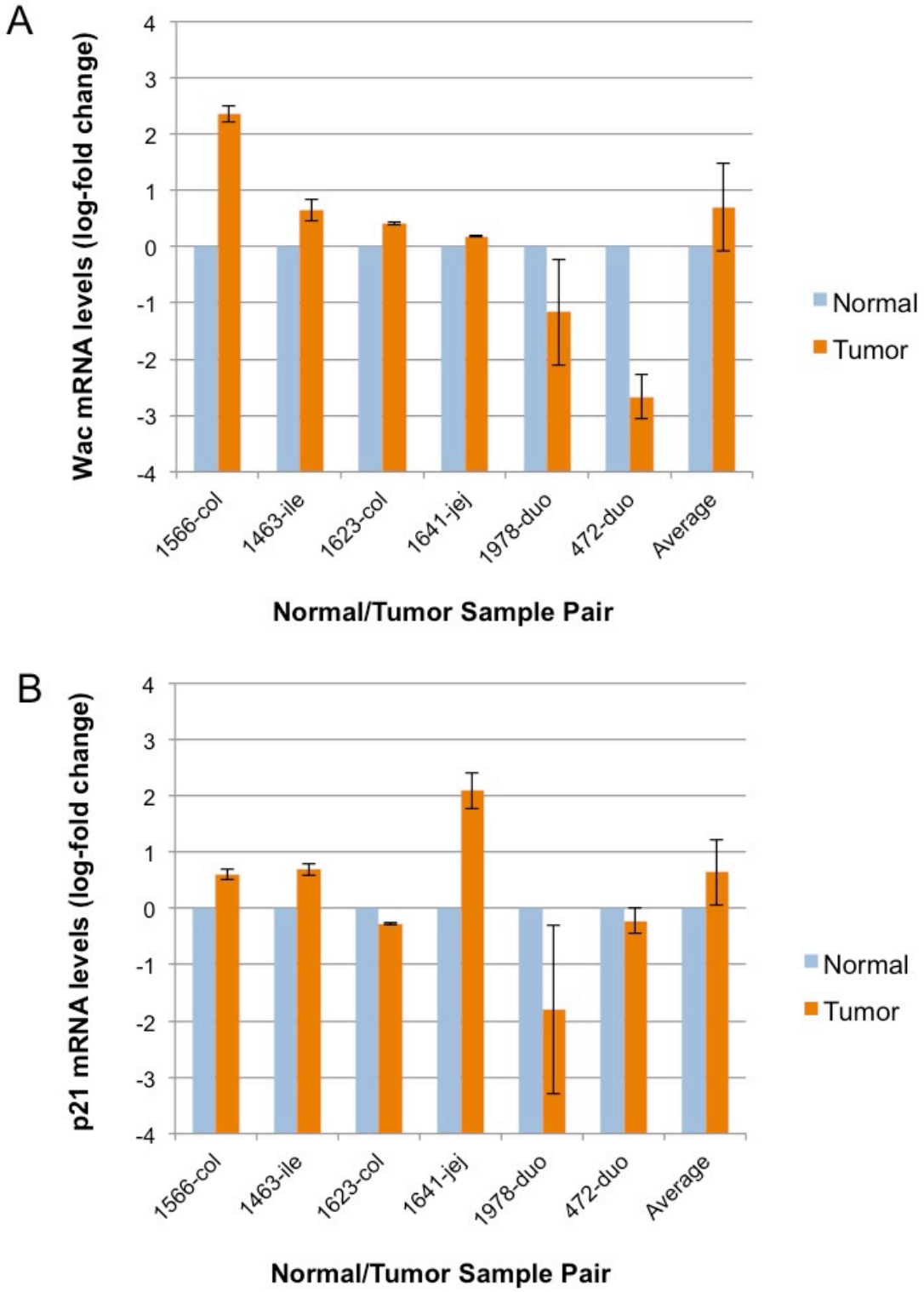
Figure 3: WAC represses anchorage independent growth in non-tumorigenic colonic epithelial cells. (A) Decreased WAC expression increased anchorage-independent growth of Immortomouse cells with mutant *Apc* (IMCE*, Apc*^{MIN}; SV40 Large T antigen *tsA58*) but not wild type *Apc* (YAMC*, Apc*$^{+/+}$; SV40 Large T antigen *tsA58*). WAC knockdown and control cells were plated in soft agar at permissive conditions (33°C, +INF-γ). Colony formation was measured at 3 weeks by staining with crystal violet and automated colony counting with ImageJ software. (B)*Wac* mRNA levels were assessed by qRT-PCR, normalized with Actb to non-silencing shRNA control. (C) Quantification of colony formation in part A. Results are representative of three independent replicate experiments. (D) Combined knockdown of WAC and p53 in human colon adenoma cells (AAC1, truncated APC, wt p53) increased colony formation compared to p53 knockdown alone.

Figure 3


A
YAMC-Nonsilencing shRNA
IMCE-Nonsilencing shRNA
YAMC-Wac shRNA
IMCE-Wac shRNA
B
Wac mRNA expression
WAC mRNA (Fold-change)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
P<0.05
shRNA:
Nons
Wac
C
Colonies per well
80
70
60
50
40
30
20
10
0
P<0.05
Cell line
shRNA
YAMC
Nons
YAMC
Wac
IMCE
Nons
IMCE
Wac
D
Colonies per well
30
25
20
15
10
5
0
P<0.05
P<0.05
Nons
shRNA
Wac
shRNA
p53
shRNA
WAC + p53
shRNA

Supplementary Figure S1: Expression levels of Wac and p21 in SB-induced murine intestinal tumors harboring transposon insertions in Wac compared to adjacent normal intestinal tissue.

Supplementary Figure S1



Supplementary Table S1: Published insertional mutagenesis screens identified *Wac* as a candidate cancer gene in multiple tissues

Cancer Type	Genetic background or treatment	Transposase expression	WAC CIS Address	CIS Rank*	Predicted Effect	Reference
Liver cancer	HBV surface antigen (HBsAg)	Liver (*Albumin-Cre*)	18:7868857-7929027	A	Not Determined	Bard-Chapeau et al, 2013
T-cell acute lymphoblastic leukemia	Wild type	Late-stage CD4+/CD8+ thymocytes (*CD4-Cre*)	18:7896837-7925450	B	Loss	Berquam-Vrieze et al, 2011
Medulloblastoma	Ptch1-lacZ/+	Ubiquitous (*Actb-Cre*)	18:7868832-7973547	D	Loss	Genovesi et al, 2013
Intestinal cancer	Apc fl/+	Intestinal tract (*Cyp1a1-Cre*)	18:7842074-7955421	A	Not Determined	March et al, 2011
Pancreatic cancer	Kras-LSL-G12D	Pancreas (*Pdx1-Cre*)	18:7892929-7892929	B	Not Determined	Perez-Mancera et al, 2012
SCC and BCC	v-Ha-ras; +/- TPA treatment	Epidermis (*K5-SB11*)	18:7868832-7973547	A	Not Determined	Quintana et al, 2012
Neurofibroma and MPNST	Cnp-EGFR +/- Trp53-R270H	Schwann Cells (*Cnp-Cre*)	18:7868832-7973547	B	Not Determined	Rahrmann et al, 2013
Intestinal cancer	Wild type	Intestinal tract (*Villin-Cre*)	18:7874333-7972742	A	Loss	Starr et al, 2009
Intestinal cancer	Apc-Min	Intestinal tract (*Villin-Cre*)	18:7855250-8046128	B	Loss	Starr et al, 2011
Medulloblastoma	Ptch+/-	Cerebellum (*Math1-SB11*)	18:7868832-7973547	Not Ranked	Not Determined	Wu et al, 2012

SCC, Squamous Cell Carcinoma; BCC, Basal Cell Carcinoma; MPNST, Malignant Peripheral Nerve Sheath Tumor

*Studies identified and CIS rank determined by the Candidate Cancer Gene Database, Starr Lab, University of Minnesota. http://ccgd-starrlab.oit.umn.edu. CIS Rank, A = Top 10%, B = 11 to 25%, C = 26 to 50% and D = Bottom 5

Supplementary Table S2: primers used for qRT-PCR

Gene	Species	Forward Primer (5'-3')	Reverse Primer (5'-3')	Amplicon length (bp)
Actb	mouse	TCCAGCCTTCCTTCTTGGGTATGGA	CGCAGCTCAGTAACAGTCCGCC	365
Wac	mouse	TGCAGATGATTGGTCTGAGC	AACTGCCAGCTTATTTGCTTC	142
Cdkn1a	mouse	TCAGAGTCTAGGGGAATTGGA	AATCACGGCGCAACTGCT	168
Bid	mouse	GCCGAGCACATCACAGACC	TGGCAATGTTGTGGATGATTTCT	226
Bad	mouse	GGAGCAACATTCATCAGCAG	GGTACGAACTGTGGCGACTC	93
Bax	mouse	TGAAGACAGGGGCCTTTTTG	AATTCGCCGGAGACACTCG	140
Bcl2l2	mouse	TGCTGAGAGTGTCAACAAAGAAA	GTATAGAGCTGTGAACTCCGC	133
ACTB	human	GCCGTCTTCCCCTCCATCGT	TGCTCTGGGCCTCGTCGC	97
WAC	human	CAACCACAGTGCTCTTCATAGTTC	AGCTAATATGCTCAGACCAGTCATC	116
TP53	human	TGTTTCCTGACTCAGAGGGG	GAGCGTGCTTTCCACGAC	123
CDKN1A	human	CATGCCAGCTACTTCCTCCT	CAGGTCTGAGTGTCCAGGAA	125

Chapter 4: Future directions

– Towards targeted therapy for RSPO2 and WAC-driven tumors –

Summary

This thesis research identified RSPO2 and WAC as regulators of cell signaling and tumorigenesis. Specifically, we found that RSPO2 activates Wnt signaling and promotes tumor formation in the colon, breast, and liver, and WAC is a tumor suppressor gene in the colon required for induction of the cell cycle regulator p21. In order to advance our understanding of RSPO2 and WAC in cancer, we propose developing additional *in vivo* models of RSPO2 activation and WAC depletion. Such models can be used to further define the signaling pathways regulated by RSPO2 and WAC, the genetic contexts in which RSPO2 and WAC perturbation contribute to tumorigenesis, and the required mediators of RSPO2 and WAC-driven tumorigenesis. These models can also be used for preclinical drug development studies. Here we outline an approach to these research questions and propose a number of strategies for therapeutically targeting RSPO2- and WAC mutation-driven tumors.

Developing in vivo models to study RSPO activation and WAC depletion in cancer

In vivo models of RSPO2 activation and WAC depletion will be useful tools for better understanding RSPO2- and WAC-driven tumor biology and for preclinical testing of targeted cancer therapies. In this thesis we developed a mouse model of RSPO2 overexpression in the liver, using hydrodynamic injection of DNA vectors encoding transposable expression constructs in a *Fah* null mouse, followed by *Fah* selection for modified hepatocytes (Chapter 2, Figure 5A). Using this model, we found that hepatic

expression of RSPO2 alone promoted tumor formation in 18% of mice, a non-significant increase over the tumor frequency seen in mice injected with shRNA against *Trp53* (4%) or a GFP control vector (5%) on day 150 post-injection. Importantly, combined overexpression of RSPO2 and knockdown of Trp53 (RSPO2/shp53) promoted tumor formation in 65% of mice (Chapter 2, Figure 6A). Tumors that formed in RSPO2/shp53 mice were adenocarcinomas with activated Wnt signaling (Chapter 2, Figure 7B and C).

In future studies, this model can be extended in several ways to answer important questions. To determine whether RSPO2 expression is required for maintenance of established liver tumors, a doxycycline-inducible promoter could be used to regulate RSPO2 expression in this model (Chin et al., 1999; Urlinger et al., 2000). This could establish proof of concept for the utility of RSPO2-targeted therapy in liver cancer. Mice injected with doxycycline-inducible RSPO2 and shRNA against Trp53 could be maintained on doxycycline to allow tumor formation. At day 150 post-injection, mice could be randomized to continue receiving doxycycline or receive no drug. Tumor burden at subsequent time points could be compared to determine the requirement for ongoing RSPO2 expression in RSPO2-initiated tumors. Alternatively, at day 150 post-injection tumors could be isolated, disaggregated, and transplanted into replicate recipient mice treated with doxycycline or no drug. This approach would have the advantage of allowing direct observation of the effect of RSPO2 withdrawal on individual tumors. These models could subsequently be used to test pharmacological inhibition of RSPO2, as discussed below.

Although our studies focused on the role of WAC in colorectal cancer, the identification of WAC by transposon insertional mutagenesis screens in six other tissues, including the liver, suggests that WAC may function as a tumor suppressor gene more broadly (Bard-Chapeau et al., 2014; Berquam-Vrieze et al., 2011; Genovesi et al., 2013; Pérez-Mancera et al., 2012; Quintana et al., 2013; Rahrmann et al., 2013; Wu et al., 2012). To test the hypothesis that WAC is a tumor suppressor in the liver, mice could be hydrodynamically injected with transposon vectors encoding shRNA against Wac, with or without shRNA against Trp53, and monitored for tumor formation at subsequent time points. This model could be used to evaluate if inactivation of Wac and Trp53 cooperate in liver as well as intestinal tumorigenesis (Chapter 3, Figure 3).

Models of RSPO2 and WAC perturbation in the colon would also be a valuable resource. Adenoviruses have been used for gene delivery in the colon (Kuhnert et al., 2004; Shibata et al., 1997). Rectal infusion of mice with recombinant adenovirus expressing RSPO2 (Ad-RSPO2) could be used to validate RSPO2 as an oncogene in colorectal cancer. Such a model could further be used to determine if canonical Wnt signaling is the critical mediator of RSPO2-driven tumorigenesis, by infusing *Ctnnb1*-deficient mice with Ad-RSPO2. Wac inactivation could be modeled by generating a floxed allele for conditional knockout of *Wac* ($Wac^{fl/fl}$). Mice harboring this allele could be rectally infused with adenovirus expressing Cre recombinase, or bred to a tissue-specific Cre recombinase allele (ex. *Villin-Cre*) to test if WAC functions as a tumor suppressor *in vivo*. Conditional *Wac* knockout mice could be bred to Apc^{Min} mice to determine how WAC inactivation modifies the Apc-driven intestinal tumor phenotype. These studies will clarify the role of

RSPO2 and WAC in tumor formation and provide preclinical models for testing targeted therapies.

Development of targeted therapies

In this thesis we have shown that subsets of human colon, breast and liver cancers express high levels of RSPO2, and that WAC is mutated or downregulated in a subset of colon cancers. Functional analysis of these alterations supports an oncogenic role for RSPO2 activation and WAC depletion in tumorigenesis. Future studies should be designed to determine whether RSPO2- and WAC-driven tumors continue to require these alterations for their sustained growth *in vivo*, and whether RSPO2 and WAC, or their associated signaling pathways, could be targeted therapeutically. Several strategies for developing RSPO2- and WAC-targeted therapy are discussed below.

Strategies for RSPO2-targeted therapy

Because RSPO2 is a secreted protein, it may be readily inhibited by monoclonal antibody or Fc-fusion-based therapies. Recombinant humanized monoclonal antibodies been successfully developed to target other secreted growth factors for cancer therapy (ex. Bevacizumab, which targets vascular endothelial growth factor) (Tol and Punt, 2010). Fc fusions proteins, in which a protein of interest is genetically fused to a human immunoglobulin Fc domain, provide an alternative approach (Huang, 2009). An Fc fusion to the extracellular domain of the RSPO receptor LGR5 (LGR5-exo-Fc) has been developed and shown to bind RSPO1 with a KD of 3.1 nM (de Lau et al., 2011). Since

LGR5 is a receptor for all four RSPO ligands (Kim et al., 2008) LGR5-exo-Fc could be tested as a therapeutic inhibitor of RSPO2.

Several questions will need to be addressed in the development of RSPO2-targeted therapy. Will RSPO-high tumors continue to rely on RSPO expression for their growth? If RSPO2-targeted therapy does inhibit tumor cell growth, will relapse occur with other activating lesions in the Wnt pathway? If RSPO2 inhibition has the potential to paradoxically activate Wnt signaling, as proposed by (Wu et al., 2014) will there be side effects of RSPO2-inhibition on normal intestinal epithelium?

An alternative approach is to inhibit the Wnt pathway rather than RSPO2 itself. Since R-spondins do not directly activate the Wnt pathway, but rather enhance signaling in the presence of Wnt ligands, RSPO2-driven tumors might be sensitive to Porcupine inhibitors that prevent the secretion of Wnt ligands (Liu et al., 2013; Proffitt et al., 2013). Wnt inhibitors that target the pathway downstream of ligand-receptor interactions, reviewed in Chapter 1, might also be effective in blocking RSPO2-initiated Wnt signaling (Chen et al., 2009; Emami et al., 2004; Huang et al., 2009; Waaler et al., 2012). However, targeting the Wnt pathway raises additional concerns. Many normal tissue stem cells rely on Wnt signaling for homeostatic growth, including normal intestinal stem cells (Barker et al., 2008). Drugs targeting the Wnt pathway will have to be assessed for toxicity in stem cell compartments. Additionally, a potential drawback of inhibiting canonical Wnt signaling in RSPO2-driven tumors is that it ignores other pathways, including non-canonical Wnt signaling, that might be activated by R-spondins (Glinka et

al., 2011b; Ohkawara et al., 2011). Future studies will need to directly test the efficacy of Wnt pathway inhibitors in RSPO2-driven tumor models.

Strategies for WAC-targeted therapy

WAC is an adaptor protein required for multiple cellular functions, including golgi biogenesis, starvation-induced autophagy, and monoubiquitination of histone H2B (H2Bub1) (McKnight et al., 2012; Totsukawa et al., 2011; Zhang and Yu, 2011). Additionally, H2Bub1 regulates expression of a large number of target genes, which in turn influence the cell cycle, apoptosis, cell differentiation, and other processes (Fuchs et al., 2012; Karpiuk et al., 2012; Shema et al., 2008). Future studies will need to be designed to determine which of these functions of WAC are tumor suppressive, and therefore should be pursued for drug development. Given that H2Bub1 regulates differentiation and expression of many cancer genes, it is tempting to hypothesize that WAC behaves as a tumor suppressor through regulation of H2Bub1. Indeed, other factors that regulate H2Bub1 have also been implicated in cancer (Johnsen, 2012). Restoring H2Bub1 in tumors with mutated or downregulated WAC would be pharmacologically challenging. It may be a more successful strategy to identify "druggable" transcriptional targets downstream of H2Bub1, for example, by screening RNAi libraries in WAC-depleted tumor models.

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