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Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA,
IRVINE

**Wnt Signaling Regulation of Colon Cancer Metabolism and
Invasion**

DISSERTATION

Submitted in partial satisfaction of the requirements
For the degree of

DOCTOR OF PHILOSOPHY

in Biomedical Sciences

by

George Tsun-Te Chen

Dissertation Committee:
Professor Marian L. Waterman, Chair
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2018

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DEDICATION

This dissertation is dedicated to

my parents,
Thomas & Yinju Chen

and

my fiancée,
Jennifer Davis

with deep gratitude for their unwavering love and support.

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My family has been tremendously supportive of my graduate school efforts, providing help and advice where they could. My parents, Thomas and Yinju Chen, have always been my biggest supporters. I’m incredibly grateful that they have always cultivated my curiosity for the world and allowed me to explore my place within it. I cannot emphasize enough how much their love and support has meant to me. This thesis is as much a celebration of work for them as it is for me. My extended family: my brothers and sister – Bill, Bob, and Patty and their families who have helped me stay sane and keep things in perspective. I must also thank my fiancée, Jennifer Davis, for putting up with my grumpiness on my hardest days and giving me her unconditional love and support as I struggled to balance the multitude of projects at any given time. Thanks as well as my future in-laws, Margaret and Calvin Davis, for their support and encouragement.

The Waterman lab, by Marian’s design, has always been composed of individuals passionately interested in pursuing science. They have been valuable colleagues, collaborators, and friends and I consider myself lucky to have been part of this tight-knit community. I must first thank Stephanie Sprowl-Tanio, who mentored me when I first joined the lab as a rotation student, and who collaborated with me on many projects during my graduate school career. Thank you also to the graduate students that preceded me: Rani Najdi, Becky Tsai, Nate Hoverter, and Kira Pate – thank you for always being open to help me figure out an errant assay, or bounce a random idea off of. I appreciated our game nights and lab outings, and am thankful that you have all been so generous with your time, even after leaving the lab. Additional thank you’s to the previous postdocs in the lab – Andria Denmon and Beibei Wu, for providing advice and insight, both in lab and in terms of life beyond graduate school. To the current members of the Waterman lab –Amber Habowski, Linzi Hosohama, and Yung Lyou, thank you for your collaboration, discussions, and scholarship. Thank you also to our frequent lab meeting collaborators – Dr. Peter Donovan and his lab, and Dr. Eric Stanbridge, for going above and beyond in advising me on my project and career trajectory, and always challenging me to be a better scientist.

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My journeys in laboratory research began as an undergraduate at UC San Diego, where as the end of my sophomore year approached, I searched for a research position by blindly emailing professors out of the blue. To my surprise, this method yielded exactly one response, from Dr. Thomas J. Kipps, who allowed me to join his lab that summer. I would like to thank him, and the members of his laboratory who mentored me – specifically Dr. Liguang Chen, Dr. Suping Zhang, Dr. Tang Li, Dr. George Widhopf, Esther Avery, and Dr. Laura Rassenti. I am extremely grateful that they were willing to take a chance on me, especially as an undergraduate student with absolutely no lab experience, to allow me to discover my love of research and to encourage me to pursue science as a career. I also want to acknowledge Dr. Heather Henter, Dr. Joshua Kohn, and Dr. Dong-Er Zhang for teaching me how to teach and helping to develop my skills as a mentor.

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4. Lee M*, **Chen GT***, Puttock E, Wang K, Edwards RA, Waterman ML & Lowengrub J (2017) “Mathematical modeling links Wnt signaling to emergent patterns of metabolism in colon cancer.” *Mol. Syst. Biol.* **13**: 912 (* Co-first author)
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5. **GT Chen**, ML Waterman. “Cancer: leaping the E-cadherin hurdle.” (2015) *The EMBO Journal*, 34(16), 2111 – 2210. doi:10.15252/embj.201592757
6. S Zhang, L Chen, B Cui, H Chuang, J Yu, J Wang, L Tang, **G Chen**, G Basak, W Frankel, T Kipps. “ROR1 Is Expressed In Human Breast Cancer And Associated With Enhanced Tumor-cell Growth” (2012) *PLoS ONE* 7(3): e31127. doi:10.1371/journal.pone.0031127

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8. L Chen, B Cui, S Zhang, **G Chen**, C Croce, T Kipps. “Association between Proficiency of B Cell Receptor Signaling and Relative Expression Levels of ZAP-70, SHIP-1, and miR-155 in Chronic Lymphocytic Leukemia.” *Blood* (ASH Annual Meeting Abstracts), 2008 112: Abstract 3155

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Abstract of the dissertation

Wnt Signaling Regulation of Colon Cancer Metabolism and Invasion

By

George Tsun-Te Chen

Doctor of Philosophy in Biomedical Sciences

University of California, Irvine, 2018

Professor Marian L. Waterman, Chair

The Wnt signaling pathway is a highly conserved cell-to-cell communication network in animals, and it regulates many gene programs essential for organism growth and development. Dysregulation of the Wnt pathway is a characteristic of many diseases, the most recognized of which is cancer. In colon cancer, Wnt-activating mutations are a key initiator of tumorigenesis, and *in vitro* and *in vivo* experiments have suggested that targeting the pathway will effectively decrease tumor burden. Despite this, years of clinical trials using Wnt pathway inhibitors in colon cancer patients have not yielded any success. As the push to develop Wnt-targeting therapies continues, a growing body of work points to a more complex understanding of Wnt signaling in colon cancer. In spite of the Wnt-activating mutations, Wnt signaling activities vary widely within a tumor, suggesting additional regulation beyond the constitutive activation. This dissertation presents two consequences of Wnt heterogeneity; first the regulation of cellular metabolism, and second, the regulation of cell migration and invasion.

Our first story builds upon our earlier work, which demonstrated that Wnt directly regulates the expression of glycolytic metabolism genes *PDK1* and *MCT1*. We find that observations of Wnt signaling heterogeneity in tumors are also manifest as heterogeneous

metabolism. We show that the spatial patterns of Wnt and metabolic heterogeneity are directly linked and a result of cell-to-cell signaling. This heterogeneity supports growth through efficient distribution of nutrients and proliferative signaling throughout the tumor. We demonstrate that this relationship between Wnt signaling and metabolism can be exploited therapeutically using a combination of Wnt and glycolysis inhibitors.

In the second story, we study heterogeneity in Wnt signaling levels over time, to show that a decrease in Wnt signaling primes tumor cells for invasion. By modulating the level of Wnt signaling in different ways, we show a graded increase of cell invasion and migration with decreasing Wnt levels, in both cell culture and orthotopic tumor models. Using sequencing data from Wnt-decreased cells and colon cancer patients, we identify potential drivers of this invasive phenotype, and suggest their use as therapeutic or diagnostic targets in colon cancer patients advancing to metastatic disease.

CHAPTER 1

Introduction

Cell signaling through the Wnt family of secreted ligands is a fundamental and highly conserved regulatory network in animals. Wnt signaling is important in directing normal tissue processes through the regulation of cellular activities and gene expression programs, including cell differentiation, embryonic development, metabolism, cell cycle progression and cell polarity. Not surprisingly, Wnt signaling deregulation via its overactivation is linked to a broad spectrum of human diseases, the most commonly recognized of which is cancer (Anastas & Moon, 2013). For this reason, development of cancer drugs that target overactive Wnt signaling to either lower its activity or block it altogether has long been a strategic goal of pharmaceutical research (Kahn, 2014). Such efforts were fraught with failure for several decades, but now there are at least two different types of small molecule Wnt inhibitors in clinical trials for the treatment of colon cancer (Kahn, 2014; Madan *et al*, 2016). Interestingly, just as these inhibitors entered the early stages of clinical trials, a few retrospective, meta-analyses were published pointing out that moderate-to-low Wnt signaling has a poorer prognosis in cancer patients compared to tumors with the highest levels of Wnt signaling (Guinney *et al*, 2015). Why, or how, a lower level of Wnt signaling tracks with a bad prognosis is not at all understood, or even largely acknowledged. Meanwhile, clinical trials with Wnt inhibitors are still ongoing; outcomes have yet to be reported. There is no clear mechanistic insight into these paradoxical observations - partly because there are no basic science studies that address the issue of high-to-low Wnt signaling activity directly and also because there are no mouse models where this correlation has been reported. In fact, the opposite has been observed in xenograft mouse models: inhibition of Wnt signaling results in smaller tumors (Pate *et al*, 2014). The work presented in this thesis addresses this paradox. First, heterogeneous Wnt

signaling from high-to-low within a single tumor is studied via mathematical modeling (Chapter 2) and second, orthotopic mouse models (colon tumors developed in the wall of the caecum) show that lowering Wnt signaling most definitely creates a more aggressive, invasive cancer (Chapter 3). Gene expression analyses provide insight into why this might be the case, and suggest considerations for therapeutic treatment.

The Wnt signaling pathway

Generally speaking, Wnt signaling is divided into two modes of signal transduction distinguished by the involvement of the ubiquitously expressed protein β -catenin. The signal transduction pathway that uses β -catenin as an effector is termed “canonical” or “ β -catenin dependent,” whereas the system that signals without β -catenin is termed “non-canonical” or “ β -catenin independent.” In the canonical Wnt signaling pathway, Wnt ligands bind to the transmembrane receptor protein Frizzled and a co-receptor, LRP5 or LRP6, initiating a signaling cascade which results in β -catenin translocating to the nucleus to bind the LEF/TCF family of transcription factors and drive expression of Wnt target genes (Behrens *et al*, 1996). It is this pathway that drives the development of colon cancer and contributes to tumorigenesis in many other tumor types. Normally, in the absence of Wnt ligands, cytoplasmic β -catenin is captured and tagged for degradation via the ubiquitin proteasome pathway by a group of proteins, including Axin, Adenomatous Polyposis Coli (APC), Casein Kinase 1alpha (CK1 α), and Glycogen Synthase Kinase-3 β (GSK3 β), that assemble into a large complex referred to as the “Destruction Complex”. Inactivating mutations in Destruction Complex components comprise the majority of Wnt-related signaling deregulation in cancer. The subunit most commonly inactivated via genetic mutations is APC (Rowan *et al*, 2000). These mutations decrease the

activity of the Destruction Complex, allowing β -catenin to accumulate and translocate to the nucleus in the absence of a Wnt activating signal.

In contrast to the canonical Wnt pathway, non-canonical, β -catenin-independent Wnt signaling broadly describes multiple signaling pathways that do not require the translocation of β -catenin. The best characterized of this type of signal transduction system are the planar cell polarity and CaMKII (calmodulin- dependent protein kinase II) pathways (Nusse, 2012). The latter pathway regulates cell migration, while the former regulates cell polarity – two important activities when immature cells are differentiating and establishing proper tissue interactions and orientations. These pathways can play a role in tumorigenesis for certain cancers, such as breast cancer, where Wnt5a-triggered non-canonical signaling has been shown to enhance tumor growth (Zhang *et al*, 2012). However, the canonical and non-canonical Wnt signaling pathways have also been observed to antagonize one another during development, though it is not yet clear at what level this occurs. General evidence suggests that specific ligands and receptors exclusively activate canonical or non-canonical Wnt signaling (in total there are nineteen human Wnt ligands and ten Frizzled receptors (Najdi *et al*, 2012; Willert & Nusse, 2012)) though emerging data suggests there are certain conditions in which canonical pathway ligands can activate non-canonical pathways and vice versa (Alok *et al*, 2017; Nalesso *et al*, 2011).

Despite the diversity in different types of WNT signaling, the majority of colon cancer cases are driven by aberrant overactivation of the canonical Wnt signaling pathway. As previously mentioned, APC, a member of the β -catenin destruction complex, is mutated in approximately 70% of all colon cancer cases (Cerami *et al*, 2012). These mutations inactivate APC to allow for accumulation of β -catenin in the nucleus independent of Wnt ligand-based activation. In a similar vein, some cancer cases have mutations in β -catenin that interfere with the ability of the destruction complex to tag it for ubiquitination and degradation, thus evading

complex activity. Another 18% of colon cancer cases harbor mutations in RNF43 (Ring Finger 43), a cell surface protein which normally targets Frizzled receptors for internalization and degradation (Giannakis *et al*, 2014). Loss of RNF43 activity causes an increased concentration of Frizzled receptors at the cell membrane, which leads to overactivation of the Wnt signaling pathway via bona fide Wnt ligand:Frizzled/LRP5/6 interactions. Altogether, between 90-97% of colon cancers have elevated Wnt signaling of the canonical type (Chang *et al*, 2013), the result of early genetic mutation events that are actually the first in a series of driver mutations fundamental to the progression of colon cancer in an intestinal stem cell organoid system (Fumagalli *et al*, 2017).

The functional consequences of activated Wnt signaling in colon cancer has been the focus of a multitude of studies, many of which have used methods that eliminate Wnt signaling in cancer cells or normal stem cells in the intestine. For example, strong overexpression of dominant negative LEF/TCF (dnLEF/dnTCF) transcription factors in colon cancer cell lines can completely interfere with oncogenic Wnt signaling. These dnLEF/TCFs lack the β -catenin binding domain and as such they bind to Wnt target genes and block β -catenin access for regulation. High levels of dnLEF/TCF expression rapidly stops cell growth of colon cancer cells *in vitro* by causing a strong stall in the G1 phase of the cell cycle (Tetsu & McCormick, 1999; Van de Wetering *et al*, 2002). Genetic deletion of the LEF/TCFs *in vivo* in stem cells of the mouse intestine leads to a similar result in that stem cell self-renewal and proliferation are halted, leading to catastrophic loss of the epithelial lining of the intestine and mortality soon thereafter (Korinek *et al*, 1998). These dramatic results demonstrate the fundamental importance of Wnt signaling in driving proliferation in stem cells and cancer, however, they used non-physiologic measures to do so and therefore reveal little of what Wnt signaling is actually doing in cells. Recently, our laboratory explored the physiology of Wnt signaling in human colon cancer cells

using less drastic measures. SW480 colon cancer cells were transduced to express moderate, physiological levels of dominant negative forms of LEF1 and TCF1 – levels that approximate the expression levels of endogenous, intact LEF/TCFs. No significant difference in cell proliferation were observed between the parental and dominant negative LEF/TCF-expressing cell lines *in vitro*, but interestingly, subcutaneous xenograft tumors developed from these cells revealed the dominant negative tumors to be significantly smaller and less vascularized in comparison to the parental (Pate *et al*, 2014). These studies revealed that Wnt signaling drives pathways for angiogenesis and metabolism in addition to cell cycle progression and proliferation. This thesis continues this line of investigation to show that cellular phenotypes of mobility, tumor heterogeneity and cellular signaling are also Wnt-regulated.

Because of the importance of the Wnt signaling pathway in colon cancer, much attention has been given to identifying small molecule agonists or antagonists of Wnt pathway components. These compounds broadly target three groups: the destruction complex, Wnt ligand:receptor interactions, and β -catenin:transcription factor interactions (Kahn, 2014). In the first group, compounds aim to enhance destruction complex activity by inhibiting the activities of negative regulators of the complex, specifically Dishevelled and Tankyrase (Dvl & Tankyrase, Figure 1). Dishevelled bridges the destruction complex to the Frizzled receptor when a Wnt ligand is bound to the cell membrane through a series of phosphorylation events that lead to a significant decrease in Destruction Complex activity. Tankyrase is a kinase that tags the Destruction Complex scaffold protein Axin for degradation, causing the complex to disassociate. Inhibitors of Dishevelled or Tankyrase therefore enable accumulation of β -catenin in cells. The second group of compounds affects the ability of Wnt ligands to activate signaling, either by preventing Wnts from being secreted in the first place, or by preventing ligands from binding to Frizzled receptors. Lastly, a third group of inhibitors interfere with interactions between β -catenin and LEF/TCF

transcription factors and/or transcription co-regulatory factors such as the co-activators CBP and p300. While these compounds have shown some efficacy in normal tissue homeostasis in animal models, little success has been seen with limiting colon cancer development in vivo. As almost all of these compounds have not advanced beyond Phase I of clinical trials, it remains to be seen if any will be clinically useful. The compound currently furthest along in clinical trials is PRI-724, which inhibits the association of transcription co-activator CBP with β -catenin, and thus decreases expression of Wnt target genes. PRI-724 has advanced to phase II clinical trials, but the study is ongoing and outcomes are not yet reported (Kahn, 2014).

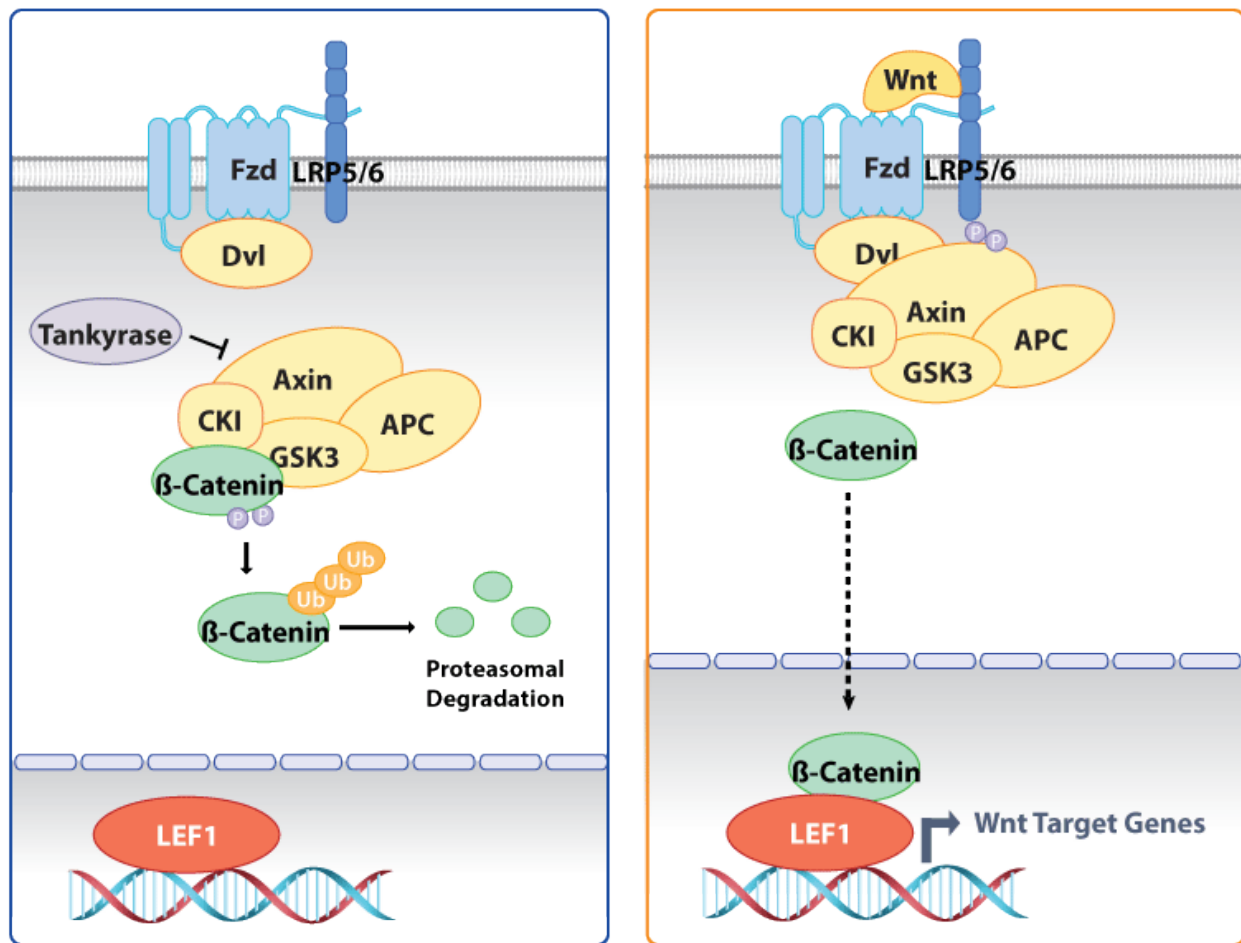


Figure 1.1 β -Catenin dependent Wnt signaling pathway.

Wnt-Off: In the absence of a Wnt ligand, β -Catenin is targeted for proteasomal degradation by a group of proteins collectively referred to as the Destruction Complex (Axin, CKI, GSK3, APC). **Wnt-On:** When a Wnt ligand binds to a Frizzled and LRP5/6 receptor complex at the cell membrane, the Destruction Complex is localized to the cell membrane and inhibited from being active, allowing β -Catenin levels to accumulate and translocate to the nucleus, where it binds to LEF/TCF transcription factors to drive expression of Wnt target genes.

The colon crypt

One key role of the Wnt signaling pathway in normal growth and development is the establishment and maintenance of the epithelial lining of the intestine from the esophagus through the small intestine and colon to the rectum. The colon part of the intestinal tract consists of five tissue layers – serosa on the outside enclosing two muscle layers, then a submucosa, and finally the mucosa. The mucosa consists of a single layer of epithelial cells that is directly in contact with the lumen. Amazingly, this single layer of epithelial cells functions as the interface-barrier to the microbiome and the outside world. The mucosal layer is predominantly comprised of secretory and absorptive epithelial cells, fully differentiated cells that are enriched at the luminal surface of the colon. The mucosal layer is also punctuated by a regular pattern of tissue invaginations known as crypts. At the base of each of these crypt structures, ten to thirteen proliferative stem cells with moderate levels of Wnt signaling activity are interspersed next to Wnt-high nurse cells - differentiated cells that are the source of important survival signals they deliver to stem cells (Barker *et al*, 2012). As the stem cells divide and give rise to progeny, the daughter cells decrease in Wnt signaling activity. They therefore lose stemness and travel upwards toward the lumen, differentiating into transit amplifying cells and then fully differentiated states and phenotypes near the surface of the lumen. Multiple specialized differentiated cell types exist in the epithelium, the main lineages being enterocytes that absorb water and nutrients, goblet cells that secrete mucin and other lubricants, and the hormone-secreting enteroendocrine cells. These differentiated cells do not live long, only a couple of days, and as they die off, they are replaced by newly differentiated cells traveling up the crypt; a remarkable conveyor-belt-like process of tissue renewal that results in the intestinal epithelium turning over completely every five to seven days. Multiple signaling systems are responsible for establishing and maintaining these highly regulated and proliferative crypt dynamics. Of these,

Wnt and Notch signaling have been shown to be the most dominant at the base of the crypt, tapering off towards the differentiated cells at the top. As mentioned above, Wnt signaling is required for establishment of the intestinal crypt; knockdown experiments of TCF4, a member of the Wnt-specific LEF/TCF transcription factor family, in mice showed that normal proliferative crypts do not form in neonatal knockout mice (Van de Wetering *et al*, 2002). Imbalance in the crosstalk between signaling pathways in the crypt, such as overactivated Wnt signaling, can lead to a hyperproliferation and blocks to differentiation. With additional driver mutations that accumulate in these cells, the accumulated damage can lead to formation of a hyperplastic growth that eventually advances to a tumor.

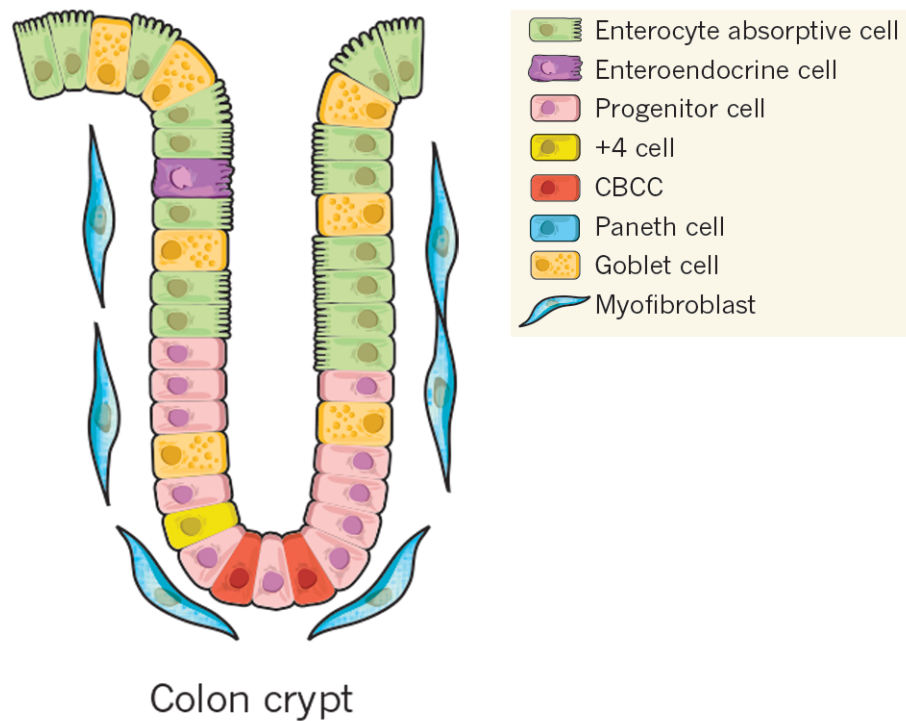


Figure 1.2. Structure of the colon crypt.

At the base of the crypt, Crypt Base Columnar Cells (CBCC), characterized to be the stem cells of the crypt, are interspersed with other supporting cell types. As they divide, daughter cells partially differentiate and are pushed up the sides of the crypt into the Transit Amplifying zone (labeled as progenitor cells) before fully differentiating at the top of the crypt. Figure adapted from (Medema & Vermeulen, 2011).

Cancer metabolism

When Wnt signaling is overactive, a number of oncogenic pathways may be activated, and among these is cellular metabolism. The metabolic state of a cell within a tissue varies based on nutrient level, oxygen tension, and signaling from other cells. For differentiated tissues, the majority of cells utilize oxidative phosphorylation as a predominant mode of metabolism where adenosine triphosphate (ATP) is efficiently produced through the conversion of glucose and oxygen in the tricarboxylic acid cycle (TCA). Low oxygen conditions force cells to switch to anaerobic glycolysis, a form of metabolism in which glucose is fermented into lactate. This process produces significantly fewer ATP per glucose molecule and is thus considered less energetically favorable. However, rapidly proliferating cancer cells, even in the presence of oxygen, predominantly utilize the glycolytic pathway. This mode of metabolism is termed aerobic glycolysis, or Warburg metabolism, named after Otto Warburg who first made this observation in the 1920's (Heiden *et al*, 2009).

The “choice” of a tumor to utilize Warburg metabolism seems paradoxical, but there are a number of reasons why cancer cells benefit from aerobic glycolysis (Cantor & Sabatini, 2012; Heiden *et al*, 2009). One reason is that the rapid proliferation rate of the cancer cells requires a source of biosynthetic intermediates necessary for production of enough protein, nucleic acids and lipids to produce a daughter cell. Thus, tumor cells upregulate pathways to intake more nutrients from the microenvironment, and they utilize catabolic pathways that do not result in the complete oxidation-destruction of their carbon resources. In utilizing the glycolytic pathway, a number of metabolic intermediates can be shunted away from energy production and towards biomass production; including ribose, a product of the pentose phosphate pathway used for nucleotide synthesis, and NADPH, used in lipid synthesis. Another reason is that oxidative phosphorylation often leads to the production of Reactive Oxygen Species (ROS), especially in

the low oxygen tension conditions of the tumor microenvironment. Avoiding the production of ROS limits DNA damage and signals for apoptosis. Lastly, increasing evidence suggests that aerobic glycolysis facilitates interaction with the tumor microenvironment. Lactate, produced by glycolytic cells, may be secreted to create an acidic tumor microenvironment that favors invasion and migration and several studies have shown that this drives angiogenesis (Porporato *et al*, 2012; Sonveaux *et al*, 2012). Additionally, secreted lactate remains a rich carbon source that can be taken up by neighboring cancer cells and utilized as metabolic fuel for biomass production. This sharing of metabolites between cells establishes a relationship that has been termed “metabolic symbiosis” (Mendoza-Juez *et al*, 2012). As discussed in Chapter 2, the metabolic program used by a cell is not a terminal decision; it changes in response to nutrient availability and specific signals from nearby cells. Thus within a tumor, despite near genetic homogeneity between cells, heterogeneous patterns of metabolism can be observed as subsets of cells adopt very different programs of metabolism.

The Waterman lab has demonstrated that Wnt signaling directly regulates metabolic reprogramming in colon cancer through the expression of target gene pyruvate dehydrogenase kinase 1 (*PDK1*) (Pate *et al*, 2014). PDK1 is a kinase that is resident in mitochondria; this kinase phosphorylates and inhibits its main target pyruvate dehydrogenase (PDH), a mitochondrial enzyme that directs the first step in oxidative phosphorylation. Inhibition of PDH shuts down mitochondria and forces cells to utilize glycolysis. PDK1 is also regulated in a β -catenin independent, non-canonical Wnt pathway, through activation of the AKT kinase (Esen *et al*, 2013). Additionally, in Sprowl-Tanio, et al, the gene *SLC16A1*, encoding lactate transporter MCT1, was also shown to be a direct Wnt target, suggesting that Wnt signaling reprograms cancer cells towards aerobic glycolysis by upregulating the expression of multiple factors in the glycolytic system (Sprowl-Tanio, 2016). The work presented here shows that heterogeneous

patterns of metabolism are resident in colon tumors (mouse and human) and that this heterogeneity tracks with patterns of high and low Wnt signaling activity – a pattern that we hypothesize is established through cell-to-cell communication via signals that include Wnt (Chapter 2). Clearly, there is potential for metabolic symbiosis between tumor cells of very different metabolic indices. The mathematical modeling presented in Chapter 2 predicts that disruption of this signaling network and the pattern it produces by combinatorially inhibiting Wnt signaling and glycolytic metabolism in concert might produce synergistic, inhibitory effects on tumorigenesis.

Colon cancer progression to metastasis

In the last few decades, a large body of research has accumulated which detail the mechanisms towards primary tumor formation in solid tumor settings. However, the biological processes driving towards metastatic disease are less well understood. Additionally, while existing methods to detect and treat early stage cancers have shown to be very effective in treating patients with solid tumors, the overwhelming majority of cancer-associated deaths are due to late-stage, metastatic disease, which remains incurable with today's treatment regimens. (Labert, et al) Therefore, understanding how patient tumors transition to a more invasive and migratory phenotype is of tremendous academic and pharmaceutical interest.

The process by which cancer cells are disseminated from the primary tumor and seeded at distant sites is broadly termed the invasion-metastasis cascade. The first step in enabling cancer cells to be more invasive and metastatic is cell-state change referred to as epithelial-mesenchymal transition, or EMT. EMT confers upon epithelial cells characteristics that allow increased motility, invasion, and extracellular matrix degradation. This process was first observed in embryonic development and has been characterized as critical in normal developmental

processes such as gastrulation, neural crest formation, and other developmental morphogenic events. In the context of cancer, tumor cells acquire some of these characteristics such as certain mesenchymal traits while maintaining certain epithelial ones, resulting in a mixed phenotype (Valastyan & Weinberg, 2011). Additionally, the stroma surrounding invasive tumors adopts a wounded tissue phenotype, reacting to physiological changes in, and signaling by the tumor cells by eliciting an immune response as well as production of signaling ligands including Wnts, TGFβs, and interleukins.

The nuclear localization of β-catenin is commonly used as a marker for Wnt signaling activity. One might predict that colon tumors that derive from genetically activated Wnt signaling might exhibit a homogeneous, uniform nuclear staining of abundant β-catenin. However, in primary colon cancer patient samples, staining for β-catenin shows significant heterogeneity in subcellular localization and overall cellular levels, despite the fact that all the tumor cells harbor the same Wnt pathway activating mutation, in what has been termed the “β-catenin paradox” (Fodde & Brabletz, 2007). Part of this heterogeneity might derive from well-differentiated tumor cells that are attempting to recapitulate the crypt architecture, with β-catenin localized to the membrane to associate with adherens junction protein E-cadherin. Other cells within the same field of the tumor express strong levels of nuclear β-catenin, particularly at the invasive edge. Studies of these Wnt-active cells at the invasive edge have shown elevated expression of Wnt target genes that associate with invasion, such as Cyclin D1, Fascin and L1 (Jung *et al*, 2001; Vignjevic *et al*, 2007; Gaver *et al*, 2005). Importantly, these Wnt-high cells have been shown in limiting dilution assays to be better able to establish new tumor colonies in vitro. Additionally, Snail & Twist, genes that are key regulators of the EMT process, have been found to be directly regulate certain Wnt targets in different model systems including cancer and organismal development (ten Berge *et al*, 2008).

These findings seem to argue that elevated Wnt signaling drives metastatic programming at the invasive edge of a tumor – and indeed, there are many reviews and studies that posit this idea. However, more recent studies challenge this conclusion, including a large-scale effort to redefine colon cancer categorization by molecular subtype (Guinney *et al*, 2015). In Guinney *et al*, datasets from multiple studies, totaling over 3000 patients in sum were re-categorized based upon their gene expression profile. The four resulting consensus molecular subtypes, referred to as CMS1, 2, 3, and 4 are: MSI (microsatellite instability) Immune, Canonical, Metabolic, and Mesenchymal respectively. CMS2, or Canonical, comprises the largest proportion of patient samples (37%), and this subtype is characterized by high Wnt signaling and Myc activity. Paradoxically, patients with CMS2 colon cancers have the best prognosis of all the subtypes. By contrast, CMS4, or the “Mesenchymal”, marks cancers with high stromal infiltration, TGF β activation, and angiogenesis – all hallmarks of the most invasive and aggressive cancers. Interestingly, CMS4 has the lowest expression of Wnt target genes of the four subtypes.

This finding has been replicated in the laboratory setting using colon cancer cell line HT29 expressing dominant negative TCF4, which serves to decrease Wnt signaling by preventing β -catenin from being recruited to Wnt target genes. These Wnt-low cells produced more metastatic nodes that were also larger than that of control cells (Seth & Ruiz i Altaba, 2016). However, the authors point out that other cell lines did not necessarily react in the same way with decreased Wnt signaling, suggesting that some heterogeneity in response exists between cell lines. Additionally, another group showed that Wnt target genes were methylated in advanced patient samples, thus decreasing Wnt signaling activity in spite of the presence of β -catenin in the nucleus (de Sousa E Melo *et al*, 2011). In Chapter 3, we will present additional evidence to argue that decreased Wnt signaling leads to more invasive and aggressive tumors using an orthotopic tumor model.

Chapter guide

This thesis will focus on how Wnt signaling influences heterogeneous patterns of metabolism and invasion in colon cancer. In chapter 2, we define a mathematical model of colon cancer metabolism that describes a three-dimensional pattern of spatial heterogeneity guided by heterogeneity in Wnt signaling activity. We validate this model and use it to make predictions of system responses to perturbations, which we confirm in *in vitro* experiments. Chapter 3 focuses on the role of Wnt signaling in colon cancer invasion. We perturb the Wnt signaling pathway both upstream and downstream of the β -catenin destruction complex and demonstrate that in an orthotopic tumor model, decreased Wnt signaling leads to a more invasive and migratory phenotype.

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CHAPTER 2

Mathematical modeling links Wnt signaling to emergent patterns of metabolism in colon cancer

Abstract

Cell-intrinsic metabolic reprogramming is a hallmark of cancer that provides anabolic support to cell proliferation. How reprogramming influences tumor heterogeneity or drug sensitivities is not well understood. Here we report a self-organizing spatial pattern of glycolysis in xenograft colon tumors where Pyruvate Dehydrogenase Kinase (PDK1), a negative regulator of oxidative phosphorylation, is highly active in clusters of cells arranged in a spotted array. To understand this pattern, we developed a reaction-diffusion model that incorporates Wnt signaling, a pathway known to upregulate PDK1 and Warburg metabolism. Partial interference with Wnt alters the size and intensity of the spotted pattern in tumors and in the model. The model predicts that Wnt inhibition should trigger an increase in proteins that enhance the range of Wnt ligand diffusion. Not only was this prediction validated in xenograft tumors but similar patterns also emerge in radiochemotherapy-treated colorectal cancer. The model also predicts that inhibitors that target glycolysis or Wnt signaling in combination should synergize and be more effective than each treatment individually. We validated this prediction in 3D colon tumor spheroids.

Introduction

A hallmark feature of many cancers is “aerobic glycolysis,” or the Warburg effect, a form of metabolism whereby cells skew their balance of cellular metabolism away from oxidative phosphorylation (OXPHOS) to favor glycolysis, despite the availability of sufficient levels of

oxygen (Warburg et al., 1956). Cellular emphasis on Warburg metabolism is intriguing since it is much less efficient than OXPHOS in producing energy (four molecules of ATP produced by glycolysis for each molecule of glucose consumed versus 36 molecules by OXPHOS). Warburg metabolism has been hypothesized to be beneficial because glycolytic intermediates can be used as biosynthetic building blocks for cell growth and proliferation, suggesting that this mode of glucose utilization is essential for actively expanding tumors (Pavlova & Thompson, 2016; Heiden *et al*, 2009). There are other effects as well: the production of lactate acidifies the tumor microenvironment, an environmental condition that can enhance tumor invasiveness (Gatenby & Gillies, 2004) and induces angiogenic responses for increased delivery of glucose, oxygen and other nutrients (Végran *et al*, 2011), effects that are growth-promoting and provide cancer cells with a fitness advantage.

Oncogenic, overactive Wnt signaling has been recently linked to metabolic and nutrient programming in tumors. For example, in colon cancer, Wnt signaling is proposed to increase expression of key glycolytic factors that enhance Warburg metabolism and angiogenesis (Pate *et al*, 2014). Oncogenic Wnt signaling most commonly derives from genetic inactivation of one or more signaling components (e.g. Adenomatous Polyposis Coli, APC), inactivating mutations that cause the pathway to become chronically activated and to trigger overexpression of Wnt target genes. One such target gene is Pyruvate Dehydrogenase Kinase 1 (PDK1), a mitochondrial kinase that inhibits the Pyruvate Dehydrogenase Complex (PDC) via phosphorylation of the component Pyruvate Dehydrogenase (PDH)(Pate *et al*, 2014). Since PDC converts pyruvate to acetyl CoA for mitochondrial respiration, phosphorylation/inhibition of PDH by PDK kinases suppresses OXPHOS modes of metabolism (ATP and CO₂ production) to favor glycolytic modes that produce lactate (Roche *et al*, 2001). Thus, at least in some tissues such as colon, Wnt

signaling elevates PDK1 to suppress OXPHOS and to encourage glycolysis and the production of lactate.

Our previous study of xenograft colon tumors established that oncogenic Wnt signals directly activate PDK1 gene transcription as well as other glycolysis-connected gene targets including the lactate transporter MCT1 (*SLC16A1*) (Pate *et al*, 2014). That Wnt signals might be directly responsible for shaping the metabolic profile of cells is a discovery from multiple studies focused on diseased (e.g. melanoma, breast (Sherwood, 2015)) and normal tissues (Esen *et al*, 2013). At least two types of Wnt signals have been defined. One signal utilizes canonical signaling and beta-catenin-regulated transcription to drive sustained expression of glycolysis regulators. A second signal utilizes a novel Rac-mTORC2 pathway to increase the protein levels of glycolytic enzymes in the cytoplasm (Esen *et al*, 2013). Both signals can be triggered by secreted Wnt ligands, and these, in addition to oncogenic Wnt pathway activities created by genetic mutations, can direct the metabolic and proliferative capacity of colon tumors. However, because metabolism is shaped by the collective activity of multiple pathways and environmental influences – including those that enhance or diminish WNT signaling - there is still much to learn about how signatures of metabolism are established.

Metabolic symbiosis has emerged as a powerful model to explain tumor heterogeneity and survival. As a concept, metabolic symbiosis means that glycolysis is not a singular metabolic choice for cells in a tumor; OXPHOS modes of metabolism may be dominant in subpopulations. The proposed outcome of this heterogeneity is that cooperation between two groups of cancer cells can maximize delivery and consumption of nutrients and minimize the environmental stresses that are imposed on a tumor. Glycolytic cells are likely the dominant consumers of glucose, and their fermentation of this carbon source produces an acidic byproduct (lactate) that must be exported to the tumor microenvironment. Lactate can be angiogenic and thus the

activities of glycolytic cells can be important for delivery of nutrients and growth factors to the tumor microenvironment (Sonveaux *et al*, 2012; Murray & Wilson, 2001). In turn, cancer cells with prominent modes of OXPHOS metabolism can uptake and utilize lactate (and other metabolic byproducts) from neighboring glycolytic cells and metabolize it as a stable source of energy over long-time scales (Epstein *et al*, 2014; De Saedeleer *et al*, 2012). An important example of this is the “Reverse Warburg” effect observed in breast cancer (Martinez-Outschoorn *et al*, 2014). Thus, not all cancer cells show a preference for glycolysis at all times because microenvironmental, spatial, and temporal factors may direct them to emphasize OXPHOS modes of metabolism (Obre & Rossignol, 2015; Sonveaux *et al*, 2008; Pavlides *et al*, 2009). Such back-and-forth influences on glycolysis and OXPHOS create non-genetic tumor heterogeneity, meaning that genetically identical cancer cells might adopt different modes of metabolism depending on cell-intrinsic and extrinsic influences. Identifying these influences and signals, and understanding the spatial and temporal forces that direct their cooperation is important, as metabolic symbiosis is not just a manifestation of tumor heterogeneity, it is likely a fundamental aspect of tumor survival.

In the course of our study of Wnt signaling and glycolysis in xenograft colon tumors, we observed heterogeneous patterns of metabolism. Heterogeneity was observed via immunohistochemical stain of PDK1 activity, a major inhibitor of mitochondrial activity, and immunohistochemical stains of Wnt signaling. In particular, these stains revealed a pattern of discrete clusters of cells, or “spots”, indicating groups of cells with different levels of glycolysis relative to OXPHOS, and differences in Wnt activity. We refer to these groups of cells as “glycolytic (P_g)” and “OXPHOS (P_o)” to indicate that they differ in the relative balance between these two modes of metabolism. As the spots of PDK activity and Wnt signaling appeared as a regular array in space, we hypothesized that metabolism was subject to rules of pattern formation

and we therefore developed a mathematical model with spatial features to study the organization of this pattern. Using reaction-diffusion equations to describe the dynamics of Wnt signaling, nutrients, cell substrates and the populations of the different metabolic cell types, we elucidate the mechanisms that underlie this spatial pattern and find good agreement between the model and experiments. We lastly exploit this knowledge to identify promising therapeutic strategies.

Results

A spotted pattern of PDK activity and LEF-1 expression in xenograft tumors

Xenograft tumors of (human) colon cancer cell line SW480 (containing homozygous loss-of-function mutations in APC and intrinsically activated Wnt signaling) were produced by subcutaneous injection of cells in immunocompromised mice. To investigate metabolic changes within the tumor, 5.0-6.0 μm serial sections of formalin-fixed, paraffin-embedded tumor were probed with antisera specific for phosphorylated-PDH (pPDH) as an indicator of PDK activity, and Lymphoid Enhancer Factor-1 (LEF-1), a Wnt signaling transcription factor and Wnt target gene (Hovanes *et al*, 2001). Both stains revealed a general, high level of pPDH and LEF-1, but also heterogeneity in the form of a prominent spotted pattern (Figure 2.1 A-B, where “mock” refers to tumors from parental SW480 cells). The pattern appeared as discrete localized clusters of cells with increased levels of pPDH, and these clusters, or spots, were detected at seemingly regular intervals. Since pPDH staining is an indicator of PDK activity, the darker stained cell clusters indicate increased rates of glycolysis relative to neighboring cells. The lighter staining, neighboring cells are likely utilizing greater levels of OXPHOS since PDH is less inhibited (less phosphorylated). Since it is known that lactate, the secreted byproduct of glycolytic cells, can be imported into neighboring cells for use as an OXPHOS metabolic fuel, this pattern points to a

potential symbiotic spatial relationship between these two cell populations, a metabolic relationship proposed by other groups studying cancer metabolism (Sonveaux *et al*, 2008; Pavlides *et al*, 2009) - glycolytic cells that are localized in distinct regions uptake glucose and produce metabolic fuel such as lactate for surrounding oxidative cells, a mode of sharing and metabolic distribution. In addition to the spotted metabolic pattern, an overlying gradient in pPDH staining level was observed wherein the spots were more densely arrayed toward the outer edges of the tumor, decreasing in frequency towards the center of the tumor, suggesting that more glycolysis occurs at the outer regions of the tumor where there is more vasculature (Pate *et al*, 2014; Appendix B Supplementary Material A1.4).

As we previously identified a link between Wnt signaling and glycolysis, we used immunohistochemistry to assess the activity of the canonical pathway. Interestingly, a spotted pattern was also evident in immunohistochemical stains of the Wnt target gene and effector, LEF-1 (Figure 2.1B), indicating that the spotted array might be linked to a pattern of heterogeneity in Wnt signaling. Automated image analysis was used to quantify the spatial parameters of each of the spotted patterns (see Appendix B Supplementary Methods A1.1-A1.3 and Figures A1.4-A1.7). Figure 2.1C shows the quantification of each spot area and distances to each nearest neighbor, showing that the parameters of the spots for pPDH and LEF-1 are very similar (data on the number of cells per spot is given in Appendix B A1.15 in Supplementary Material). We found that the total area fraction of tumor covered by each set of spots in Figures 1A and B was nearly the same (pPDH: 21.2%; LEF-1: 20.2%). To assess the overlap between the pPDH and LEF-1 spots in the serial sections in Figures 1A-B (see Appendix B A1.2 in Supplementary Material), we counted spots that partially overlap and found that there was a significant overlap of 65-77% in the spatial arrangement of the pPDH and LEF-1 spots (Figure EV2.1). We also found that the area fraction of tumor covered by the overlapping region (pPDH

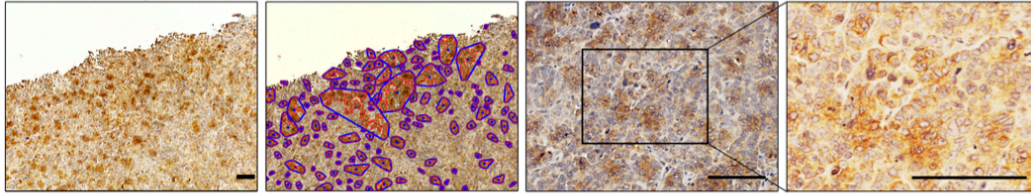
spots that are contained in LEF1 spots and LEF1 spots that are contained in pPDH spots) is 7.4%.

To determine the significance of the association between the spots (see Appendix B A1.2 for details), we analyzed staining in pairs of pixels, assuming that each pixel location in one section corresponds to the same pixel location in the other section. We performed a Cochran-Mantel-Haenszel test (Cochran, 1954; Mantel & Haenszel, 1959) and found that the pPDH and LEF-1 spots are significantly associated with one another ($p < 0.0001$). While this analysis is not definitive because it does not guarantee that the paired pixels are in the same cell (we found it difficult to directly match cells in the serial sections) and also does not take into account spatial variation in spot densities, it suggests that the patterned heterogeneity of metabolism and Wnt signaling are linked.

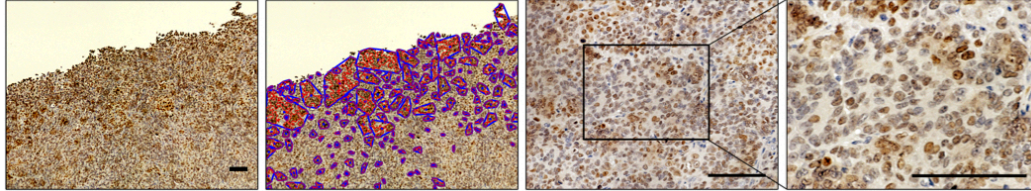
Xenograft tumors from colon cancer cell lines are different from primary human colon cancers, the latter of which develop in immunocompetent patients and contain a greater variety of cell types and stromal involvement. We asked whether PDK activity and Wnt signaling were uniform or heterogeneous in primary human colon tumors. In Figure 2.1D, pPDH and LEF-1 stains in primary human colon tumors compared to normal colon tissue demonstrate that there is indeed significant spatial heterogeneity in human tumors. In addition, serial sections of a primary human colon tumor stained with pPDH and LEF-1 antisera show a striking concordance in expression pattern (Figure 2.1E). While a regular spotted array is not apparent in primary tumors like it is in xenografts, the heterogeneous pattern of clusters of cells with high glycolysis and high LEF-1 in the epithelial portion of the tumor suggests that although xenograft tumors are artificial and have a different microenvironment, understanding the mechanisms underlying the observed spatial patterning in xenograft tumors can provide insight into the forces that create non-genetic heterogeneity in primary human colon tumors.

SW480 Mock Xenograft Tumor

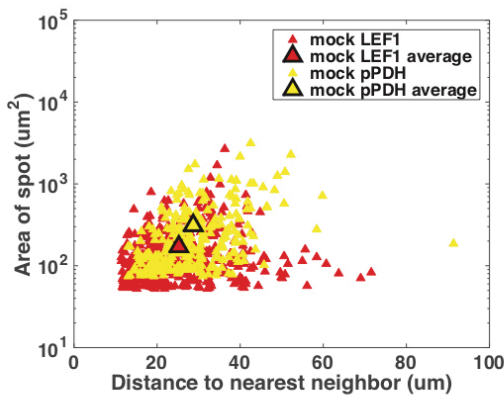
A. Phosphorylated PDH



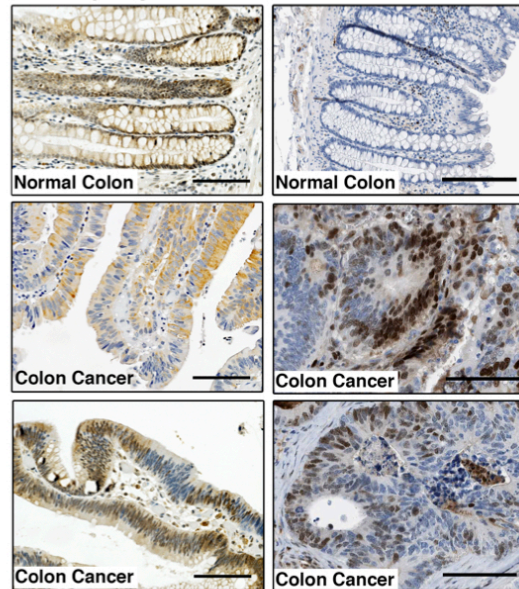
B. LEF1



C.



D. Phosphorylated PDH LEF1



E. Phosphorylated PDH

LEF1

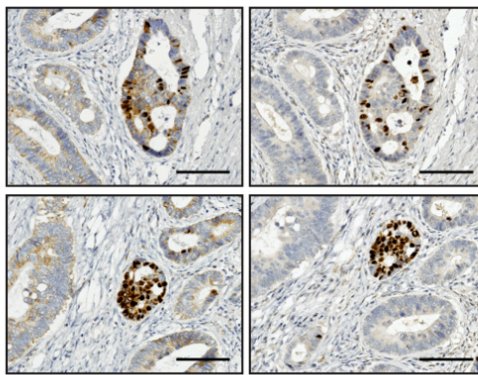
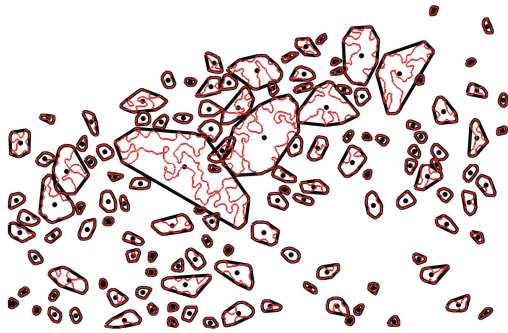
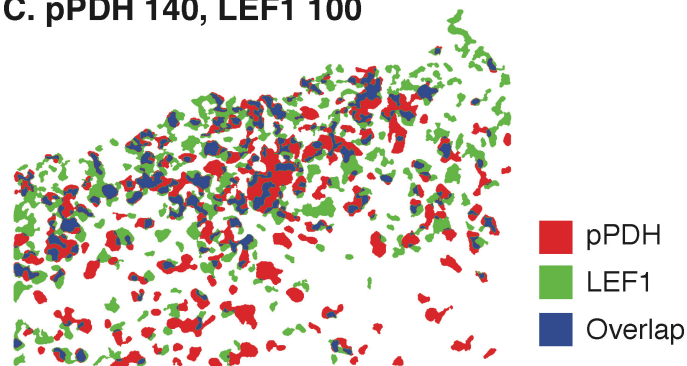
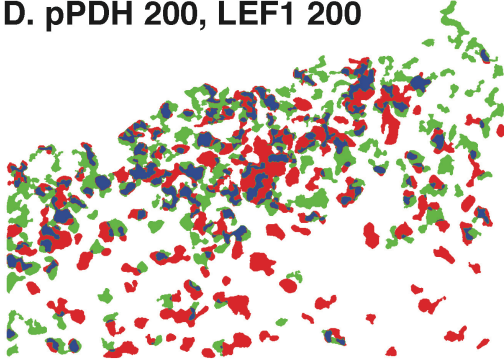
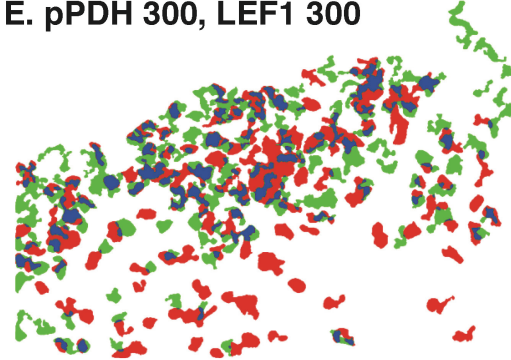


Figure 2.1. SW480 xenograft tumors reveal a spotted pattern of metabolic heterogeneity.

SW480 cells lentivirally transduced with empty pCDH vector (mock) were subcutaneously injected in immunocompromised mice. The resulting tumors were stained for (A) phosphorylated pyruvate dehydrogenase (pPDH) and counterstained with hematoxylin or (B) lymphoid enhancer factor 1 (LEF-1). Scalebars indicate 100 μm in the series of 4X, 20X and 40X images. (C) Image analysis of spot size versus distance of spot to nearest neighbor, using analyzed 20x images (second panels of A and B). The outlined data points indicate the average distance and area for pPDH and LEF-1 spots. Results show that quantifiable features of the spotted patterns in pPDH and LEF-1 are similar. (D) Colorectal carcinoma patient samples (tumors 1, 2, and 3) stained for pPDH (top) and LEF-1 (bottom) show spatial heterogeneity in expression levels. Scalebars are 100 μm (LEF-1 samples from (Uhlen et al, 2015)). (E) Serial section of human colorectal carcinoma stained with pPDH and LEF-1 antisera. Scale bar is 100 μm . In (A) and (B), the red curves denote spot contours and the blue curves denote convex hulls, which group together spots that are sufficiently close to one another (see Appendix B A1).

A. Mock pPDH contours**B. Mock LEF1 contours****C. pPDH 140, LEF1 100****D. pPDH 200, LEF1 200****E. pPDH 300, LEF1 300**

F.	Threshold		pPDH			LEF1		
	pPDH	LEF1	# of spots	# over-lapping	%	# of spots	# over-lapping	%
	140	100	131	99	75.57%	173	114	65.90%
	200	200	108	79	73.15%	111	81	72.97%
	300	300	80	59	73.75%	84	65	77.38%

Expanded View Figure 2.1. Overlap of pPDH and LEF1 spots in xenograft tumors

Convex hull image analysis of serial sections of SW480 Mock xenograft tumors stained for pPDH and LEF1, as shown in Figure 2.1A and 1B, second panels. (A & B) Isolated contour maps with convex hull outlines for pPDH and LEF1. (C-E) pPDH and LEF1 contour maps were overlaid on each other and overlapped regions highlighted in blue. Different thresholds for spot detection were tested; each threshold condition revealed between 65-77% overlap between pPDH and LEF1 spots. (F) Summary of overlap results.

Reaction-Diffusion Modeling Mimics the Self-Organizing Patterns of PDK activity and Wnt Signaling in Xenograft Tumors

The regular spotted pattern in the xenograft IHC stains suggests the development of a mathematical model consisting of reaction-diffusion equations similar to those first described by Alan Turing (Turing, 1952). Turing's equations describe how an initial perturbation in the concentrations of chemicals, or morphogens, can grow in the presence of diffusion (the Turing instability) and self-organize into a spatial pattern. Because diffusion is normally a stabilizing process, diffusion-driven instabilities occur only under certain conditions (Murray, 2003; Kondo & Miura, 2010). Recently, Marcon et al. (Marcon *et al*, 2016) performed an automated analysis of Turing-type reaction diffusion equations and identified general conditions for which instabilities could occur. When two species are considered (e.g., activator-inhibitor models), the species need to diffuse at sufficiently different rates as observed previously (e.g., short-range activator, long range inhibitor). However, when multiple diffusing species are present, instabilities can be obtained even for arbitrary diffusivities. Here, we focus on reaction-diffusion models that link cell metabolic phenotypes with Wnt signaling and argue that conditions for instability are met in colon cancer.

Despite the fact that colon cancers are most often driven by genetically activated Wnt signaling, a cell-autonomous condition, there are numerous studies that highlight that secreted Wnt ligands and their bona fide signaling through Frizzled receptors on the plasma membrane are abundantly active in human colon cancer and that they influence colon cancer biology (Voloshanenko *et al*, 2013; Giannakis *et al*, 2014; Seshagiri *et al*, 2012; Holcombe *et al*, 2002). Importantly, Wnt ligands are highly constrained in their diffusion, traveling only 1-2 cells from the origin of their secretion, meaning that the range of their influence is highly localized (Farin *et al*, 2016). This is in contrast to the longer-range diffusion properties of known, secreted inhibitors

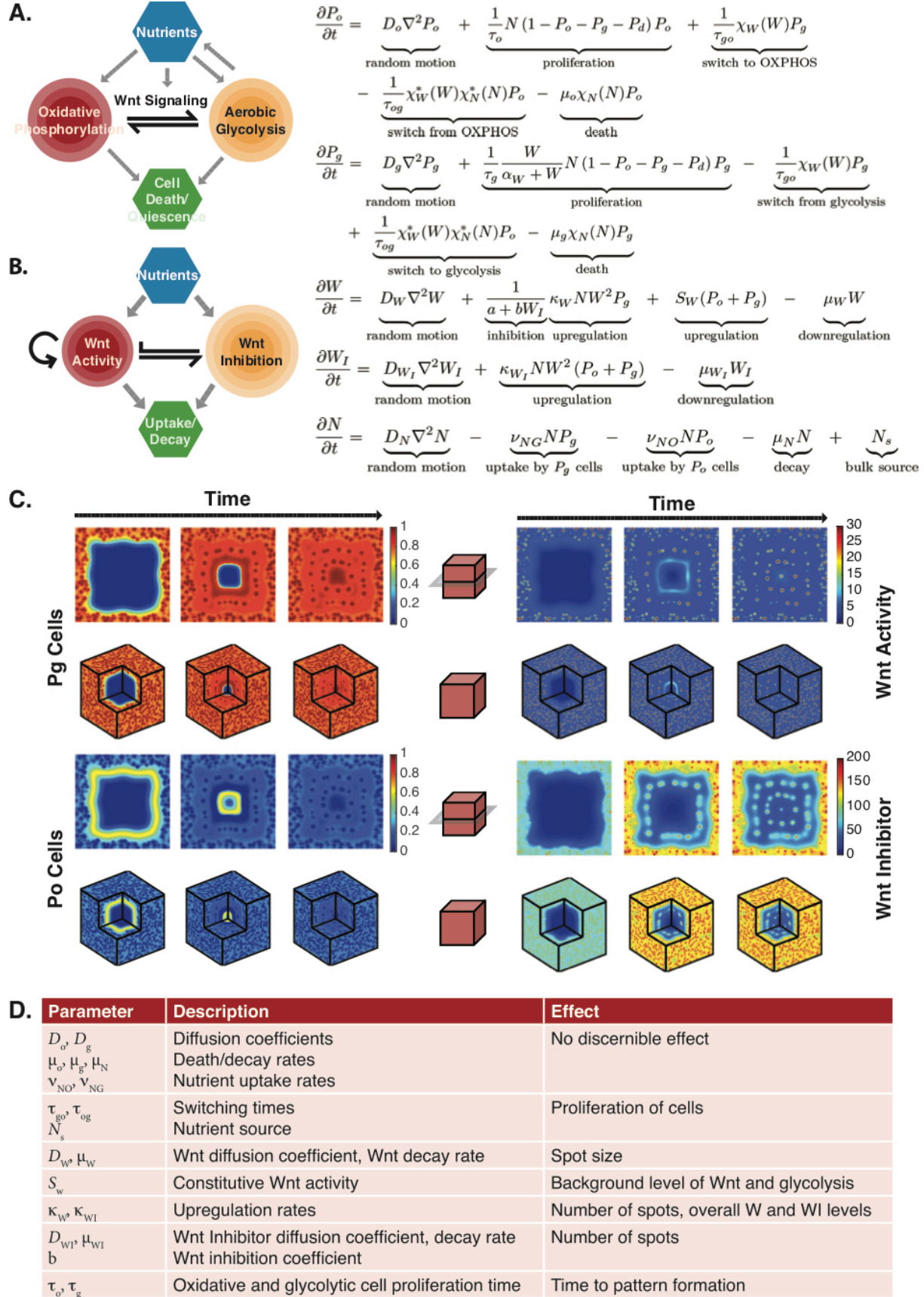


Figure 2.2. A mathematical model for Wnt signaling regulation of metabolism.

This set of reaction-diffusion equations describes the change over time of oxidative (Po) and glycolytic (Pg) cell populations, Wnt signaling activity (W), and Wnt inhibitor activity (WI). (A) The cells can diffuse, proliferate, “switch” metabolism programs depending on Wnt signaling activity and nutrient levels, and die from lack of nutrient (N). (B) Wnt and Wnt inhibitor activity equations are based on the Gierer-Meinhardt activator-inhibitor model. The Wnt signal diffuses short range relative to the longer-range diffusion of the Wnt inhibitor. Wnt also auto-upregulates its activity in glycolytic cells at a rate proportional to nutrient level, is inhibited by a Wnt inhibitor, is constitutively upregulated in both cell types, and decays (downregulation term). The Wnt inhibitor diffuses long range, is nonlinearly upregulated by Wnt, and decays. Equations for nutrient and dead cells (Pd) are not shown; their descriptions are in the main text. (C) Three-dimensional numerical simulations that model the spatial distribution and level of glycolytic and oxidative cells, Wnt, and Wnt inhibitor reveal an emergent self-organizing pattern of metabolic heterogeneity (spots). The simulations shown depict the heterogeneity in a 3D and 2D representation. The 3D representation includes a portion of the “tumor” removed to visualize the interior of the domain. The 2D representation is a horizontal slice of the respective 3D simulation in the center of the domain. (D) Summary of parameter effects on the spotted pattern.

that bind to Wnt ligands and/or interfere with receptor binding (i.e. DKK, SFRPs)(Mii & Taira, 2009, 2011). Some of these inhibitors are Wnt target genes, for example DKK4, an inhibitor that is expressed in human colon cancer, and SFRP2, a secreted Wnt inhibitor induced by Wnt4 in the developing kidney (Lescher *et al*, 1998). Thus, a Turing-type model, wherein short-range nonlinear activation by Wnt ligands and long-range inhibition of their activities, fits well with the known physical and regulatory properties of Wnts and their inhibitors. Moreover, this type of model is capable of forming patterns (Murray, 2003; Kondo & Miura, 2010). Previously, Turing models have been used to describe Wnt-directed patterns in a variety of contexts including hair follicles (Sick *et al*, 2006; Kondo & Miura, 2010), colon crypts (Zhang *et al*, 2012), limb development (Raspopovic *et al*, 2014), and stem-cell driven cancers (Youssefpour *et al*, 2012; Yan *et al*, 2016). Additionally, the BMP family, known to be Wnt signaling antagonists, have been recently described to direct murine intestinal patterning (Walton *et al*, 2015).

We therefore developed a Turing-type model for simulating the spatial and temporal dynamics of different metabolic phenotypes, nutrients, and Wnt signaling activity through a system of reaction-diffusion equations (Figures 2A and 2B; Appendix B Supplementary Methods A2 and A3). We included populations of cells that perform less glycolysis, which we refer to as oxidative (P_o) cells, and those that perform more glycolysis, which are termed glycolytic (P_g) cells. Both types of cells may divide, die and undergo random movement. Depending on local environmental conditions, the cells may switch from one phenotype to the other. A diffusible substrate (N), which accounts for concentrations of nutrients such as glucose and growth factors, regulates cell division and death rates (χ_N), switching rates between the two cell types (χ_w from glycolysis to oxphos and $\chi_w^* \chi_N^*$ from oxphos to glycolysis), and the ability of cells to generate Wnt (W) and Wnt inhibitor (W_i) activities. The Wnt and Wnt inhibitor equations are based on

the Gierer-Meinhardt activator-inhibitor model (Gierer & Meinhardt, 1972), where Wnt is the short-range activator which produces a long-range factor that inhibits Wnt activity (e.g. SFRP2). Because Wnt signaling is assumed to be constitutively active, both oxphos and glycolytic cells are assumed to upregulate Wnt activity at the rate S_W . In the model shown in Figures 2A and 2B, the glycolytic cell proliferation rates and the metabolic switching rates (χ_W and χ_W^*) also depend on Wnt activity where a higher activity level increases cell propensities for glycolysis over OXPHOS, if sufficient nutrients are available (χ_N^*). To model the angiogenic response of the mouse vasculature to the lactate produced by the glycolytic cells and the accompanying increased delivery of nutrients, we introduced sources N_S that increase the amount of nutrient in the system proportionally to the amount of glycolytic activity of the cells. We also assumed that the vascular density was largest at the domain boundary and thus we modified the boundary conditions for nutrients analogously. See Appendix B Supplementary Methods for the precise functional relationships (Appendix B A2).

We also considered a more general in vivo model, which accounted for PDK activity, hypoxia-inducible transcription factor concentrations (HIF1 α), lactate concentration and cross-feeding between glycolytic and oxphos cells (Appendix B Supplementary Material A3). Assuming that Wnt and HIFs promote PDK expression/activity (Kim *et al*, 2006; Prigione *et al*, 2014; Pate *et al*, 2014), that PDK activity promotes lactate production (Pate *et al*, 2014), that lactate increases HIF1 α expression levels and provides a source of fuel for oxphos cells (Epstein *et al*, 2014; De Saedeleer *et al*, 2012), we obtained results that were qualitatively similar to the simpler model shown in Figures 2A and 2B where these additional processes were not considered directly. In particular, the effects of Wnt signaling dominate those of cross-feeding between the cell types and the positive feedback loop between Wnt and PDK (high PDK implies more P_g cells; higher

numbers of P_g cells imply more Wnt activity; more Wnt activity means increased PDK) has been distilled in the simpler model so that Wnt activity level, rather than PDK levels, provides an effective metabolic switch between relative amounts of OXPHOS and glycolysis. Because PDK drives the switch in metabolism in SW480 cells, we use the P_g and P_o spatial distributions to compare to our xenograft stains.

The model equations were solved in nondimensional form using a characteristic proliferation time T of 1 day to rescale time and a characteristic diffusion length ℓ of the Wnt inhibitor to rescale space. Since we did not know ℓ (in fact there may be many factors that contribute to Wnt inhibition) we varied ℓ and found good agreement between the experimental and numerical patterns when $\ell \approx 40\mu m$. A full description of the both models, boundary conditions, and the nondimensionalization can be found in Materials and Methods and in the Supplementary Material. The parameter values can be found in Table 1 (Rockne *et al*, 2010; Jiang *et al*, 2005; Mendoza-Juez *et al*, 2012).

Figure 2.2C presents the numerical results for the fractions of glycolytic and oxidative cells and the concentrations of Wnt and Wnt inhibitor, where each two-dimensional plot is a horizontal slice through the center of the three-dimensional spatial domain (nutrient distributions can be found in Appendix B Figure S15, Appendix B A4). The cells were initially seeded randomly near the boundary of the domain to reflect the fact that the cells that survive the initial implantation are likely close to nutrient sources (alternative initial distributions of cells give similar results). The cells then proliferate and grow inwards towards the center of the domain with angiogenesis-induced nutrient sources fueling the growth. Consistent with the xenograft data, a distinct spotted pattern in the population of glycolytic cells is produced by the model over time. Over the entire domain, there is a high level of glycolytic-dominant cells with localized

areas of highly active glycolytic cells (dark red spots). Similar to the xenograft tumors, the spots are denser toward the boundary of the tumor space, where there is a higher density of vasculature, a spatial pattern that agrees with the overall pattern of pPDH staining of the mock tumors in Figure 2.1A. The oxidative cell fractions are close to 0 in the same spots where glycolysis is high, and their levels are relatively higher in regions surrounding these spots. Wnt and Wnt inhibitor activity show a similar pattern, with high levels distributed in a spotted array throughout the domain, surrounded by lower levels in the neighboring regions. Like the pattern of glycolysis, the frequency of spots is higher near the boundary relative to the interior. The square symmetries in the simulated spot distributions are due to the use of a cubic spatial domain in the simulations. Quantitative and comparative analysis of the patterns in the xenograft tumors to the simulated pattern generated by the model indicates that the model predicts similar dimensions for the size of the spots and distance between the spots (see Figure 2.3D, E), although there is significant scatter in the data.

Because the model parameters were largely unknown, we investigated their influence on the results through a parameter study (Appendix B A5, Supplementary Material). Using a diffusive stability analysis to determine the ranges of values for which patterns were predicted to occur (see Appendix B A6, Supplementary Material), we modified the parameters one by one within the pattern forming range and tested for phenotype changes in metabolism and patterning. The results are summarized in Table in Figure 2.2D. Increasing the Wnt diffusion coefficient or decreasing the Wnt decay rate increased the extent of Wnt activity, so that the spots of glycolysis increased in size. Increasing the Wnt inhibitor diffusion coefficient, or decreasing the decay of the Wnt inhibitor caused the inhibitor to stay within the system for longer times, and resulted in fewer spots. Modifying the switching times between the phenotypes changed the proportion of P_o and P_g . Increasing or decreasing the Wnt switch changed the background levels of P_g cells

Table 1: Model parameters for mock and dnLEF/dnTCF simulations

Parameter	Description	Mock value	dnLEF value	Reference
D_o	Diffusion coefficient of oxidative cells	0.01	0.01	Rockne et al. (2010)
D_g	Diffusion coefficient of glycolytic cells	0.01	0.01	Rockne et al. (2010)
D_W	Diffusion coefficient of Wnt	0.004	0.008	Chosen to be small
D_{W_I}	Diffusion coefficient of Wnt inhibitor	1	1.5	Chosen to be large
D_N	Diffusion coefficient of nutrient	100	100	Jiang et al. (2005)
τ_o	Oxidative cell proliferation time	1	1	Non-dimensionalization (time scale)
τ_g	Glycolytic cell proliferation time	1	1	Non-dimensionalization (time scale)
τ_{og}	Switch time from OXPHOS to glycolysis	1/24	1/24	Mendoza-Juez et al. (2012)
τ_{gc}	Switch time from glycolysis to OXPHOS	1	1	Mendoza-Juez et al. (2012)
α_W	Constant for Michaelis-Menten dynamics	1	1	Parameter estimation
κ_W	Rate of nonlinear Wnt production	5	5	Parameter estimation
κ_{W_I}	Rate of Wnt inhibitor production	1	1	Parameter estimation
μ_o	Decay rate of P_o cells	1	1	Parameter estimation
μ_g	Decay rate of P_g cells	1	1	Parameter estimation
μ_d	Decay rate of P_d cells	1	1	Parameter estimation
μ_W	Decay rate of Wnt	2	2	Parameter estimation
μ_{W_I}	Decay rate of Wnt inhibitor	3	3	Parameter estimation
μ_N	Decay rate of nutrient	0.1	0.1	Parameter estimation
S_W	Rate of Wnt production through cells	7.5	6.5	Parameter estimation
a	Constant of inhibition	10^8	10^8	Parameter estimation
b	Constant of inhibition by W_I	1	1	Parameter estimation
γ_W	Sensitivity level of Wnt switch functions	1	1	Parameter estimation
γ_N	Sensitivity level of nutrient switch function	100	100	Assumed to be high
ν_{NG}	Uptake of nutrient by P_g cells	10	10	Parameter estimation
ν_{NO}	Uptake of nutrient by P_o cells	10	10	Parameter estimation
N_s	Parameter for nutrient source	2	2	Parameter estimation
W^*	Wnt level at which 50% of cells switch metabolism	5	5	Parameter estimation
N^*	Nutrient level below which cells die	0.07	0.07	Parameter estimation
N_g^*	Nutrient level below which P_o cells cannot switch to glycolysis	0.1	0.1	Parameter estimation
α_N	Value of scaling function when $\int P_g = 0$	0.025	0.025	Parameter estimation
S_x	Horizontal length of spatial domain	12	12	
S_y	Vertical length of spatial domain	12	12	

and spot sizes without affecting the number of spots. Small reductions to S_W , which can be thought of as reducing overall Wnt signaling, reduced the background levels of glycolytic cells without much effect on the sizes or numbers of spots. Sufficiently reducing S_W resulted in all terms (P_o , P_g , W , and W_I) decreasing to 0. Decreasing κ_W (nonlinear Wnt activity) or increasing b (Wnt response to inhibition) paradoxically increases the number of glycolytic cells because nonlinear interactions actually result in a decreased amount of W_I . Analogously, when κ_{W_I} (nonlinear Wnt inhibitor activity) decreases, the number of glycolytic cells decreases. Modifying the cell diffusion coefficients, death and decay rates and the nutrient uptake rates did not significantly influence the self-organization of a spotted array. Similarly, varying the proliferation times only changed the time it took to reach a steady state but otherwise had no effect on pattern formation.

Interfering with Wnt signaling Alters Colon Cancer Metabolic Patterns *in vivo*

Since our model utilizes Wnt signaling, we tested how interference of this pathway would alter metabolic patterning. To disrupt the pathway, we used lentiviral transduction to express dominant negative LEF-1 (dnLEF-1), or dominant negative TCF-1 (dnTCF-1) transcription factors. Both dominant negative versions are naturally occurring LEF/TCF isoforms that lack the β -catenin binding domain and therefore interfere with the activation/expression of Wnt target genes. Expression of moderate, physiological levels of dnLEF-1 or dnTCF-1 expression, partially, but not completely, disrupts Wnt target gene expression in the xenograft tumors (Van de Wetering *et al*, 2002; Hoverter *et al*, 2012; Pate *et al*, 2014). Partial disruption is necessary because complete inhibition of Wnt activity would block cell cycle progression and the formation of tumors altogether.

SW480 colon cancer cells that had been lentivirally transduced and selected for dnLEF-1 or dnTCF-1 expression were subcutaneously injected in immunocompromised mice for tumor formation. Experiments showed that, as a result of dnLEF-1 expression, PDK1 activity was reduced, Warburg metabolism was diminished, and tumor mass was reduced approximately 4-5-fold (Pate *et al*, 2014). Immunohistochemical staining of the levels of phospho-PDH in these tumors (Figure 2.3A) revealed a lighter background and lower pPDH level overall. Interestingly, pPDH positivity remained easily visible in clusters of cells, but there were striking changes in the spotted pattern. Each pPDH-positive cluster was comprised of a larger number of cells (Mock average: ~7 cells/spot and dnLEF average: ~17 cells/spot; see Appendix B A1.15, Appendix B Figure S11) and there was a greater distance between each spot (compared to parental, mock transduced cells; Figure 2.3D). We also utilized immunohistochemical staining for the Wnt-mediating factor β -catenin in the dnLEF tumors (Figure 2.3E) (dnLEF-expressing tumors cannot be stained for LEF-1). These stains revealed a spotted pattern, with clusters of cells having higher levels of β -catenin in the nucleus than neighboring cells, although because of the very high levels of β -catenin in SW480 cells, there was an overall strong intensity of the IHC stain. Image analysis showed that while the β -catenin^{HI} spots are, on average, smaller than the pPDH-positive spots, they too had increased in size and distance relative to the pattern of β -catenin^{HI} cell clusters in the Mock/parental tumors. Additional image analyses of staining for pPDH and β -catenin in dnLEF-1-, and dnTCF-1-expressing tumors are provided in Appendix B A1 (Supplementary Appendices A1.8-A1.12), together with a quantification of these staining patterns (Appendix B A1, Supplementary Material A1.13-A.1.15). In summary, there were significant changes in both the intensity and distribution of the spotted patterns for pPDH and β -catenin when Wnt signaling was reduced by dnLEF-1/dnTCF-1 expression.

Reaction-Diffusion Modeling of Metabolic Patterns under Partial Disruption of Wnt Signaling Predicts Expression of Factors that Increase the Range of Wnt Signaling

To understand the phenotypic changes in the spotted patterns when Wnt signaling was partially disrupted, we used our model to identify changes in parameters that could recapitulate the experimental observations. The simplest change was to reduce S_w , which mimics dnLEF-1 and dnTCF-1 expression in lowering intrinsic Wnt activity throughout the domain, a manipulation that represents the cell autonomous effect of expressing Wnt-interfering, dominant negative LEF/TCF factors in the nucleus of every cell. However, as described earlier in our parameter study, decreasing S_w lowers the overall background levels of P_g cells, but does not affect the spotted pattern (unless it is taken to be too small in which case the pattern disappears). Thus, solely lowering overall Wnt signaling (S_w) in the model produces outcomes in pattern that are inconsistent with the experimental data.

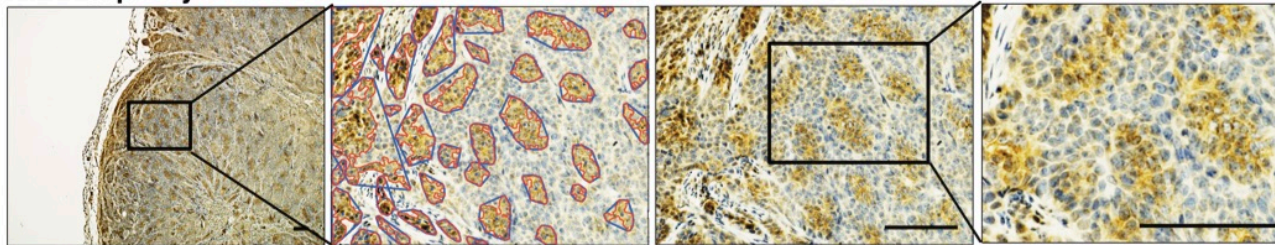
Clearly, the effects of dnLEF-1/dnTCF-1 expression are more complex than the cell-autonomous manipulation of only decreasing Wnt pathway activity in the nucleus. We considered the possibility that dnLEF-1/dnTCF-1 might also be triggering a cell-extrinsic response that connects collections of cells in the microenvironment. Specifically, our parameter study suggested that the increase in the sizes of pPDH-positive cell clusters might be due to extracellular soluble factors that increase the range of the activator (Wnt ligands) and that the decrease in the number of pPDH-positive cell clusters could be due to factors that increase the range of inhibition. Therefore, we included two additional parameter modifications: increasing D_w , which increases the range of Wnt ligands and makes the spots larger, and concomitantly increasing D_{wI} , which increases the range of Wnt ligand inhibitors and reduces the number of

spots. Changing these two parameters and decreasing S_w simultaneously resulted in a striking recapitulation of the changes in the spotted pattern observed in the dnLEF-1/dnTCF-1-expressing tumors: lower background levels of P_g cells and larger, fewer spots of P_g -glycolysis (Figure 2.3C). The average sizes and centroid distances of the P_g spots in the simulated tumors correlated very well with the experimental observations (Figure 2.3D). Further, the simulation showed a decrease in nutrient concentrations throughout the tumor (Supplementary Appendix B A4), a result that is consistent with our previous experimental data as we observe significantly fewer blood vessels in the dnLEF-1 and dnTCF-1 tumors (Pate *et al*, 2014). This is because the nutrient concentration N is linked to the proportion of P_g cells, which are decreasing.

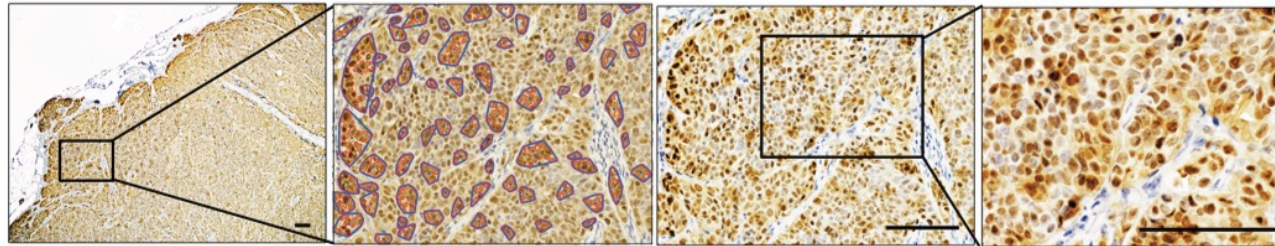
Since in the experiments, we used IHC staining of β -catenin as a direct assessment of patterns in Wnt signaling, in the simulations we analogously examined patterns of Wnt activity in the model. The results show very good agreement between the simulations and the experiments: the spots of Wnt activity are smaller than the P_g spots but the Wnt-activity spots were increased in size and distance relative to the pattern of Wnt activity in the simulations of the Mock tumors (Figure 2.3E). In summary, our results suggest that stressing the colon cancer cells by interfering with Wnt signaling triggers changes in the expression of factors that increase the diffusion range, or “spread”, of Wnt ligands and extend the range of Wnt inhibition.

SW480 dnLEF1 Xenograft Tumor

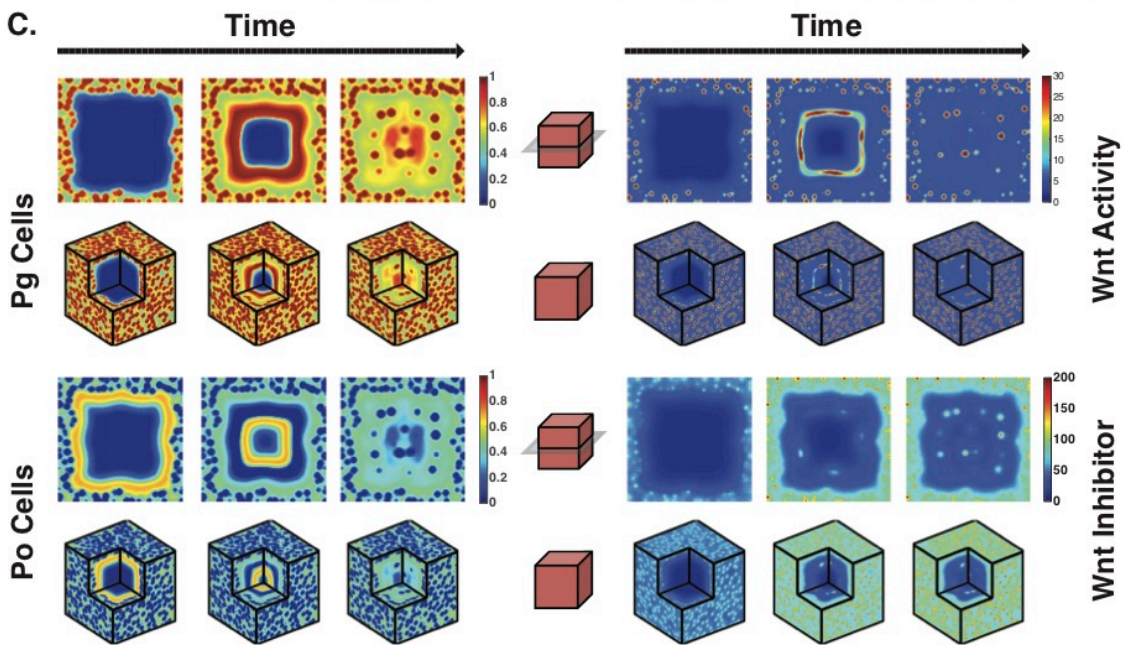
A. Phosphorylated PDH



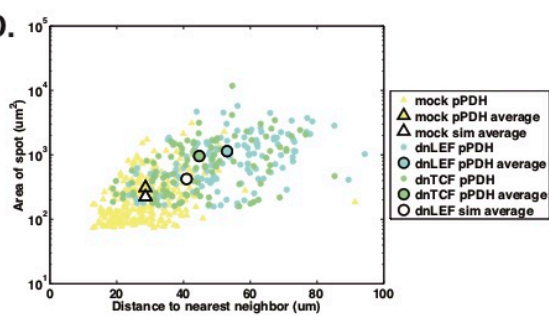
B. β -catenin



C.



D.



E.

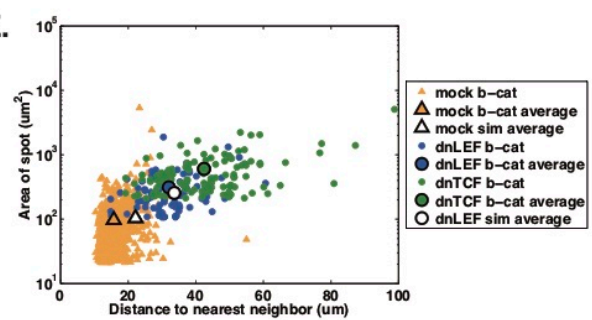


Figure 2.3. Decreasing Wnt signaling leads to changes in metabolic patterning in xenograft tumors.

SW480 cells were lentivirally transduced to express dominant negative LEF-1 (dnLEF-1), and transduced cells were injected subcutaneously into immunocompromised mice. Tumor sections were stained for phosphorylated PDH (A) and beta-catenin (B) and counterstained with hematoxylin. Compared to mock tumors, the spots are larger and more heterogeneous and the background staining is lighter, which reflects an overall decrease in Wnt signaling. Scalebars are 100 μm . (C) Numerical simulations that lower Wnt signaling activity in the model show an overall decrease in glycolysis and a change in the spotted pattern that closely mimics that observed in the dnLEF tumors. (D) Image analysis of spot size versus distance of spot to nearest neighbor, using analyzed images. Averages for Mock and dnLEF spot simulations are denoted in white outlined symbols (pPDH: Spot sizes/Inter-spot distances: Mock simulation average - $225 \pm 278 \mu\text{m}^2 / 29 \pm 12 \mu\text{m}$; Mock xenograft tumor average - $309 \pm 367 \mu\text{m}^2 / 29 \pm 10 \mu\text{m}$; dnLEF-1 simulation average: $423 \pm 327 \mu\text{m}^2 / 41 \pm 14 \mu\text{m}$; dnLEF-1 xenograft tumor average: $1139 \pm 1042 \mu\text{m}^2 / 53 \pm 15 \mu\text{m}$). Results are also shown for dominant negative transcription factor 1 (dnTCF-1) tumors (see Appendix B A1.10-A1.12, Appendix B Figures S8-S10). The metabolic pattern in dnTCF-1 tumors is consistent with that in dnLEF-1 tumors. The analysis and model predict that the changes in the metabolic spotted pattern (larger spots, greater distance between spots) are due to an increase in the diffusion of Wnt and the Wnt inhibitor. (E) Comparison of Mock beta-catenin spots to dnLEF-1 and dnTCF-1 β -catenin spots from image analysis. Averages for Mock and dnLEF-1 spot simulations are denoted in white outlined symbols. (β -catenin Spot sizes/Inter-spot distances: Mock simulation average - $139 \pm 145 \mu\text{m}^2 / 26 \pm 11 \mu\text{m}$; Mock xenograft tumor average - $97 \pm 209 \mu\text{m}^2 / 16 \pm 4 \mu\text{m}$; dnLEF-1 simulation average: $342 \pm 221 \mu\text{m}^2 / 39 \pm 14 \mu\text{m}$; dnLEF-1 xenograft tumor average: $312 \pm 277 \mu\text{m}^2 / 32 \pm 9 \mu\text{m}$; dnTCF-1 xenograft tumor average: $603 \pm 578 \mu\text{m}^2 / 42 \pm 14 \mu\text{m}$). The analysis and model predict that Wnt signaling diffuses further with dominant negative LEF-1 expression.

In Vivo Validation of Model Predictions

Only a few studies have directly examined the diffusion range of Wnt ligands in any tissue, a range which is extremely limited, in part because the ligands are post-translationally modified by palmitoylation and are highly lipophilic for membranes and extracellular matrix proteins (Farin *et al*, 2016; Willert *et al*, 2003). There is a growing awareness of proteins that modify the range of ligand diffusion, although their actions and impact are not very well characterized (Figure 2.4A). Perhaps the best-characterized factors that influence Wnt ligand diffusion are the SFRP protein family, secreted inhibitors that bind directly to Wnt ligands and interfere with receptor binding. Importantly, several studies have shown that even though SFRPs can interfere with Frizzled receptor binding, they are bimodal in their actions, repressing Wnts at high concentrations of ligand but also promoting Wnt actions by increasing their range of diffusion and, in essence, delivering the ligands to cells that are further away (Mii & Taira, 2009, 2011). Given that our mathematical model predicts the diffusion of Wnt ligands and their inhibitors have increased in the dnLEF-1 and dnTCF-1 xenograft tumors, we tested the prediction that one or more candidate regulators of Wnt diffusion were elevated in their expression. Using RNA-seq data as a guide for identifying candidates expressed in SW480 cells, we designed human-specific primers for both diffusion regulators and inhibitors that were detectably expressed in this cell line. Expression analysis of mRNA purified from 2D cultures and 3D xenograft tumors revealed that the Wnt diffusers SPOCK2, GPC4, and SFRP5 are upregulated specifically in dnLEF-1 and dnTCF-1 xenograft tumors but not 2D culture (Figures 4B and 4C). Since the primers are human-specific, the expression changes derive specifically from the human cancer cells and not mouse-derived cell types in the tumor microenvironment.

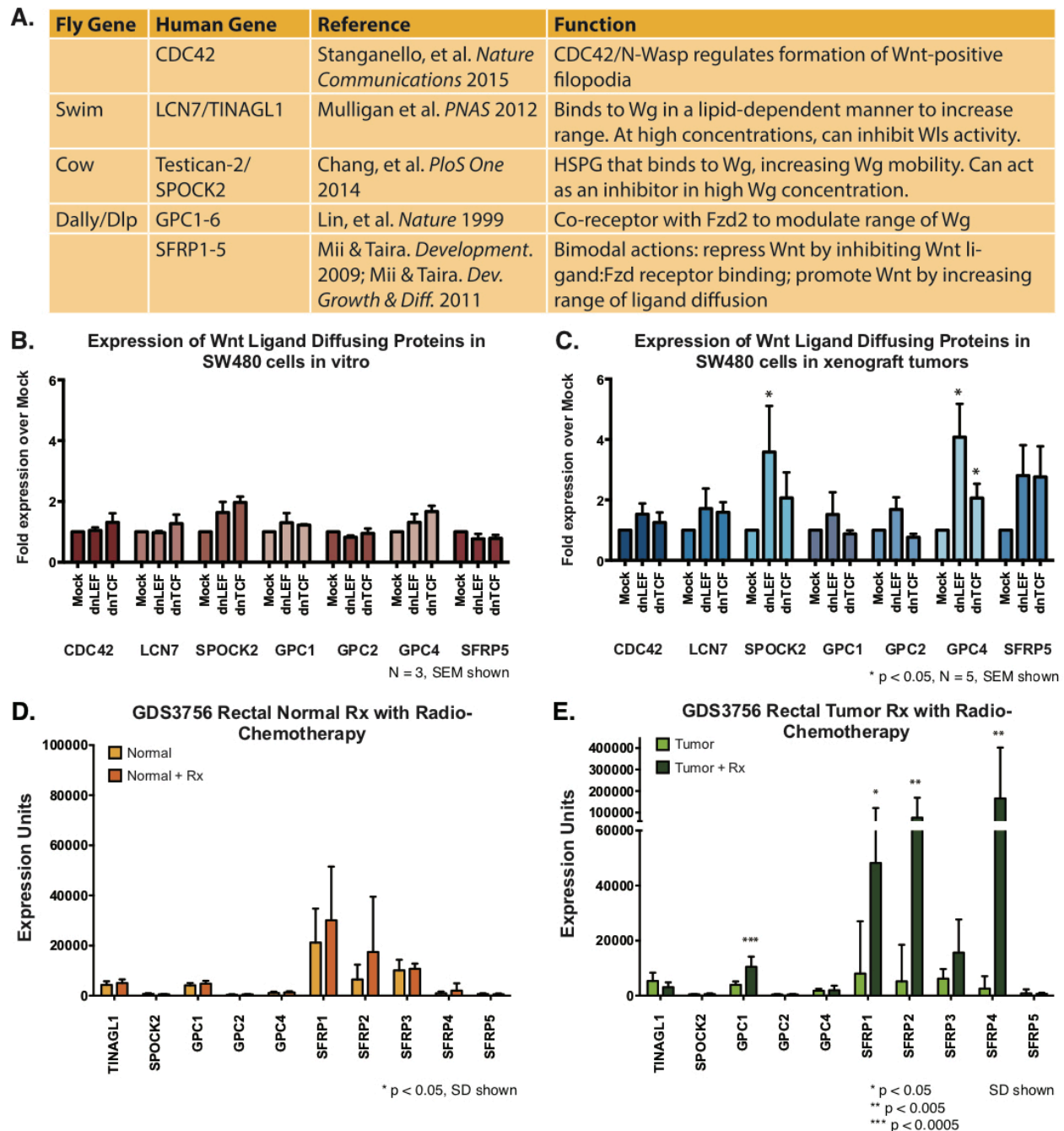
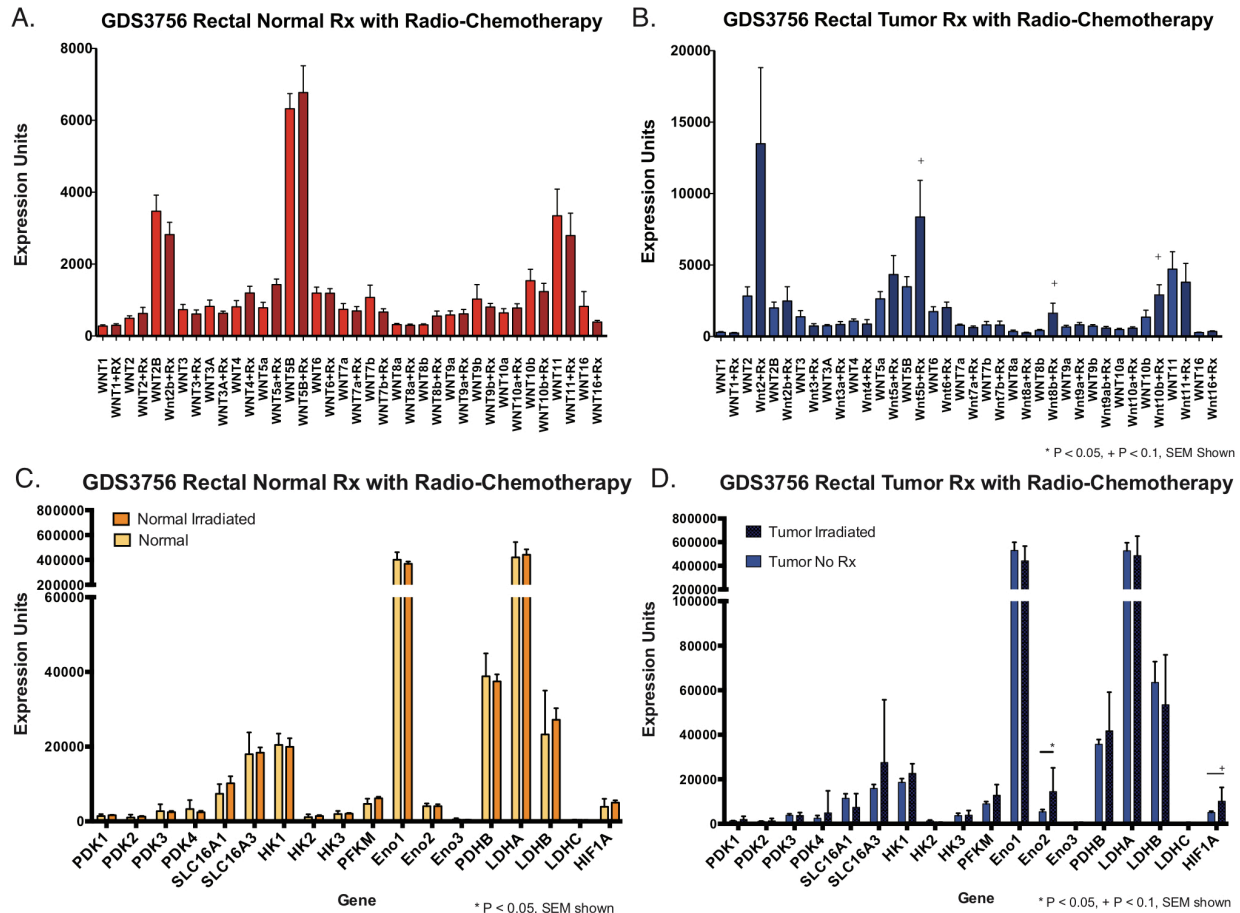


Figure 2.4. Model predictions revealed in xenograft tumors and human colorectal cancer.

The model predicted that lowering Wnt signaling results in an increase in the expression of factors that increase the range of diffusion of Wnt and Wnt inhibitors. (A) Known regulators of Wnt ligand diffusion. (B & C) Quantitative PCR of diffusion regulators in SW480 Mock, dnLEF-1, and dnTCF-1 (B) transduced cells and (C) xenograft tumors show human SPOCK2, GPC4, and SFRP5 mRNA are notably upregulated in xenograft tumors but not 2D in vitro culture. In vitro data represent an average of three sample sets (+/- SEM), xenograft tumor data represents the average of five independent tumor sets (+/- SEM); * denotes $p < 0.05$. Statistical significance was determined using Student's two-tailed t-test. (D & E) Gene expression data from GEO dataset GDS3756, of 21 rectal cancer patient tissue with or without radio-chemotherapy (Snipstad *et al.*, 2010). Significant changes in expression levels of GPC1 ($p = 0.00019$), SFRP1 ($p = 0.016$), SFRP2 ($p = 0.0006$) and SFRP4 ($p = 0.0006$) are observed in post-therapy tumor cells compared to before treatment. SD shown. Statistical significance was determined using the Mann-Whitney U test with Benjamini-Hochberg correction for multiple hypotheses (RStudio).



Expanded View Figure 2.2. Wnt ligand and glycolysis gene expression in rectal cancer patients post-radiochemotherapy

Gene expression data from GEO dataset GDS3756 of 21 rectal cancer patients before and after radio-chemotherapy (Snipstad, et al. Mol Oncol 2010). (A, B) Expression of Wnt ligands WNT5B, WNT8B, and WNT10B show trends toward increased expression in rectal tumor tissue treated with radiochemotherapy, but these changes do not reach statistical significance when $p < 0.05$ is used as a cut-off (specific p -values are 0.10, 0.10, and 0.08 respectively). (C, D) Expression of the glycolytic enzyme ENO2 is specifically increased in tumor tissue after radiochemotherapy ($p=0.008$); HIF1A expression also shows a trend in increased expression ($p=0.06$). * denotes adjusted p -value < 0.05 ; + denotes adjusted p -value < 0.10 . Statistical significance was determined using the Mann-Whitney U test with Benjamini-Hochberg correction for multiple hypothesis testing (RStudio).

While small molecule Wnt inhibitors that mimic the effects of dnLEF-1 and dnTCF-1 are working their way through pre-clinical testing and early phase clinical trials, there are not yet any available data from patient studies that profile gene expression changes in primary colorectal cancers treated with Wnt inhibitors. However, there are limited data available from patients treated with radio- and chemotherapy regimens, treatments that induce stress and loss of nutrient delivery to the tumor. We analyzed one dataset ((Snipstad *et al*, 2010) NCBI GEO GDS3756), which provided gene expression profiles of a group of colorectal cancer patients before and after radio- and chemotherapy treatment. Figures 4D and 4E show that, while the treatment had no significant effect on expression of Wnt ligand regulators in normal rectal tissue, the expression of GPC1 and three SFRP family members (SFRP1, SFRP2, SFRP4) were strongly and specifically increased in the tumor following treatment (Figure 2.4E). We checked for changes in expression of Wnt ligands and although there was a trend toward significantly increased expression of Wnt2, Wnt5b, Wnt8b, and Wnt10b specifically in the tumor and not the neighboring normal tissue, the changes did not quite reach statistical significance (Figures EV2.2A, EV2.2B). Interestingly, one glycolytic gene (ENO2), was significantly increased in radiochemotherapy-treated tumor tissue (Figure EV2.2D) and the glycolytic regulator HIF1A was increased but not to the same level of significance. This suggests that radiochemotherapy may trigger increased expression of proteins that increase the range of Wnt diffusion; a response that we predict might serve to maintain a critical level of glycolytic cells in the tumor.

Modeling a Therapeutic Treatment for Cancer: Metabolic Targeting

To test whether glycolytic cells are the important subpopulation of cells to target in the tumors, we compared the effectiveness of a hypothetical therapy program that selectively

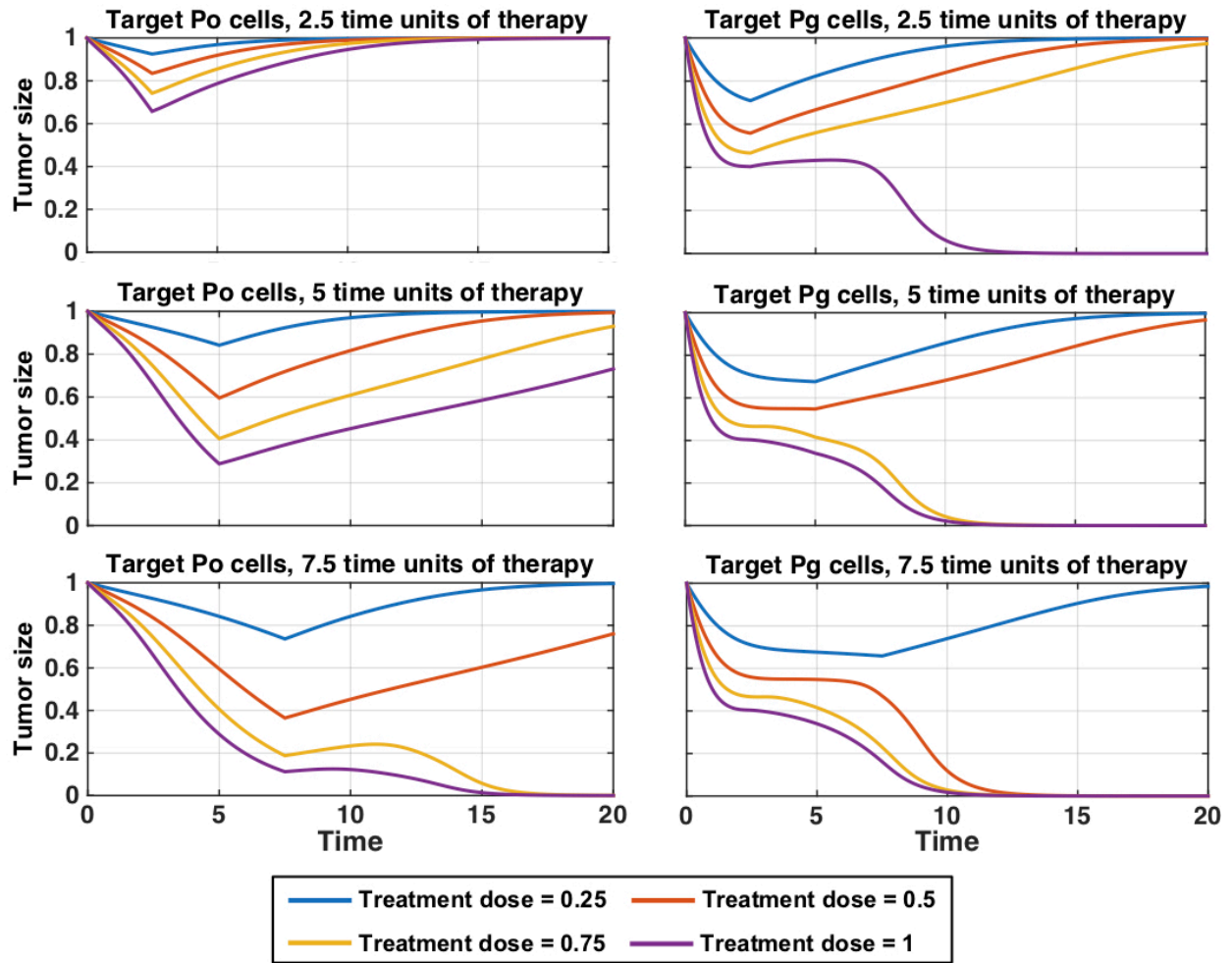
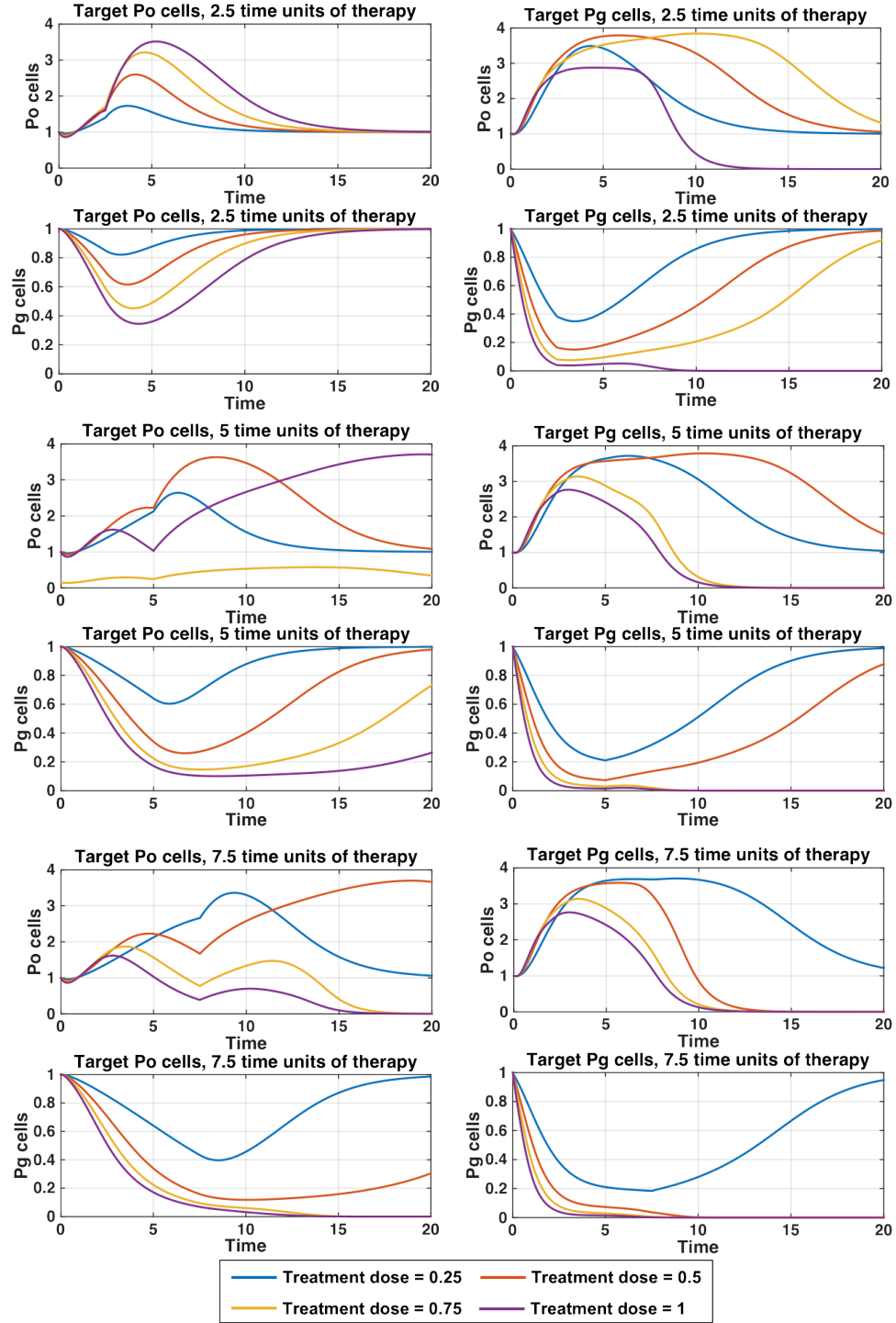


Figure 2.5. Simulations identify the glycolytic cell population as a sensitive drug target. We target either cells with more oxidative phosphorylation (Po; left) or cells with more glycolysis (Pg; right) selectively, starting from a metabolically patterned state, for 2.5, 5, or 7.5 (arbitrary) time units, with a treatment dose between 0.25 and 1. After therapy is halted, the cells are allowed to evolve according to the original model (Figure 2.2). The total cell populations, relative to the initial, starting cell population are shown. Corresponding populations of Po and Pg cells can be found in Figure EV2.3.



Expanded View Figure 2.3. Targeted therapy simulations for P_g and P_o populations

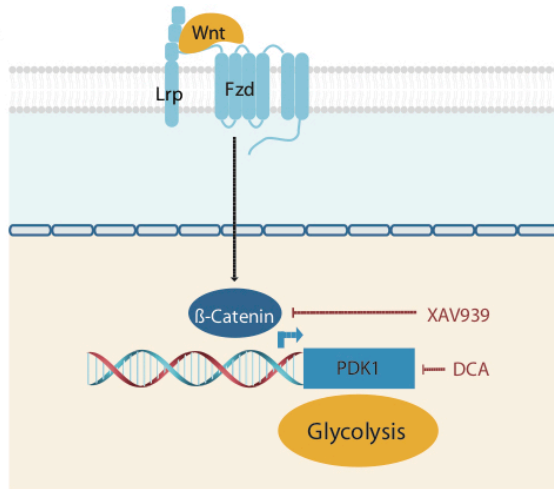
Figure 2.5 in the main text gives the results for total tumor size after individually targeting either P_g or P_o cells with given treatment doses and treatment times. Here are the effects for the individual P_g and P_o populations in those targeted therapy simulations. Simulations suggest that the glycolytic cell population is a more sensitive drug target than the oxidative cell population. We target either P_o (left) or P_g (right) cells selectively, starting from a metabolically patterned state, for 2.5, 5, or 7.5 (arbitrary) time units, with a death rate between 0.25 and 1. After therapy is stopped, the cells are allowed to evolve according to the original model (Figure 2.2). The P_g and P_o cell populations, relative to their initial cell populations, are shown.

targeted each population by independently varying the death rates of P_o and P_g cells (we introduced additional death terms $\mu_{P_o}P_o$ and $\mu_{P_g}P_g$ in the P_o and P_g equations in Figure 2.2A). The simulation applied the targeted therapy to a fully developed tumor at steady state for different lengths of time (days), followed by removal of the therapy and a recovery time for tumor development (Figure 2.5). In this figure, the tumor size (integral of $P_g + P_o$ over the entire domain) is shown relative to that of the untreated tumor (see Figure EV2.3 for the dynamics of the individual cell populations). The treatment dose refers to cell death rates μ_{P_o} or μ_{P_g} and targeting means that the death rate is non-zero only for the target cell population. These simulations revealed that regardless of the targeted population, modest rates of cell death suppressed tumor development transiently, followed by full recovery of the system once therapy was removed, a pattern more evident and more robust when cell killing was directed towards the P_o population. At sufficiently large death rates, complete loss of the tumor could be achieved. However, targeting the P_g population led to a complete loss of the simulated tumor at shorter treatment times and smaller death rates than when P_o cells were targeted. Thus, the simulation predicts that P_g cells are the more sensitive population and that targeting these cells could more effectively lead to a full regression of the tumor.

Since selective targeting resulted in full recovery of the simulated tumors unless death rates were sufficiently high and treatment was sufficiently long, we considered dual targeting of two features of cancer cell metabolism as a mechanism for more effective killing. Specifically, we targeted canonical Wnt signaling and PDK enzyme activity, both of which act as regulators of glycolysis (Figure 2.6A). Dichloroacetate (DCA) inhibits PDK activity and therefore targets cell metabolism directly by releasing inhibition of PDC, which increases OXPHOS capacity.

Tankyrase inhibitors such as XAV939 reduce β -catenin levels and hence reduce canonical Wnt

A.



B.

	XAV939	DCA
Function	Tankyrase inhibitor. Prevents dissolution of β-catenin destruction complex, increasing degradation of β-catenin.	Pan-PDK inhibitor, prevents shutting down mitochondrial respiration, encourages OXPHOS
Model	Decreased Wnt signaling (S_W), increased diffusion coefficients of W & WI (D_W, D_{WI})	Increased switching rate from glycolysis to OXPHOS ($1/t_{go}$)
Clinical	Preclinical	Phase II trials in glioma patients. (Michelakis, 2010)

C.

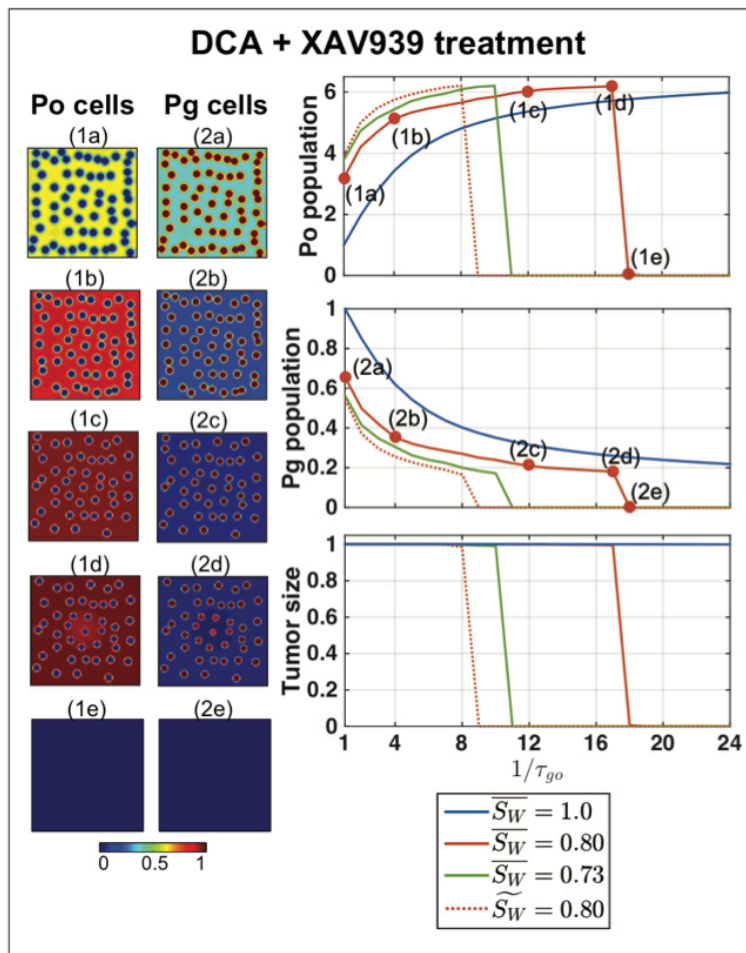


Figure 2.6. Therapies targeting metabolism and Wnt synergize for tumor death in mathematical simulations.

(A & B) Modeled therapies, their targets and the model parameters influenced by therapy. (C) Starting with a metabolically patterned state, treatment of tumors with dichloroacetic acid (DCA) and XAV939 combined leads to an effective crash in the system, as shown by the complete loss of cells (C.1e and C.2e). The panels on the left show the cell arrangements for the oxidative (Po, top) and glycolytic (Pg, bottom) populations (metabolic patterning) and the three graphs on the right show the fractions of Po or Pg cells relative to their initial values, after applying the therapy for 50 (arbitrary) time units. The effects of therapy on the total cell population, relative to the initial cell population, for the same D_W and $1/\tau_{go}$ values, are shown in the third graph. XAV939 treatment is modeled by decreasing S_W and increasing D_W and D_{WI} linearly with respect to the decrease in S_W ; legend values are listed relative to mock S_W . The dashed curves labeled $\tilde{S}_W = 0.80$ correspond to the case in which $\bar{S}_W = 0.80$ but D_W and D_{WI} are unchanged and take the values used in the mock tumor simulations. The panels on the left correspond to the red curve in the graphs and show the effect on patterning for $1/\tau_{go}$ values 1, 4, 12, 17, and 18 respectively (denoted by labels (1a) through (2e)).

signaling. Compounds that target Wnt and PDK are currently being tested in preclinical studies as individual agents in clinical trials but they have not been tested in combination (Figure 2.6B). We asked whether targeting glycolytic cells using anti-Wnt and anti-PDK therapies in combination is more effective than single agent therapy.

Because the inhibition of β -catenin by XAV939 is similar to the effects of dnLEF-1 and dnTCF-1, we modeled treatment by XAV939 using an analogous approach. In particular, we assumed that XAV939 decreases the general Wnt signaling term S_w and increases the ranges of Wnt and its inhibitor (due to upregulation of Wnt and Wnt inhibitor diffusers), which we modeled by increasing D_w and D_{wI} proportionally. To model the effects of DCA, we increased the rate at which cells switched from a glycolytic metabolic phenotype to an OXPHOS phenotype (e.g., $1/\tau_{go}$ is increased) to reflect the tendency of cells to perform OXPHOS when PDK is inhibited.

In Figure 2.6C, we simulated a combination therapy applied to a fully developed tumor at steady state. At a fixed dose of XAV939, coupled to increasing doses of DCA, the simulation predicts that the population of oxidative cells will increase initially as cells switch from a glycolytic state (P_g) to an OXPHOS state (P_o) until there is an insufficient level of glycolytic cells to sustain the tumor and all the tumor cells die. Furthermore, the treatment simulations indicate that a combination of the two therapies will be more effective than single therapies as long as one or the other has adequately been applied. For example, a value of $\frac{1}{\tau_{go}} = 12$ is effective in eradicating the cells as long as Wnt signaling has been reduced by more than about 27% (e.g., $\bar{S}_w = 0.73$). In other words, if β -catenin expression has not been sufficiently suppressed by XAV939, then PDK inhibition by DCA must be adequately increased, and vice versa. Similar results are obtained when an in vitro version of the mathematical model is used to simulate the growth of colonies in

fibrin gels (Figure 2.7C; see also Appendix B Supplementary Materials A7, Appendix B Figure S17). In the in vitro case, however, the colony does not die out as the DCA concentration is increased. Instead the effect saturates because sufficient nutrients are available to diffuse throughout the spheroid to maintain cell viability in the absence of glycolytic cells. Additionally, in the in vitro version of the model, there is no angiogenesis, but there is cross-feeding between the oxphos and glycolytic cells (Appendix B Supplementary Material A7.1-A7.3).

Given that factors that increase Wnt diffusion were increased in the dnLEF/dnTCF tumors, we also tested the effect of increases in Wnt diffusion. We determined that increased expression of Wnt diffusers decreases the sensitivity of the tumors to treatment. For example, decreasing S_W but maintaining D_W and D_{WI} at their pre-treatment values to model the inhibition of Wnt diffusers results in more efficient treatment—tumor eradication occurs at smaller concentrations of XAV939 and DCA (e.g., compare the red solid and dashed curves in Figure 2.6C (right panels), which correspond to a 20% reduction of Wnt signaling; the dashed curve shows the result when the production of Wnt diffusers is inhibited and is labeled as $\tilde{S}_W$).

We performed a preliminary experimental test of model predictions using 3D colony growth of colon cancer cells. 200 single cells were seeded in fibrin gels and cultured under drug treatment for 14 days (Figure 2.7A). Over this time the single cells proliferated to give rise to tumor spheroids and we used image analysis to quantify the increase in colony size as a proxy for proliferation (Figures 7B-C). Treatment with low doses of DCA (0.5mM, 2mM) or XAV939 (5 μ M) as single agents had no effect on the development and growth of colonies over the two-week treatment period - in fact, DCA treatment appeared to increase colony size. By contrast, combination therapy had a significant inhibitory effect on colony growth, indicating a strong, negative and synergistic effect on proliferation.

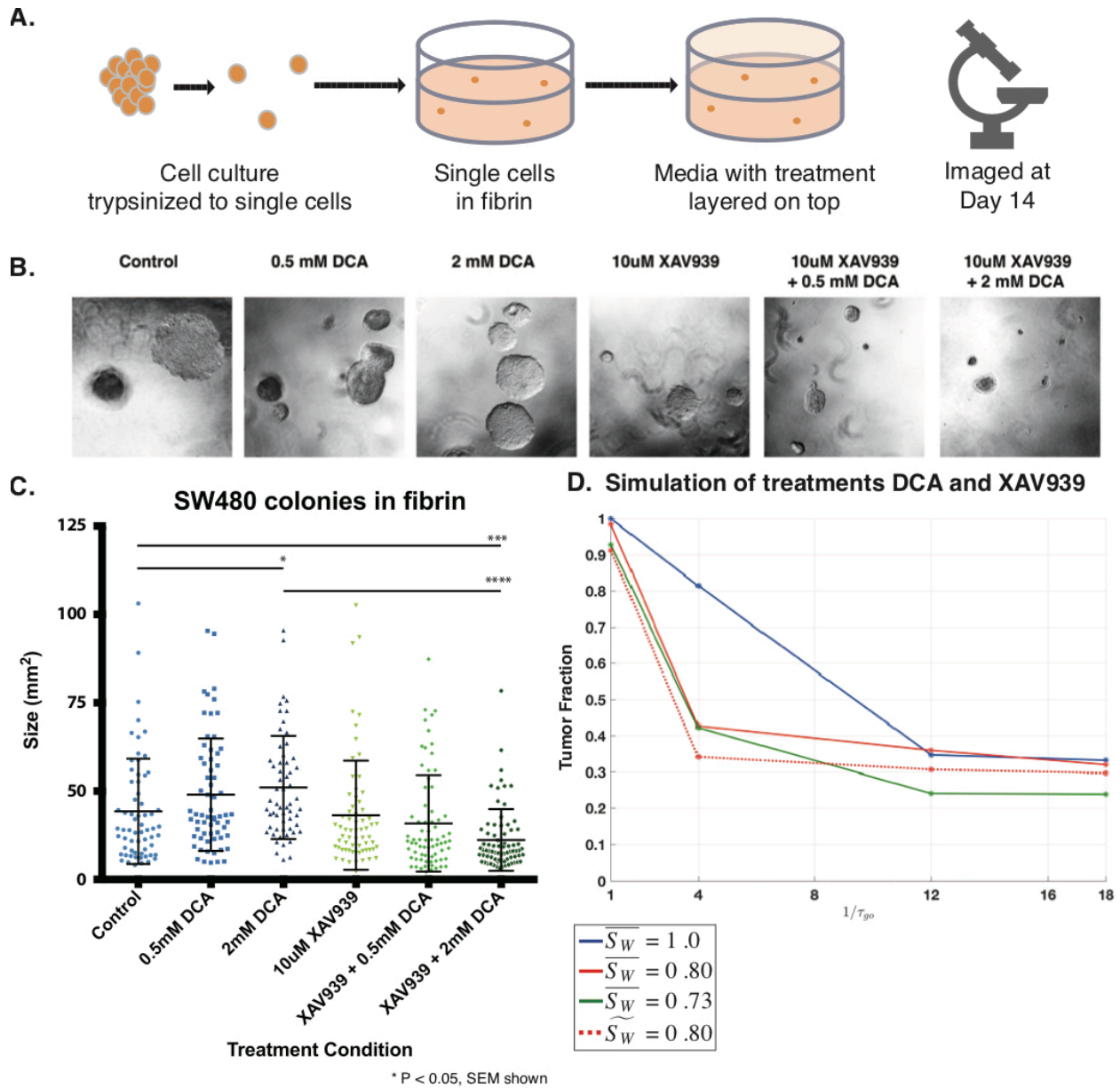


Figure 2.7. Targeted therapy significantly decreases SW480 tumor spheroid size *in vitro*

Combined Wnt signaling and glycolysis targeting therapies significantly decrease SW480 spheroid size *in vitro*. SW480 cells were embedded in a fibrin gel using a method described in (A). Media containing a MOCK treatment, 0.5 mM DCA, 2 mM DCA, 10 μ M of XAV939, or a combination of DCA and XAV939. (B) Representative 4x images of spheroids each condition, imaged 14 days after treatment, is shown in. (C) Analysis of 75 spheroids per condition shows 2mM DCA significantly increases SW480 spheroid size, while combined 2mM DCA treatment with 10 μ M XAV939 significantly decreases their size. Statistical significance was determined using Student's Two-Tailed T-test. (D) The effects of therapy on the total cell population, relative to the initial cell population, of combined XAV939 and DCA treatment were simulated using an *in vitro* version of the model (Appendix B A7). As in Figure 2.6, DCA treatment was modeled by increasing the rate at which cells switched from a glycolytic metabolic phenotype to an OXPHOS phenotype (e.g., $1/\tau_{go}$ is increased) to reflect the tendency of cells to perform OXPHOS when PDK is inhibited. XAV939 treatment was modeled by decreasing the general Wnt signaling term S_W and increasing the range of Wnt and its inhibitor (due to upregulation of Wnt and Wnt inhibitor diffusers), which we modeled by increasing D_W and D_{WI} proportionally. The dashed curves labeled $\bar{S}_W = 0.80$ correspond to the case in which $\bar{S}_W = 0.80$ but D_W and D_{WI} are unchanged.

Synergy can be quantified using the Bliss Independence Model Combination Index (Foucquier & Guedj, 2015), which assumes that XAV939 and DCA treatments act independently (e.g., XAV939 targets Wnt signaling and DCA targets PDK activity). In particular, if the Combination Index is less than one, this indicates synergy (see Appendix B A8 for the definition of the Combination Index and further details). In the in vivo simulation and the in vitro experiments, the Combination Index is zero because neither XAV939 nor DCA treatment separately affects tumor sizes (provided the concentrations of XAV939 and DCA are not too large; Appendix B A8). In the in vitro model, the Bliss Combination Index is 0.3462 (using $\bar{s}_w = 0.80$, $1/\tau_{go} = 1/4$), although the Combination Index does depend on the drug concentrations and increases towards one as the DCA concentration increases because the responsiveness to DCA treatment saturates (Appendix B A8). Since the Combination Indices in all cases are less than one, this indicates synergy of the XAV939 and DCA combinatorial treatments. As predicted by both the in vivo and in vitro models, these results suggest that combining Wnt inhibitors and metabolic targeting agents is a promising strategy for treating colon tumors.

Discussion

In this study we generated colon cancer xenograft tumors and examined changes in cellular metabolism by immunohistochemical staining for phospho-PDH (a marker of PDK activity), and markers of Wnt signaling (LEF-1, β -catenin). We observed that the tumors exhibit a pronounced spotted pattern of metabolic states where the spots indicate clusters of cells in which their mitochondria are inhibited (by PDK action) and thus where glycolysis was likely to be highly active. This is in contrast to the cells in the regions surrounding the spots. In these regions,

mitochondria are more active (not inhibited by PDK), and therefore utilize more oxidative phosphorylation. Although we cannot rule out that the spotted pattern is due to the emergence of genetically distinct, clonal populations, the short timescale of the xenografting (14-21 days) and the reproducibility of the pattern in another cell line (SW620) as well as site of injection (subcutaneous and orthotopic within the colon caecum), suggests that what we have observed is a fundamental pattern of tumor heterogeneity that is not genetic in nature, but non-genetic and dynamic (Appendix B A9, Appendix B Figure S20).

Metabolic patterning had been previously proposed as a mechanism to facilitate transport of glucose into hypoxic regions of tumors (Sonveaux & Végran, 2008). In particular, cells performing OXPHOS would be located near blood vessels and, rather than fueling respiration using glucose, these OXPHOS cells would instead use lactate produced by the hypoxic (glycolytic) cells as an alternative nutrient source. Utilization of lactate frees the glucose to travel farther into the hypoxic regions of the tumor where it would be used during glycolysis. Recent studies propose that cancer cell subpopulations segregate and re-organize to survive the sudden loss of nutrients due to anti-angiogenic therapy and that this leads to the development of resistance (Jiménez-Valerio *et al*, 2016; Allen *et al*, 2016; Pisarsky *et al*, 2016). However, in our xenograft images, we did not observe this type of spatial relationship between metabolism and vasculature. In fact, we observed that the spotted pattern was denser at the tumor margin where the vascular density was highest.

To investigate the mechanisms underlying the patterning we observed, we proposed a mathematical model based on Turing-Gierer-Meinhardt activator-inhibitor equations that simulated a symbiotic spatio-temporal relationship between these two cell populations (an oxidative population and a glycolytic population). Our model incorporated terms for Wnt as an activator with a short range of diffusion and Wnt inhibitors (e.g., SFRP, DKK) with longer

ranges of diffusion. The Wnt and Wnt inhibitor equations describe a feedback relationship and this lies at the crux of a spotted pattern that emerges in our simulations. Our equations also describe activities of metabolic reprogramming through changes in Wnt levels and availability of nutrients and cell substrates. The model describes a mutualistic interaction between the glycolytic and OXPHOS cells because the glycolytic cells induce the delivery of nutrients from blood vessels to mimic the effects of lactate-induced angiogenesis and these nutrients benefit both cell types. More generally, we can also interpret these nutrients as mutually beneficial cell substrates produced by the glycolytic cells. When we considered the effects of symbiosis by explicitly incorporating cross-feeding between glycolytic and oxphos cells in a more general model (Appendix B A3, Supplementary Material) and an in vitro model (Appendix B A7, Supplementary material), we found that Wnt signaling dominates the behavior and the patterning is robust to this form of symbiosis. Although mathematical models have been developed previously to investigate metabolic symbiosis, including spatially homogeneous (Mendoza-Juez *et al*, 2012) and heterogeneous models (McGillen *et al*, 2014; Phipps *et al*, 2015), to our knowledge the model presented here is the first to describe a pattern for heterogeneity in tumors that derives from an intricate spatial relationship between metabolic types and Wnt signaling. Though the role of Wnt signaling in cancer growth and development has been studied for many years, only recently has its regulation of Warburg/glycolysis metabolism been described. A better understanding of the link between Wnt and metabolism is crucial for defining how this overactive pathway drives tumorigenesis and progression, and for developing novel cancer treatments that target its key oncogenic actions.

Our mathematical model demonstrated strong qualitative and quantitative (spatial) agreement with the spotted patterns of activity detected in the tumors. The model also predicted that interference with Wnt signaling is not solely the result of a decrease in overall Wnt activity.

Making the simple parameter change of decreasing Wnt signaling throughput leads to overall less glycolytic activity (lower background of P_g cells), a prediction that was validated in our xenograft experiments in which Wnt transcription was partially blocked by the overexpression of dominant negative LEF-1. However, the phospho-PDH stains also revealed fewer, but larger regions of PDK activity (i.e., larger, fewer cell clusters). To simulate these observations, coefficients of diffusion for Wnt and its secreted inhibitors were increased - parameter changes that resulted in a “spreading out” of Wnt and Wnt inhibitor activity. This model prediction prompted us to investigate whether the expression of proteins known to increase the range of Wnt ligand and Wnt inhibitor diffusion was increased when dnLEF or dnTCF-expressing colon cancer cells were developed into xenograft tumors. Consistent with the mathematical model, we observed that the diffusers SPOCK2 and GPC4 were overexpressed in our xenografts but interestingly, not in our 2D *in vitro* culture conditions. The expression of SFRP5, which acts simultaneously as a Wnt inhibitor and diffuser by preventing binding to Frizzled receptors, also shows somewhat higher expression in our Wnt-low xenografts (dnLEF-1). It is important to emphasize that it is human-specific oligonucleotide primers that detect these changes in expression of Wnt ligand modifiers. Thus, the implanted human colon cancer cells appear to adapt to interference with Wnt signaling by directly increasing expression of Wnt ligand regulators. Our analysis of primary human colorectal tumors stressed by radiochemotherapy treatment, shows that one consequence of therapy could be a similar increase in the distribution of Wnt ligands through upregulated expression of glypicans (e.g. GPC1) and SFRP proteins (Figure 2.4E). These observations suggest that there are likely to be significant changes in Wnt signaling dynamics and metabolic programming of treated tumors, perhaps as a means of coping and surviving the loss of nutrient and cellular damage.

The model also suggested that the tumor is most reliant on the glycolytic cells and we found that inhibitors that target glycolysis and Wnt signaling in combination are more effective than treatments that only target one of these features. We simulated the actions of XAV939, which lowers β -catenin levels; and DCA, which inhibits PDK activity, thereby decreasing glycolysis. Since standard of care treatments for colon cancer have not changed significantly for decades, the novel combination of an inhibitor of glycolysis (e.g. DCA) with a Wnt pathway inhibitor (e.g. XAV939) might be an effective treatment to consider. We validated this prediction using 3D colony growth of SW480 colon cancer cells, which have high, intrinsic Wnt signaling and high levels of glycolytic activity. However, given that primary human colon cancers are more complex with respect to intrinsic Wnt pathway activity and crosstalk with the microenvironment, determining which tumor subtypes will be the most sensitive to this drug combination and how in vivo tumors respond to treatment will be important issues to resolve in developing effective drug combinations.

Our mathematical model is an abstract idealization and simplification of tumor proliferation and metabolism, and real tumors are much more complex than modeled here. For example, it is likely that Wnt and a Wnt inhibitor are not the only factors contributing to the pattern, although it is clear that Wnt has a strong influence, since the spots change significantly when Wnt signaling is interfered with. In the Supplementary Materials, we developed a more complete model that simulated PDK activity, hypoxia-inducible transcription factor concentrations ($\text{HIF1}\alpha$) and lactate concentration (see Appendix B A3, Supplementary Material), and linked these factors to cross-feeding, Wnt signaling and metabolism. In this more detailed model, the switch between metabolic phenotypes depends on PDK activity, rather than on Wnt directly. However, since Wnt and $\text{HIF1}\alpha$ promote PDK expression and activity (Kim *et al*, 2006;

Pate *et al*, 2014; Prigione *et al*, 2014), we found that the spatiotemporal distributions of PDK, Wnt and lactate track closely together and so the results from the more detailed model are qualitatively similar to those presented in the main text for the simplified model.

While we have used a Turing-type model to simulate the spotted pattern, a decision well-supported by data, we acknowledge that this type of model is only one possible mechanism to explain the patterning in the tumors. Other alternatives include differential adhesion or cell sorting—a process where cells of different adhesion potential sort away from each other (Amack & Manning, 2012; Foty & Steinberg) and bet-hedging—a process where different cells are differentially sensitive to external stresses, so that no matter the condition, at least some cells thrive (de Jong *et al*, 2011; Vogt, 2015; Starrfelt & Kokko, 2012). For bet-hedging to generate patterns, cell state changes must be reversible on a time scale slower than cell division, so that cells of like state end up clustered by default. Further experiments are needed to definitively distinguish among these processes. For example, these three types of processes (Turing, differential adhesion, bet-hedging) would be expected to be driven by different types of molecular signals and so these signals would need to be identified and tested.

Finally, although we focused on xenograft tumors, artificial constructs that only partially model tumorigenesis, we observed heterogeneity in metabolism and Wnt signaling in other settings as well. For example, we generated orthotopic tumors by implanting colon cancer cells (Mock-parental SW480 and SW620, as well as dnLEF- or dnTCF-expressing variants) in the wall of the mouse caecum and observed patterning (Appendix B A9, Supplemental Figure S19). In normal colon epithelia, pPDH stains show a gradient with high PDK activity in the base of the crypt where there is strong Wnt signaling and less PDK activity at the top of the crypt near the mucosal surface where Wnt signaling is not active (Pate *et al*, 2014; Figure 2.1D). In primary human patient colon tumors, our analysis revealed clear and striking heterogeneity in PDK

activity and LEF-1 expression in the epithelial portion of the tumor (Figure 2.1D). While this heterogeneity was not apparent as a regular array of cell clusters like the xenograft patterns, groups of cells with markedly different activities are clearly evident. Since the tumor microenvironment is more complex in human tumors than xenograft tumors, an inherent pattern of metabolic and Wnt activity may be modified by additional structural and cellular components as well as nutritional stresses and a changing microenvironment. Understanding how these additional components influence metabolic heterogeneity and the symbiosis between neighboring cells, and how they might create more complex pattern-on-pattern activities is a challenge going forward.

Materials and methods

Numerical simulations

The nondimensionalized equations for the rate of change in the population of oxidative (P_o) cells and glycolytic (P_g) cells respectively are shown in Figure 2.2A. See Appendix B A2 in Supplementary Materials for details on the nondimensionalization. Figure 2.2B shows the rate of change in the concentration of Wnt and Wnt inhibitor (W and W_I) activity respectively.

The first term on the right side of the equality in the P_o and P_g equations refers to diffusion, or random motion of the cells. The next terms are standard logistic proliferation terms, with proliferation dependent on nutrient level N , cell type, and the current total population of cells in the domain. The model sets the proliferation of glycolytic cells to be dependent on Wnt activity according to Michaelis-Menten dynamics, given by the term $\frac{W}{\alpha_W + W}$, which saturates at high levels of W . The parameters $\frac{1}{\tau_o}$ and $\frac{1}{\tau_g}$ are proliferation rates and $\frac{1}{\tau_{go}}$ and $\frac{1}{\tau_{og}}$ are switching rates. The

last terms in these equations are cell death terms; death is modeled such that it occurs if the nutrient supply N drops below a threshold N_d . The death rates are given by μ_o and μ_g .

The model is designed such that glycolytic and oxidative cells can emphasize, or “switch”, metabolism programs depending on W , Wnt activity, which is reflected in the third and fourth terms of each equation, where χ_w and χ^*_w are switch functions. Each switch function is defined by a modified hyperbolic tangent function, such that if Wnt activity falls below a parameter W^* then the cells utilize a more dominant OXPHOS program, and if Wnt activity is above W^* , then cells are more likely to utilize a greater level of glycolysis.

We assume that oxidative cells can switch to utilizing more glycolysis only if sufficient nutrient is present, given by parameter N^*_g . Cells will die if nutrient is below the parameter N_d . The steepness of the functions can be adjusted so that they are more step-like and hence more sensitive to W and N . Since we use large values for the steepness of the functions, we could alternatively have used piecewise functions for χ_N and χ^*_N .

The dynamics of dead cells (not shown) is described by a similar reaction-diffusion equation. This is the population of cells that have died from lack of nutrient. These cells can also diffuse and decay. The equations in Figure 2.2 describe W (Wnt) and W_I (Wnt inhibitor, e.g., DKK or SFRP) activity. D_W and D_{W_I} are constant diffusion coefficients. It has been shown in epidermal cells that Wnt target genes produce Wnt signals as well as long-range secreted Wnt inhibitors (Lim *et al*, 2013), so the inhibitor is assumed to diffuse much longer range than Wnt, i.e., D_W must be significantly smaller than D_{W_I} . Wnt signaling activity is assumed to be nonlinear with respect to Wnt and is inhibited by the Wnt inhibitor through the term $\frac{1}{a+b W_I}$. We assume the Wnt inhibitor is being produced by Wnt activity through both cell types. The terms μ_w and

μ_{WI} are decay rates. The term $S_W(P_o + P_g)$ in the Wnt equation refers to constitutive Wnt signaling through the cells.

The equation for nutrient (Eq. 1h in Appendix B A2, Supplementary Methods) describes the diffusion and uptake, decay, and source of nutrient. The nutrient term has Dirichlet (fixed) boundary conditions and diffuses in from the boundary of the spatial domain, so that the boundary can be considered as regions where vasculature is high. The second and third terms refer to uptake of nutrient by the two different cell types. The term $\mu_N N$ is a natural decay term. The last term, N_s , refers to the nutrient source, which is a small source term applied to the entire domain. This source term is based linearly on the glycolytic activity of the cells and is given by $N_s = N_s(\int P_g) = \gamma_N[(1 - \alpha_N) \int \frac{P_g}{S_x S_y} + \alpha_N]$ where α_N and γ_N are parameters, $\int P_g$ is the integral of P_g cells and S_x and S_y are the lengths of the sides of the spatial domain. This function was chosen so that $N_s(0) = \gamma_N^{\alpha_N}$ and $N_s(S_x S_y) = \gamma_N$ ($S_x S_y$ is the maximum that $\int P_g$ can reach). We chose to have the nutrient source N_s depend on P_g cells because glycolysis induces angiogenesis (Dhup *et al*, 2012; Porporato *et al*, 2012; Ruan & Kazlauskas, 2013), allowing more nutrients and growth factors to be delivered to the tumor (Pate *et al*, 2014). We use a linear function in the model as the simplest form for the dependency between N and P_g , which is consistent with experimental observations.

To summarize, in addition to consideration of Wnt signaling dynamics, biological assumptions for the model include terms for random motion in space (diffusion), terms for each cell type (oxidative and glycolytic, or P_o and P_g respectively) and their propensity to proliferate, die, and switch to the other cell type. Equations were included to account for dead cells, which consists of P_o and P_g cells that have died from lack of nutrient, and which can diffuse and decay. Terms for Wnt (W) and Wnt inhibitor (W_I) activity were made nonlinear with respect to Wnt,

meaning that their rates are proportional to Wnt activity. Nonlinear Wnt activity is dependent on P_g levels while nonlinear Wnt inhibitor activity is proportional to both P_g and P_o levels. A term for constitutive Wnt signaling was included for both cell types as well as decay terms for W and W_I . The general nutrient term N can diffuse, decay, and be taken up by the different cell populations. A bulk source was included for the nutrient as well as a Dirichlet boundary condition, both of which are dependent on the average level of glycolytic cells in the domain, a simplified way to incorporate increased angiogenesis driven by glycolysis (Pate *et al*, 2014). This relationship was included to take into account our observation that there is considerably less vasculature in tumors in which Wnt signaling has been blocked by dominant interfering forms of the Wnt transcription factors Lymphoid Enhancer Factor 1 (dnLEF-1) or T Cell Factor 1 (dnTCF-1)(Pate *et al*, 2014). Finally, there is a baseline assumption that there is sufficient oxygen available throughout the domain for OXPHOS to operate, even at a minimal level.

In the numerical results presented here, no-flux boundary conditions were used for all terms except N , which is governed by Dirichlet boundary conditions (N at the boundary is equal to the value $\frac{1}{\gamma_N} N_s$ where N_s is described above). Initial conditions were set for a random distribution of P_g cells located near the boundary, and small random values of W and W_I in the same areas where initial P_g cells are located. A constant high level of nutrient throughout the domain was provided, and the initial condition contained no P_o cells. All parameters are given in Table 1, and a sensitivity analysis is provided in Appendix B A5 in Supplemental Materials.

Numerical simulations were performed in MATLAB, using a forward difference method for each time derivative. P_o , P_g , W , and W_I equations were solved implicitly in centered diffusion terms. The nutrient equation was solved implicitly in uptake, decay, and centered diffusion terms.

The initial condition for the P_g cell population was a random distribution near the boundary of the domain. W and W_I initial conditions were random distributions in the same locations where initial P_g cells were located. Initially N was 1 everywhere in the domain; results did not change qualitatively if N was solved as a quasi-steady state equation. Initial conditions for P_o and P_d terms were 0.

Animal Protocols for Xenograft and Orthotopic tumors

SW480 stable transductants for xenograft or orthotopic injection were prepared through lentiviral infection with pCDH vector from System Biosciences: empty vector (Mock), or vector expressing dnLEF-1 or dnTCF-1, followed by selection with 500 $\mu\text{g}/\text{ml}$ G418. Transduced cells were collected as a pool for confirmation of expression, and Wnt signaling activity was measured by a SuperTOPFlash luciferase reporter (Pate *et al*, 2014). 2.5×10^6 cells were injected into immunodeficient NSG mice (male and female, 2 month old NSG male and female mice were used for the subcutaneous xenograft tumors (JAX™ Mice from Jackson Labs); male and female NSG mice, approximately 3 months old were used for orthotopic tumors (injection of $5\text{-}10 \times 10^3$ cells into thecaecum wall)). Tumors were removed (subcutaneous after 3 weeks, orthotopic after 4 weeks), fixed in paraformaldehyde overnight, and paraffin embedded four weeks after injection. All experiments involving animals were approved by the UCI IACUC (Protocol 2002-2357-4 to R. Edwards).

Immunohistochemistry

Deparaffinized 5-6 μm sections of formalin-fixed-paraffin-embedded (FFPE) mouse xenograft tumor and human colorectal carcinoma tissues followed by pressure cooker antigen

retrieval in citrate buffer, sections were blocked in 3% H₂O₂, goat or horse serum plus MOM block reagent (if mouse primary antibody was used on mouse tissue), avidin, and biotin blocking reagents (Vector Labs). Sections were incubated in primary antibodies: anti-phospho-PDHPSer293 (Calbiochem; 1:50-1:100), anti- β catenin (BD; 1:500), anti-LEF-1 (Cell Signaling; 1:100) anti-HIF1 α (ThermoScientific; 1:1000) followed by biotinylated secondary antibodies and visualization using a peroxidase-conjugated avidin-based Vectastain protocol. Slides were then counterstained with hematoxylin and mounted using PermOUNT mounting medium (Fisher). Images were captured using an Olympus FSX100 system and processed in Adobe Photoshop.

Quantitative PCR

RNA was extracted from xenograft tumors and cells using TRIzol (Invitrogen) following manufacturer's instructions. cDNA was synthesized with 1 μ g of total RNA with the High Capacity cDNA Reverse Transcription Kit (Invitrogen), per manufacturer's instructions. qPCR was performed in triplicate for each experimental condition using Maxima SYBR green qPCR Master Mix (Invitrogen), according to the manufacturer's instructions. To normalize mRNA levels, GAPDH probes were used. Primer pairs as follows: GAPDH Forward:

TCGACAGTCAGCCGCATCTTCTT, Reverse: GCGCCCAATACGACCAAATCC.

TINAGL1 Forward: ACCAGGTCACCTCCTGTCTACC, Reverse:

GATGCCTCCCTTGTATAGGAAG. CDC42 Forward: CCATCGGAATATGTACCGAC,

Reverse: CTCAGCGGTTCGTAATCTGTC. SPOCK2 Forward:

CCCGGCAATTTTCATGGAGG, Reverse: GCGGTTCCAGTGCTTGATC. GPC1 Forward:

GGCTGGTGGCTGCTATGT, Reverse: CAGGTTCTCCTCCATCTCGC. GPC2 Forward:

CACCTGCTGTTCCAGTGAGA, Reverse: AGAGAGTGCTGGGCTACTGA. GPC4

Forward: GTGGGAAATGTGAACCTGGAA, Reverse: CGAGGGACATCTCCGAAGG.

DKK4 Forward: GGGACACTCTGTGTGAACGA, Reverse:

TGGTTTTTCCTGGACTGGGTG. SFRP5 Forward: CTGTACGCGTCATCCTAGCC,

Reverse: CGGACCAGAAGGGGGTCTAT.

Fibrin gel assay

200 trypsinized SW480 cells were mixed with 100 μ L of 2.5 mg/mL bovine fibrinogen (MP Biomedicals) in DMEM plus 10% FBS and 1% Penicillin-Streptomycin-Glutamine and 1 μ L of thrombin (Sigma). The fibrin gels were seeded in 96 well, flat bottom plates. After the gels solidified, 100 μ L of DMEM media containing the desired drug treatment was layered on top (DCA was obtained from Sigma, XAV939 from Stemgent). Wells were imaged after 14 days of incubation. Size measurements were taken using Adobe Photoshop. Data was analyzed using Prism (Graphpad).

Image processing of spots

Image processing (overlay of spot contours and convex hulls) was done using built-in functions in MATLAB's Image Processing Toolbox. Briefly, a color channel of an image was converted to a binary image based on a manually chosen threshold dependent on staining intensity. A noise filter was applied to reduce background staining. Thresholds were then chosen to define cutoff values of spot boundaries. Parameters for built-in tools were chosen manually to give the best fit for pattern contours. Details for this method are provided in Appendix B A1, Supplementary Materials A1.1-A1.3, and Appendix B Figures S1 – S10.

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CHAPTER 3

Disrupting β -catenin dependent Wnt signaling activates an invasive gene program predictive of colon cancer progression

Abstract

The recent classification of colon cancer into four molecular subtypes revealed that patients with the poorest prognosis harbor the lowest levels of Wnt signaling. This is contrary to the long-standing understanding that overactive Wnt signaling promotes tumor progression from early initiation stages through to the later stages including invasion and metastasis. Here, we lower the levels of Wnt signaling in SW480 and SW620 colon cancer cell lines via interference with two different steps in Wnt signaling that lie upstream or downstream of the effector protein β -catenin. We find that these Wnt-reduced lines exhibit a more aggressive disease phenotype, including increased mobility *in vitro* and localized invasion in an orthotopic mouse model. RNA sequencing reveals that interference with Wnt signaling leads to an upregulation of gene programs that favor cell migration and invasion. We identify a set of upregulated genes common among the Wnt perturbations, and find that elevated expression of this gene set is strongly predictive of poor patient outcomes in early-invasive colon cancer. These genes may have clinical applications as either patient biomarkers or new drug targets to be used in concert with existing therapies.

Introduction

Globally, colorectal cancer (CRC) is one of the leading causes of cancer-related deaths. Though increased screening, changes in lifestyle habits associated with CRC risk, and

improvements in treatment have decreased the CRC mortality rate over the past few decades, metastatic disease continues to be a significant challenge to treat. Approximately 50-60% of CRC patients will develop metastatic disease, and the five-year relative survival for these patients is 13.9% (Davis, 2018).

Mutations in the Wnt signaling pathway are a hallmark of CRC, with 80-90% of patients harboring pathway-activating mutations. In normal colon epithelia, the β -catenin dependent Wnt signaling pathway is important in regulating the renewal of the epithelial layer, which turns over in its entirety every five to seven days. The epithelium consists of a regular repetition of invaginations known as crypts, at the base of which are highly proliferative intestinal stem cells. These cells respond to a high level of Wnt ligand secretion by their neighboring cells to activate the Wnt pathway and drive cell proliferation and self-renewal gene programs through the nuclear localization of β -catenin and subsequent binding to LEF/TCF transcription factors. The absence of Wnt ligands allows β -catenin to be targeted for phosphorylation and degradation by the destruction complex, a group of proteins including the scaffold protein Axin2, the tumor suppressor APC, and the kinases CK1 and GSK3 (Gammons & Bienz, 2018). In CRC, the most common mutations target adenomatous polyposis coli (APC), which then limits the cell's ability to sequester and degrade β -catenin, allowing β -catenin to translocate to the nucleus in the absence of a Wnt ligand binding to a Frizzled:LRP receptor complex. Though the general consensus has been that these activating mutations relieve CRC cells from responding to Wnt ligands, recent evidence has shown that Wnt ligands are still capable of modulating Wnt activity, even in APC-mutant cells (Voloshanenko *et al*, 2013; Seshagiri *et al*, 2012).

In addition to tumor growth, it is thought that the overactive Wnt pathway also drives disease progression through the latter stages of invasion and metastasis. Some of the findings that support this include a direct link between Wnt regulation of Snail and Twist expression (ten

Berge *et al*, 2008), two genes important in epithelial to mesenchymal transition. Both genes have been found to be direct transcriptional targets of β -catenin:LEF/TCF regulatory complexes. Other observations include the fact that the invasive edge of CRC tumors are frequently enriched in nuclear-localized β -catenin, suggesting high Wnt pathway activity in this region (Brabletz *et al*, 2001; Jung *et al*, 2001). Also, efforts to drug or decrease Wnt signaling have led to reductions in rates of cancer cell proliferation *in vitro* and tumor proliferation in *in vivo* xenograft models (Polakis, 2012). More recently however, several studies have challenged the dogma surrounding Wnt-driven disease progression. For example, one study reported that advanced colon cancers and metastases have overall decreased Wnt target gene expression relative to early stage tumors (de Sousa E Melo *et al*, 2011). Though nuclear β -catenin is observed in these patients' tumors, de Sousa E Melo et al found that several Wnt target genes, including *ASCL2* and *LGR5*, are methylated in advanced disease samples. Additionally, a different group showed that repression of Wnt signaling through expression of dominant negative TCF4 in colon cancer cells increased their metastatic potential when introduced into mice via tail vein injection (Varnat *et al*, 2010). But perhaps the most convincing evidence thus far is that advanced, poor prognosis colon cancers feature decreases in Wnt signaling - a finding that came from a recent effort to classify colon cancer patients by molecular subtype (Guinney *et al*, 2015). Combining data from six previous studies that collectively analyzed over 3000 patient tumors and associated clinical data, a four-cluster categorization scheme was developed. Of these four groups, the tumors with the highest Wnt gene signature (labeled CMS2, or Canonical) exhibited the best overall clinical outcome, while the patients with most aggressive disease, the poorest prognosis (CMS4, or mesenchymal) had the lowest Wnt signature expression. CMS4 additionally has a marked upregulation of gene programs associated with matrix remodeling, EMT activation, and stromal infiltration. Following up on this finding, Smedt et al analyzed patient samples of both total

tumor and portions of small budding cell clusters at the invasive edge and found that the invasive clusters demonstrated a shift from CMS2 to CMS4 – meaning, an overall lower level of Wnt target gene expression (Smedt *et al*, 2016). The invasive cells additionally showed upregulation in gene expression programs of EMT, cell movement, cell morphology, and cell survival - and downregulation of programs for cell growth and proliferation. In a following study that aligned PDX models and a large panel of colon cancer cell lines to the CMS system, a clear difference in response to chemotherapy emerged between CMS2 and CMS4 samples; chemotherapy induced apoptosis in CMS2 samples, but CMS4 samples were largely resistant (Linnekamp *et al*, 2018). Interestingly, the researchers were able to classify a majority of the traditional colon cancer cell lines tested into different CMS groups, even though these cells have been maintained in an *in vitro* system for decades. This suggests at least some level of intrinsic stability on the part of cancer cell lines in preserving CMS status *ex vivo*.

Here we primarily utilize two colon cancer cell lines, SW480 and SW620. These lines were derived from the same patient, the former from the primary tumor and the latter from a metastatic node. In line with the CMS2 to CMS4 observation described above, the baseline Wnt signaling level of SW620 cells is much lower than that of the SW480 line. We downregulate Wnt signaling activity in multiple ways, and utilizing an orthotopic mouse model, observe an increase in tumor invasion in all downregulated lines. Comparing the differentially expressed genes between the parental and downregulated lines, we identify a 12-gene signature correlated with increased tumor cell invasion. We validate a number of these genes by protein expression, and demonstrate in multiple patient datasets that overexpression of this gene signature is significantly correlated with poor prognosis. We conclude that decreasing Wnt signaling causes invasion-associated genes to be expressed, and we therefore further suggest that at least in mid-stage CMS4 tumors, patients would not benefit from Wnt inhibitor therapies.

Materials and Methods

Cell lines

SW480, SW620, COLO320, and HCT116 were obtained from ATCC. Cell lines were cultured in Dulbecco's Modification of Eagle's Medium (DMEM, Hyclone) or RPMI-1640 (Hyclone) supplemented with 10% Fetal Bovine Serum (Atlas Biologicals), 1% Penicillin/Streptomycin (Mediatech), and 2mM Glutamine (Mediatech). Unless otherwise stated, all assays were conducted in the same media. dnLEF1 cell lines were prepared as previously described (Pate *et al*, 2014). LRP6 knockout (LRP6KO) cell lines were created by co-transfecting cell lines with 3 μ g LRP6 CRISPR and 300ng eGFP-Puro using BioT transfection reagent (BioPioneer). After selection with Puromycin, cells were sorted for LRP6 knockdown using flow cytometry (anti-human LRP6-APC, R&D Systems MAB1505) and successful knockdown was confirmed by Western blot.

LRP5 and LRP6 CRISPR constructs

The CRISPR kit used for constructing multiplex CRISPR/Cas9 vectors was a gift from Takashi Yamamoto (Addgene kit #10000000054). Guide RNAs targeting LRP5 and LRP6 were designed using the Zhang Lab Optimized CRISPR Design Tool (<http://crispr.mit.edu>). A Cas9 nickase system was used such that two closely aligned guides per gene were developed. For LRP5, the primer pairs used were: LRP5-Cas9n-1-Fwd: CACCGCTCGGTCCAGTAGATGTAGC, LRP5-Cas9n-1-Rev: AAACGCTACATCTACTGGACCGAGC, LRP5-Cas9n-2-Fwd: CACCGCGGCAAGCCGAGGATCGTGC, LRP6-Cas9n-2-Rev: AAACGCACGATCCTCGGCTTGCCGC. For LRP6, the primer pairs used were: LRP6-

Cas9n-1-Fwd: CACCGTTGGCCAAATGGTTTAGCCT, LRP6-Cas9n-1-Rev:
AAACAGGCTAAACCATTGTTGGCCAAC, LRP6-Cas9n-2-Fwd:
CACCGAAGTGTTAACCAATACTACA, LRP6-Cas9n-2-Rev:
AAACTGTAGTATTGGTTAACACTTC. Guides were inserted into vectors using T4 polynucleotide kinase (New England Biolabs), and the final combined guide RNA vector was assembled by Golden Gate Assembly using Quick Ligase (New England Biolabs). The final vector was electroporated into DH5a competent cells and purified using a Nucleospin Maxiprep kit (Macherey Nagel). Restriction enzyme digested products were run out on an agarose gel to confirm insert size and plasmid quality. Correct assembly was verified by Sanger Sequencing.

Western blotting

Eighty micrograms of total cell lysates were analyzed by Western blot using the following antibodies and concentrations: LRP6 (1:1000, Cell Signaling Technology 3395), LRP5 (1:1000, Cell Signaling Technology 5731), Beta-Catenin (1:1000, Cell Signaling Technology 8480), Beta-Tubulin (1:2000, Genetex GTX101279). All blots were incubated overnight in primary antibody, washed, and then incubated for two hours in secondary antibody – anti-rabbit-HRP (1:5000, GE Healthcare) or anti-mouse-HRP (1:2000, GE Healthcare). Blots were imaged using a Syngene G-Box system. Bands were quantified using Adobe Photoshop. Statistical evaluation of three or more independent biological replicates was performed using Student's Two-Tailed T-Test.

Luciferase assay

Cells were seeded at a density of 1×10^5 cells per well in a 12-well tissue culture plate using antibiotic-free media 24 hours prior to transfection. Cells were transfected with 100ng SuperTOPFlash or SuperFOPFlash (Gifts from Dr. R.T. Moon, Addgene plasmid 12456) and

100ng CMV- β -Galactosidase using BioT (Bioland Scientific B01-02) per manufacturer's instructions. Cells were harvested 24 hours post-transfection and assayed for luciferase activity and β -galactosidase activity (as a normalization control). Statistical evaluation was performed using Student's unpaired T-test.

Scratch assay

Cells were seeded at a density of 2×10^6 cells per well in a 6-well plate. After 24 hours, 2 crosses were scratched into the cell monolayer with a P1000 tip. Each well was washed once with PBS before incubating in media. Images were taken at 0 hours, 24 hours, and 48 hours post-scratch. Measurements were obtained using Adobe Photoshop image analysis. Statistical evaluation was performed using Student's unpaired T-test.

Flow cytometry

One million cells per sample condition were collected and washed with FACS buffer (3% FBS in PBS). If needed, cells were fixed in 4% paraformaldehyde for 15 minutes at room temperature, and permeabilized by 0.1% saponin in HBSS for 15 minutes on ice. Cells were incubated with primary antibody for 1 hour at room temperature in the dark. If secondary antibody was needed, cells were washed twice with FACS buffer prior to incubating in secondary antibody for 30 minutes at room temperature, in the dark. Cells were finally washed and resuspended in 300-600 μ L FACS buffer per sample and analyzed on either a BD FACS Aria or Acea Biosciences Novocyte. Data was analyzed using FlowJo (Treestar).

Orthotopic injections

Immuno-deficient NSG mice (Jackson labs) were anaesthetized with 300µl of 100mg/kg ketamine/10mg/kg xylazine. The surgical site was shaved and cleaned with ethanol. After an incision was made, the caecum was drawn out and washed with sterile PBS. Cells (5×10^5) were injected in the caecum wall at six to eight sites using a Hamilton syringe (Hamilton 80301), three to four injections per side. The peritoneal wall and skin were sutured separately, the former with resorbable sutures, the latter with nylon. 5mg/kg carprofen was injected as an analgesic. Mice health was monitored for 28 days prior to harvest.

Immunohistochemistry

Excised caecums were fixed in 10% formalin, cut on the latitude, and mounted on edge in paraffin. 10µm FFPE sections were cut onto SuperFrost Plus slides. For antigen retrieval, slides were deparaffinized and rehydrated, followed by antigen retrieval in a pressure cooker using 10mM sodium citrate for five minutes at pressure. Slides were then stained by hematoxylin and eosin, dehydrated and mounted using Permount mounting medium (Fisher). Slide images were captured with a Keyence BZ-X700 system and processed in Adobe Photoshop.

Pathology scoring

A blinded evaluation of stained slides was performed by pathologists who scored each section of caecum on the absence, low presence, or high presence of phenotypic features. Additional measurements were taken of each section by comparing the total pixels of a section to pixels of individual components (eg. extracolonic tumors, intact epithelia, et cetera) and normalizing by percentage of total section. Approximately three to five sections per caecum from at least three mice per cell line were analyzed. A complete set of analyzed images is in Appendix C

Supplemental Materials Figure 6.

Quantitative PCR

RNA was extracted from cells using TRIzol (Invitrogen) and DirectZol RNA Extraction Kit following the manufacturer's instructions (Zymo Research). RNA was extracted from flash-frozen tissue samples by using a mortar and pestle to crush tissue into fine powder, homogenizing in TRIzol using a Precellys 24 (Bertin), and extracting the RNA with the DirectZol kit. cDNA was synthesized from 1 µg of total RNA using the High Capacity cDNA Reverse Transcription Kit (Invitrogen), as per the manufacturer's instructions. qPCR was performed in triplicate for each experimental condition using Maxima SYBR Green qPCR Master Mix (Invitrogen), according to the manufacturer's instructions. Primer pairs used are as follows: *AXIN*: Forward:

CTGGCTTTGGTGAAGTGTG Reverse: AGTTGCTCACAGCCAAGACA

, *MYC*: Forward: CTACCCTCTCAACGACAGCA Reverse:

AGAGCAGAGAATCCGAGGAC, *SP5*: Forward: AATGCTGCTGAACTGAATAGA

Reverse: AACCGGTCCTAGCGAAAACC, *GAPDH*: Forward:

TCGACAGTCAGCCGCATCTTCTT Reverse: GCGCCCAATACGACCAAATCC.

RNA Sequencing

RNA sample quality was tested using an Agilent Bioanalyzer 2100. Libraries were prepared using TruSeq Stranded mRNA kit (Illumina) and sequenced on an Illumina HiSeq 2500. The resulting sequencing data was trimmed with Trimmomatic (Bolger *et al*, 2014) prior to alignment to GRCh38 using Bowtie2 (Langmead & Salzberg, 2012) and Tophat2 (Kim *et al*, 2013). Counts matrices were generated using HTSeq (Anders *et al*, 2015). Differential gene expression analysis was performed using DESeq R package (Anders & Huber, 2010). Dataset overlap was performed using the online Venn diagram tool at <http://bioinformatics.psb.ugent.be/webtools/Venn/>.

Gene set expression analysis (GSEA) was performed using GSEA (Mootha *et al*, 2003; Subramanian *et al*, 2005).

Patient survival analyses

Kaplan-Meier analyses of publically available colon cancer datasets were performed using PROGgeneV2 (Goswami & Nakshatri, 2014) and SurvExpress (Aguirre-Gamboa *et al*, 2013).

Results

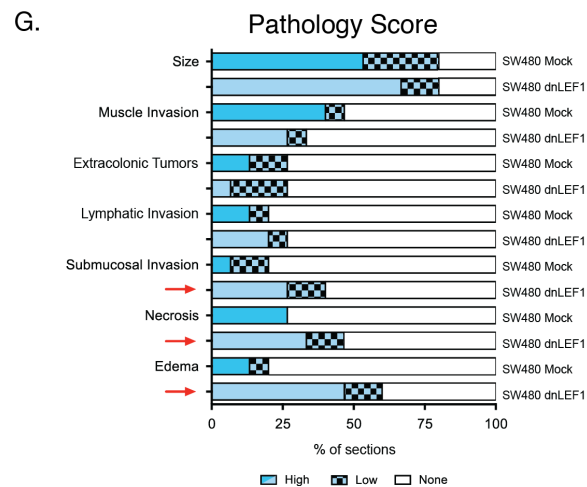
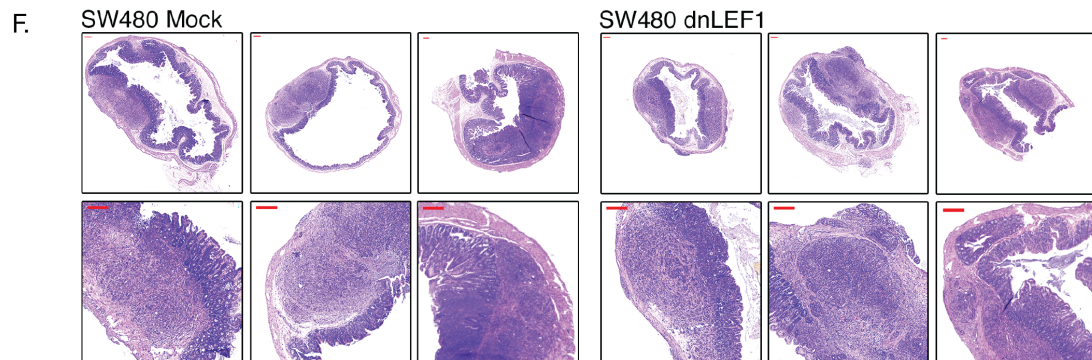
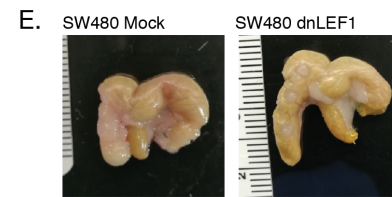
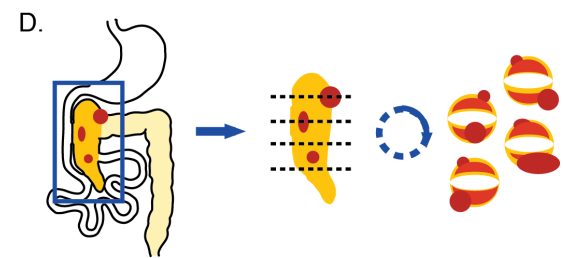
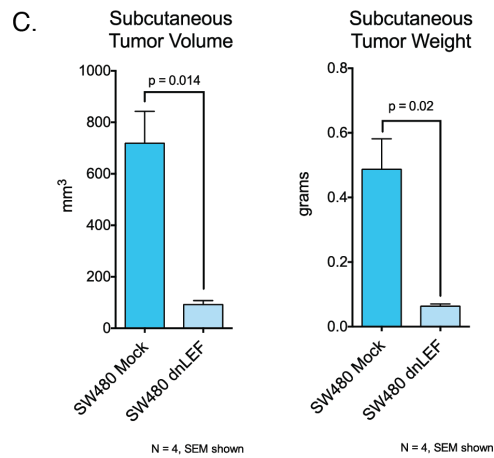
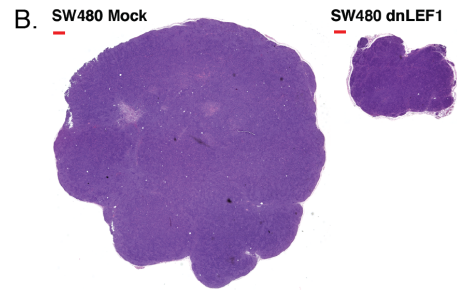
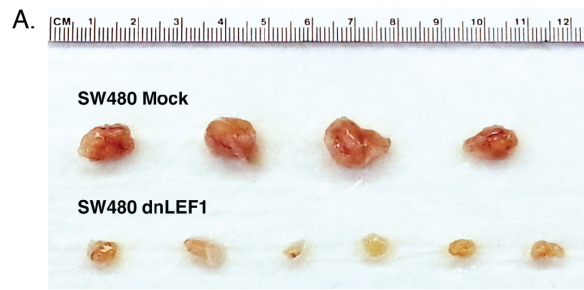
Decreasing Wnt signaling in SW480 cells increases cell invasiveness

Our previous studies probed how decreases in Wnt signaling affected in a subcutaneous xenograft tumor model (Pate *et al*, 2014). To interfere with Wnt signaling, we lentivirally transduced an expression vector for dominant negative LEF1 (dnLEF1) into the cells prior to subcutaneous injection (Figure 3.1A-C). The dnLEF1 expression construct lacks coding sequences for the N-terminal β -catenin binding domain of full-length LEF1, but retains all other sequences, including the HMG-box DNA binding domain and nuclear localization signal. This truncated transcription factor therefore can localize to the nucleus and displace endogenous full-length LEF/TCFs from their occupancy of Wnt Response Elements (WREs) at bona fide Wnt target genes. We used this mode of interference as it had been first developed by Van de Wetering and Clevers and used by multiple groups to effectively interfere with canonical Wnt signaling in the nucleus (Van de Wetering *et al*, 2002). Indeed, using a Wnt-luciferase assay, we find that dnLEF1 transduction-expression decreases Wnt signaling activity 90% compared to Mock-transduced cells in SW480. (Figure 3.2B) Three weeks after subcutaneous injection, we harvested the tumors and examined them histologically. We observed that tumors expressing

dnLEF1 exhibited a significant decrease of in tumor weight and volume (4-5 fold) and a 50% decrease in angiogenic vasculature (Figure 3.1A-C)(Pate *et al*, 2014). These data are in line with the overall understanding that high levels of Wnt signaling promote tumor growth and development. However, the subcutaneous microenvironment on the back flank of a mouse is not the endogenous location for colon tumor development and further, the subcutaneous environment lacks the nutrient availability of the richly vascularized intestinal epithelium. We therefore expanded our animal studies to include an orthotopic injection model whereby tumors were developed in the mouse colon.

To study tumorigenesis in a more representative microenvironment, we injected the SW480 Mock or SW480 dnLEF1 cells into the stroma between the epithelial and muscle wall layers of the caecum. After four weeks of development, the caecums were harvested and sectioned for immunohistochemistry. Unlike our findings with subcutaneous tumors, we observed no gross deficit in tumor size when comparing the SW480 Mock and SW480 dnLEF1 orthotopic tumors implying that the high-density vasculature in the colon wall was able to compensate for the deficits in angiogenic development in the subcutaneous-dnLEF1 tumors (Figure 1F). A blinded evaluation of physiological features for each tumor by pathologists revealed that SW480 dnLEF1 orthotopic tumors exhibit higher rates of invasion into the submucosa, higher prevalence of intratumor necrosis, and much more edema (Figure 1G). When each component of the caecum and tumor was quantified by area on a per-section basis, there was no significant difference in amount of caecum space filled by tumor, but we observed a significant increase in edema induced by the SW480 dnLEF1 tumors. Along with a near-significant difference in the degree of intact epithelium, these results point to a more aggressive tumor formed by SW480 dnLEF1 cells, extravasating and clogging the lymphatic network, and likely leading to the increase in edema (Appendix C Supplemental Figure 7). These data very clearly suggest that

SW480 dnLEF1 tumors are more invasive and their cells more migratory. To evaluate the intrinsic migratory potential of the cells, we plated parental and dnLEF1 expressing cells at high density in a tissue culture plates and wounded the cell monolayer by introducing a “scratch-wound” track. After 24 hours, we observed that SW480 dnLEF1 cells were significantly more migratory into the scratch-wound than SW480 Mock cells (Figure 3.1I). We conclude that the decrease of Wnt signaling in colon cancer cells creates a more aggressive tumor both because the cells are intrinsically more migratory and because given the correct microenvironmental context they exhibit invasive behaviors.



H. Wound Closure SW480 Mock/dnLEF1

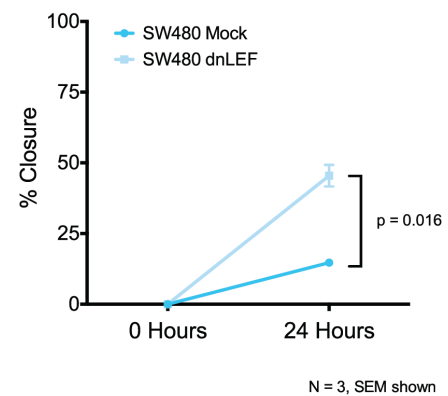


Figure 3.1. Decreasing Wnt in SW480 colon cancer xenografts increases cell migration and invasion

A-C. dnLEF1-transduced SW480 cells show a significant decrease in tumor burden compared to Mock-transduced SW480 cells in subcutaneous tumors in immunocompromised mice. Representative tumors shown at harvest (A), FFPE sections stained with hematoxylin & eosin (B). Scale bar is 500 μ m. Quantitation of both tumor volume and weight show statistically significant differences between SW480 Mock and SW480 dnLEF1.

D. Schematic of orthotopic injection and harvest. Tumors are injected into the caecum wall and allowed to grow for 3-5 weeks. At harvest, the caecum is cut latitudinally and mounted on edge.

E-F. Representative images of caecums at harvest (E) and FFPE sections stained with hematoxylin and eosin (F). Top row: full caecum section, bottom row: magnified inset. All scale bars 200 μ m.

G. Blinded pathologist scoring of orthotopic tumor sections shows a greater number of sections ranked high in submucosal invasion, necrosis, and edema in SW480 dnLEF1 tumors compared to SW480 Mock.

H. Scratch assay of SW480 Mock and SW480 dnLEF cells on plastic showed that dnLEF1 expression significantly increases cell migration after 24 hours of culture.

LRP6 is required for β -catenin-dependent Wnt signaling in colon cancer cells

Because several groups have reported that Wnt ligands are capable of enhancing Wnt signaling activity, even in APC-mutant colon cancers (Voloshanenko *et al*, 2013; Seshagiri *et al*, 2012; Giannakis *et al*, 2014; Jung *et al*, 2015; Nishioka *et al*, 2011), we hypothesized that autocrine-acting Wnt ligands from the cancer cells and paracrine Wnt signals from the surrounding microenvironment might contribute to maintaining colon cancer cells in a non-invasive state in the orthotopic condition. The dominant negative LEF1 constructs do not directly address this hypothesis, as their effect is to interfere with β -catenin actions in the nucleus, not the cell membrane. Therefore, to more directly probe how Wnt ligands in the tumor microenvironment might affect β -catenin-dependent signaling and colon cancer cell behavior, we used a CRISPR/Cas9 system to genetically knockout the essential Wnt ligand co-receptors LRP5 and LRP6. We tested the efficacy of this manipulation by transiently co-transfecting HEK293 cells with the Cas9 nickase and guide RNA expression constructs with various Wnt ligand expression plasmids for ligands known to be upregulated in colon cancer. Using the SuperTOPFlash luciferase reporter we assayed for Wnt signaling activity. Knockdown of LRP5 or LRP6 expression effectively interfered with Wnt ligand activation of the luciferase reporter. For example, we noted that the Wnt1 ligand required both LRP5 and LRP6 for reporter activation (Supplemental Figure 2), in line with observations from other groups (Goel *et al*, 2012). We tested other Wnt ligands and found that while a number of Wnt ligands required both LRP5 and LRP6, others required solely LRP6 while none of the ligands required only LRP5 (Appendix C, Supplemental Figure 2). In part, this may be due to LRP6 influencing LRP5 expression, as the knockout of LRP6 also reduced LRP5 protein (Appendix C, Supplemental Figure 4). To confirm that this was not an off-target effect of our chosen guide RNAs, we also silenced LRP6 using an shRNA construct, which yielded the same result. Given that the LRP6 co-receptor

appears to be a uniformly required co-receptor for β -catenin-dependent signaling, we focused our remaining efforts using LRP6 knockout cells.

LRP6 modulates Wnt signaling levels in APC-mutant colon cancer

We noted that SW480 cells do not innately express much LRP6 protein and that what residual LRP6 protein is detectable, is not detectable on the cell membrane where Frizzled receptors reside (Appendix C, Supplemental Figure 1). We therefore used SW620 cells, which are derived from an invaded lymph node of the same patient as SW480. LRP6 is robustly expressed on the cell surface of these cells and when we transfect these cells with the LRP6 CRISPR expression construct, LRP6 protein levels drop and Wnt signaling activity is decreased by ten to twenty percent compared to expression of Cas9 alone (Figure 3.2B). As expected, compared to the dominant negative LEF1-expressing lines, the effect of a genetic LRP6 knockout on overall Wnt signaling is not as strong. Using flow cytometry, we were able to specifically sort-isolate a population of LRP6 knockout cells from the total transfected SW620 cell population (Figure 3.2C). We validated that LRP6 expression is strongly decreased in the sorted SW620 LRP6KO cells by Western blot (Figure 3.2D). Using a quantitative PCR assay, we measured the mRNA expression levels of Wnt target genes *AXIN2*, *MYC*, and *SP5* in our three sets of manipulated cell lines and detected significant decreases in expression of these Wnt target genes in both SW480 dnLEF1 and SW620 dnLEF1 cell lines. Only *AXIN2* was significantly downregulated in SW620 LRP6KO, although *MYC* and *SP5* trend towards significant downregulation. These results demonstrate that decreasing expression of LRP6 moderately decreases Wnt signaling, pointing to real possibilities for ligand-based Wnt pathway activation, even in the presence of a downstream APC mutation. Additionally, we found that β -catenin protein levels significantly decreased when *LRP6* was knocked out, but not with dnLEF1 expression (Figure 2F). In part, this can be

explained by a decrease in β -catenin (*CTNMB1*) mRNA expression (data not shown), though notably total cellular protein level of β -catenin decrease more than its *CTNMB1* mRNA, suggesting that β -catenin protein degradation is enhanced. This is entirely plausible and in line with findings from Saito-Diaz et al (Saito-Diaz *et al*, 2018), which suggest that LRP6 associates with the β -catenin destruction complex in the absence of a Wnt ligand, partially inhibiting its activity. Thus the loss of LRP6 allows the destruction complex to be more active, decreasing overall β -catenin protein. We confirmed this by decreasing LRP6 expression in colon cancer cell line HCT116, which has a mutant β -catenin and wildtype APC. LRP6 knockout did not significantly decrease Wnt signaling activity in these cells, suggesting that the release of the destruction complex from the plasma membrane did not increase β -catenin degradation (Appendix C, Supplemental Figure 3). We conclude that knocking out LRP6 in APC-mutant colon cancer cell lines destabilizes β -catenin and moderately decreases Wnt signaling.

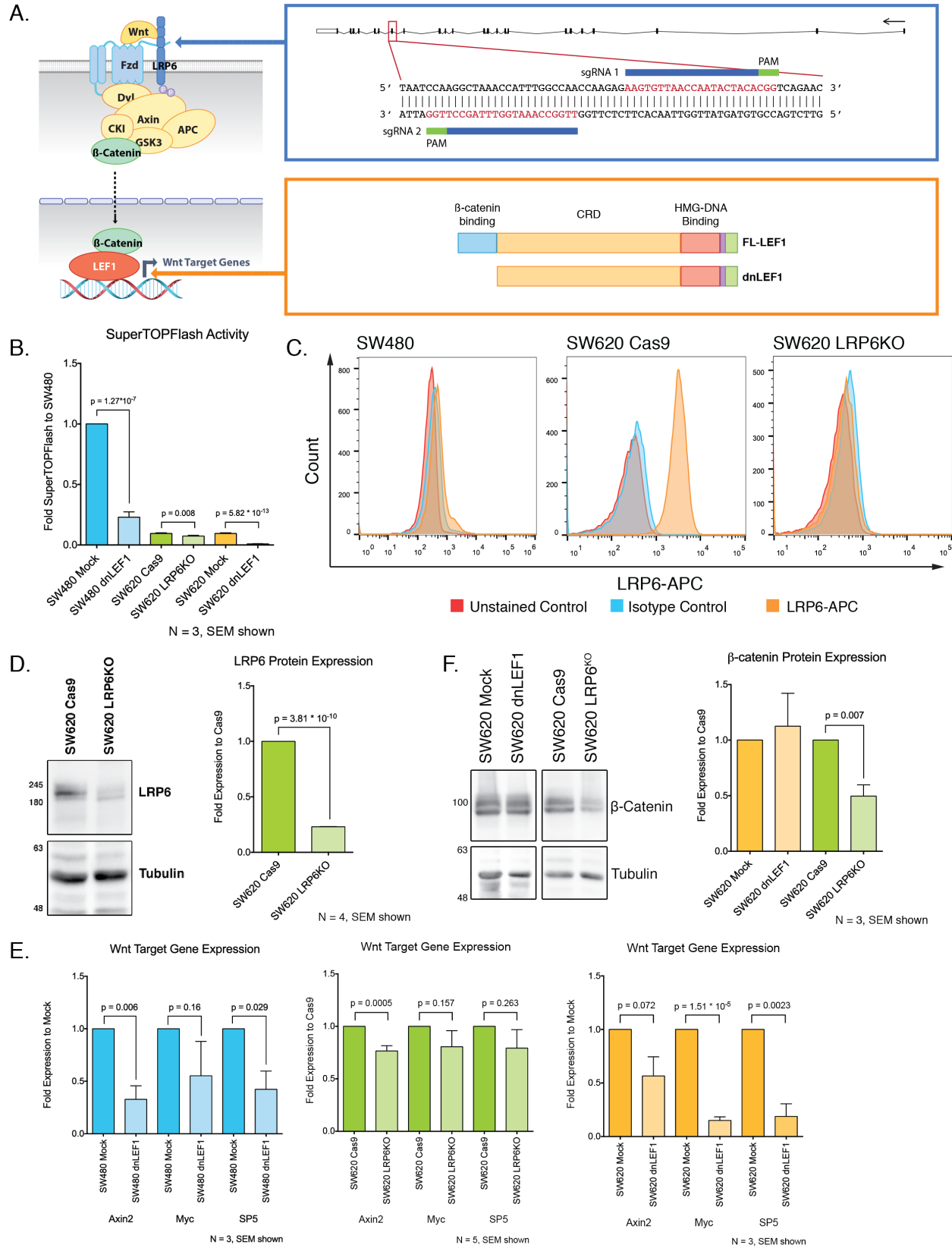


Figure 3.2. Modulating Wnt signaling upstream and downstream of β -catenin.

A. Schematic for dnLEF1 and LRP6 CRISPR effects on β -catenin dependent Wnt signaling. Two CRISPR guide RNAs were developed to target LRP6, their alignments to the nucleotide sequence along with the PAM sites are highlighted in the blue box. Both guide RNAs were used with a Cas9 nickase for specific targeting of LRP6. dnLEF1 is missing the β -catenin binding domain, preventing β -catenin from activating Wnt target gene expression.

B. Wnt signaling levels as measured by SuperTOPFlash luciferase reporter activity, normalized to SW480 Mock. SW620 cells exhibit significantly lower levels of Wnt signaling; in both cell lines expression of dnLEF1 further reduced Wnt activity by 80-90%. LRP6 knockout in SW620 cells induced a modest, but statistically significant decrease in Wnt signaling activity.

C. Live cell flow cytometry reveals minimal expression of LRP6 in SW480 cells at the cell membrane, but is visible when cells are fixed (Appendix C, Supplemental Figure 1). In contrast, SW620 cells robustly express LRP6, and LRP6 CRISPR expression, coupled with FACS sorting and clonal expansion, successfully eliminated LRP6 protein from the cell membrane.

D. Validation of LRP6 knockout in SW620 by Western blotting shows a significant decrease in total protein level.

E. Expression of Wnt target genes Axin2, Myc, and SP5 in SW480 dnLEF1, SW620 dnLEF1, and SW620 LRP6KO normalized to parental control. Significant decreases in Wnt target gene expression levels are observed in all three cell line pairs.

F. Total β -catenin protein level is significantly decreased in SW620 LRP6KO, but not SW620 dnLEF1. The loss of LRP6 affects the stability of β -catenin due to the release of the destruction complex from the plasma membrane. (Saito-diaz *et al*, 2018)

Decreasing Wnt signaling in SW620 cells leads to an invasive phenotype

In comparison to SW480 cells, SW620 cells are more invasive in the orthotopic tumor condition where we observe tumor cells invading the epithelial layer and coursing through the muscle layer to form tumors on the outer wall of the colon (Figure 3.3A). Interestingly, lentiviral transduction-expression of dnLEF1 in SW620 cells increased the invasive phenotype even more (Figure 3.3A & B). Blinded scoring of SW620 Mock and dnLEF1 orthotopic tumor sections confirmed a higher percentage of SW620 dnLEF1 sections with extracolonic tumors (Figure 3.3C). Additional sections revealed an increase in tissue edema, a likely result of tumor cells filling and plugging the lymphatic network to prevent drainage. In both SW620 Mock and SW620 dnLEF1 tumors, more sections contained necrotic areas compared to SW480 Mock or SW480 dnLEF1. This may suggest an innate, higher sensitivity of the SW620 cells to nutrient levels in the surrounding microenvironment. Orthotopic tumors of SW620 Cas9 and SW620 LRP6KO were largely indistinguishable from one another by pathology scoring, except for again, a higher prevalence of large extracolonic tumors in LRP6KO sections (Figure 3.3E). While the number of extracolonic tumors per section was similar between the two tumor types, the size of the LRP6KO tumors were dramatically larger than the Cas9 tumors (Figure 3.3F), suggesting that the SW620 LRP6KO have an improved ability to migrate, survive, and/or proliferate outside the colon. This does not appear to be the case in SW620 dnLEF1 orthotopic tumors, which grow to fill the epithelial space significantly more than SW620 Mock, but do not produce significantly larger extracolonic tumors (Appendix C, Supplemental Figure 7). In vitro, SW620 dnLEF1 was found to be significantly more motile than SW620 Mock after 24-hour incubation. The SW620 LRP6KO cells were also more migratory, but this enhanced phenotype only began to show significant differences after 48 hours (Figure 3.3G & H). This suggests that there may be a graded effect to Wnt signaling inhibition of cell migration, with decreasing Wnt signaling levels yielding

an increase in the rate of migration. Using immunohistochemistry, we probed orthotopic tumors for phosphorylated Histone H3 as a marker of proliferation, and found no measurable difference between SW620 Cas9 and SW620 LRP6KO orthotopic tumors (Data not shown). Therefore, we conclude that the larger extracolonic tumors from SW620 LRP6KO cells are more likely the result of increased survival rather than increased migration.

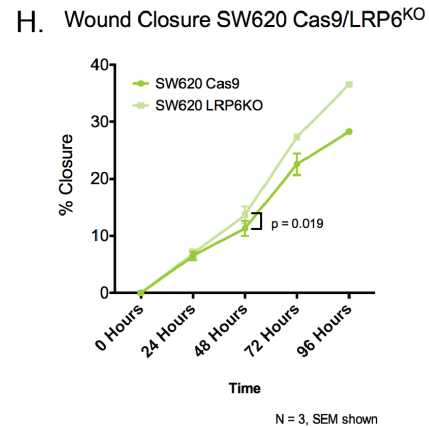
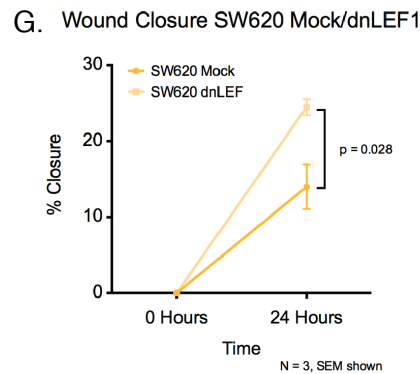
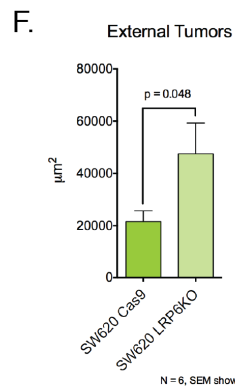
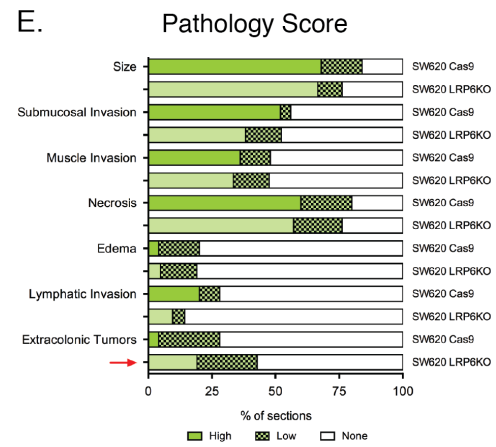
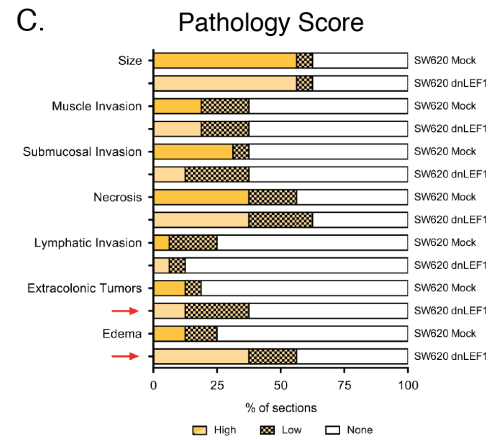
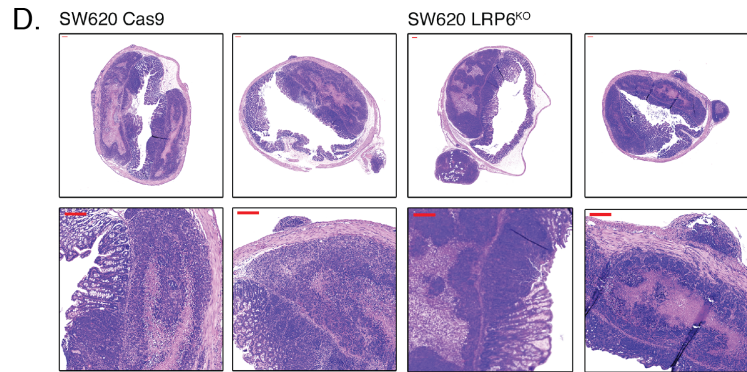
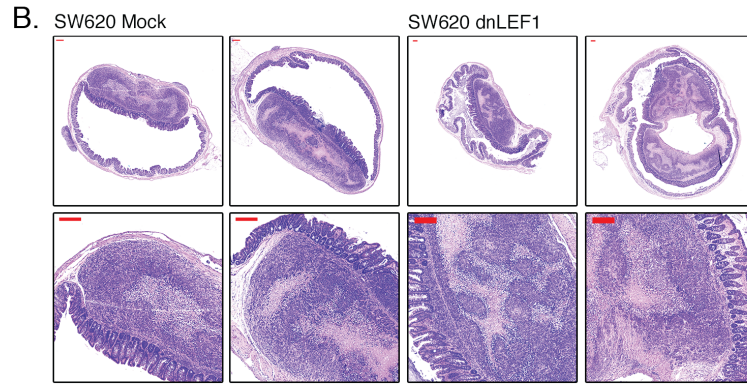
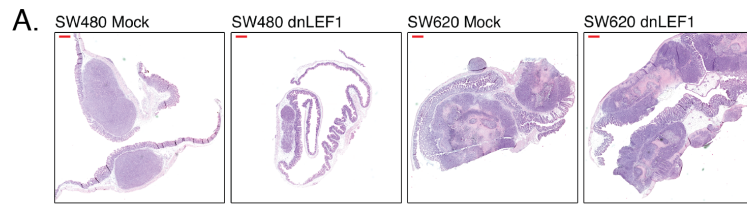


Figure 3.3. Wnt-reduced cell lines are more locally invasive than their parental counterparts

- A. Hematoxylin and eosin stains of orthotopic tumor sections. SW480 orthotopic tumors are less aggressive than SW620 orthotopic tumors, expression of dnLEF1 enhances invasion. Scalebars are 500 μ m.
- B. Representative hematoxylin & eosin images of SW620 Mock and SW620 dnLEF1 orthotopic tumors. Scalebars are 200 μ m.
- C. Blinded pathologist scoring of SW620 Mock and dnLEF1 orthotopic tumor sections. A greater number of dnLEF1 tumor sections ranked “high” or “low” for extracolonic tumors and edema compared to Mock tumor sections.
- D. Representative hematoxylin & eosin stained sections of SW620 Cas9 and SW620 LRP6KO orthotopic tumors. Scalebars are 200 μ m.
- E. Blinded pathologist scoring of SW620 Cas9 and SW620 LRP6KO orthotopic tumor sections. A greater number of LRP6KO sections had extracolonic tumors compared to Cas9 sections.
- F. Area measurements of extracolonic tumors show a significantly higher average extracolonic tumor size in LRP6KO samples.
- G-H. Scratch assay of SW620 Mock versus dnLEF1 shows significant difference in cell motility by 24 hours post-scratch, while SW620 Cas9 versus LRP6KO shows a significant difference in cell motility only after 48 hours, suggesting with a more dramatic Wnt inhibition, there is an increase in cell motility.

RNA Sequencing reveals a common invasive gene signature for decreased β -catenin-dependent Wnt signaling

To identify drivers of this invasive phenotype, we assessed global mRNA levels in our SW480 and SW620 cell lines and found that expression of dnLEF1 induced dramatic changes in gene expression in both SW480 and SW620 cell lines compared to the Mock-transduced cells. Not surprisingly, the more moderate change in Wnt signaling induced by knocking out LRP6 led to fewer and smaller, but still-significant changes in gene expression in SW620 (Figure 3.4A). Paradoxically however, we noted that the number of genes that were significantly different in expression between the low-Wnt signaling SW620 Mock cells and the even lower Wnt signaling SW620 dnLEF1 cells was several orders of magnitude greater than the number of genes with differential expression between SW480 Mock and SW480 dnLEF1. We hypothesize that this difference derives from the more plastic state in SW620 compared to SW480 cells, and a state that is more sensitive and responsive to genetic changes. As SW620 cells were derived from a more advanced, metastatic site (lymph node) compared to the SW480 line, which was derived from the primary tumor, these cells would have proceeded through an epithelial-to-mesenchymal transition (EMT), a process that requires de-differentiation. In fact, poorly differentiated tumors are known to be more invasive than well-differentiated tumors, and as such, this state correlates with poor patient prognosis. To address this, we used Gene Set Enrichment Analysis (GSEA, (Subramanian *et al*, 2005; Mootha *et al*, 2003)), and found that compared to SW620, SW480 cells were significantly enriched in a number of differentiation and development gene sets - including stem cell differentiation, positive regulation of cell development, and tissue morphogenesis (Figure 3.4B). Additionally, GSEA of pluripotency markers identified in Mallon *et al* (Mallon *et al*, 2013) showed a significant enrichment in SW620 compared to SW480 (data not shown). This

suggests that the more advanced and invasive SW620 cell line is characterized by increased cell plasticity and de-differentiation to a more stem cell-like state.

To more definitively address the question of decreasing Wnt signaling in invasive colon cancer, we conducted GSEA comparing SW480 Mock and SW480 dnLEF1. We find that regulation of the canonical Wnt signaling pathway is significantly enriched in SW480 dnLEF1, which serves as an embedded positive control. Among the gene sets that were enriched, we find that regulation of epithelial to mesenchymal transition and negative regulation of cell substrate adhesion to both be significantly enriched in SW480 dnLEF1, suggesting a transition to a more invasive cell phenotype (Figure 3.4C). Interestingly, the shift to a de-differentiated state is not significant; with neither sample being enriched for markers of pluripotency (data not shown). This suggests that while the decrease in Wnt signaling is not sufficient to drive cells to a more plastic state, it is sufficient to enable cell migratory phenotypes. To identify genes whose expression is correlated with an invasive phenotype, we compared the lists of differentially upregulated genes from each of our cell line pairs: SW480 Mock and SW480 dnLEF, SW620 Mock and SW620 dnLEF, and SW620 Cas9 and SW620 LRP6KO (Figure 3.4D). This comparison showed that 21 genes were commonly upregulated. Several of these genes have been characterized as having a role in cell migration, but only one (*TNC*) has been previously linked to Wnt signaling (Saupe *et al*, 2013; Beiter *et al*, 2005). We also compared the lists of differentially downregulated genes from our cell line pairs, but found no common mechanism among the 16 genes (Appendix C, Supplemental Figure 10). A heatmap of the 21 upregulated genes in individual RNA samples shows consistent enrichment in SW480 Mock versus SW480 dnLEF1 and SW620 Mock versus SW620 dnLEF1, but a more heterogeneous expression in SW620 Cas9 versus SW620 LRP6KO, perhaps due to a less significant change in Wnt signaling (Figure 3.4E). Western blotting of AXL, TEM4 (*ARHGEF17*), and Vimentin (*VIM*) showed significant

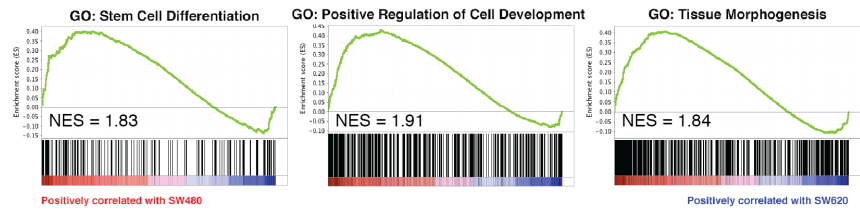
upregulation at the protein level in the Wnt-reduced cell lines, consistent with the RNA sequencing results (Figure 3.4F). We conclude that a decrease in Wnt signaling induces gene expression changes in line with an increase in cell migration, and we identify a list of potential invasive gene signature candidate members.

A.

	UP	DOWN
SW480 Mock vs dnLEF1	1941	1955
SW620 Mock vs dnLEF1	4424	4585
SW620 Cas9 vs LRP6KO	121	118

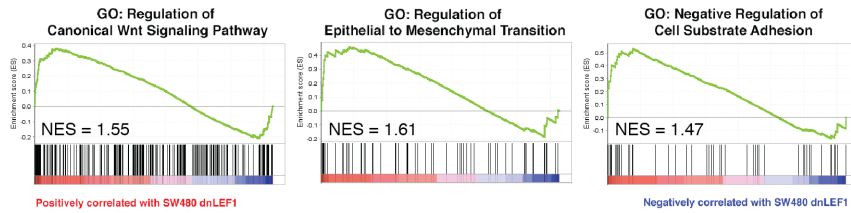
B.

Selected gene sets enriched in SW480 vs SW620

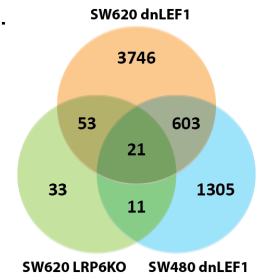


C.

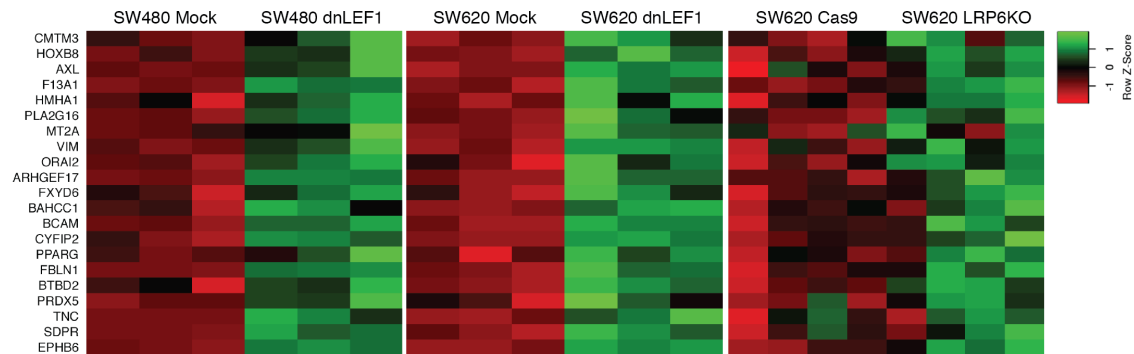
Selected gene sets enriched in SW480 dnLEF1 vs SW480 Mock



D.



E.



F.

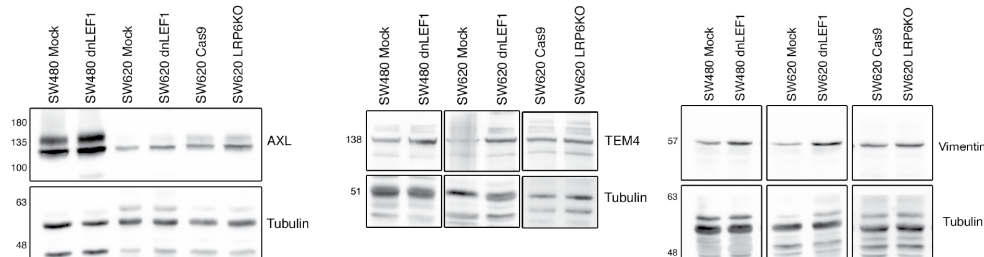
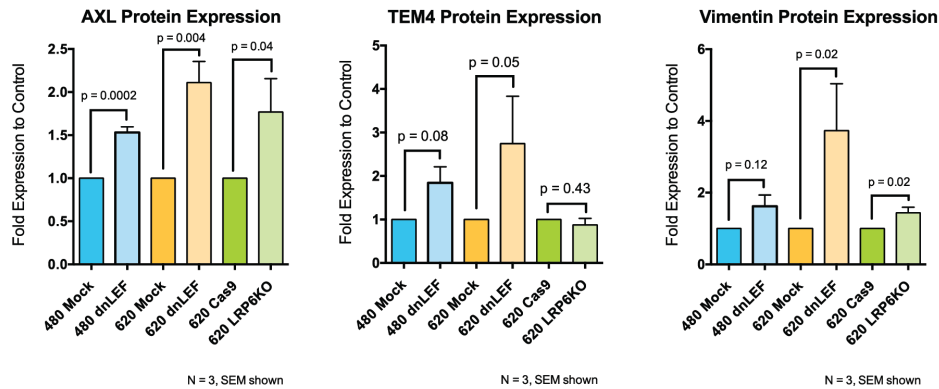


Figure 3.4. RNA sequencing reveals a common set of upregulated genes in Wnt-reduced cell lines

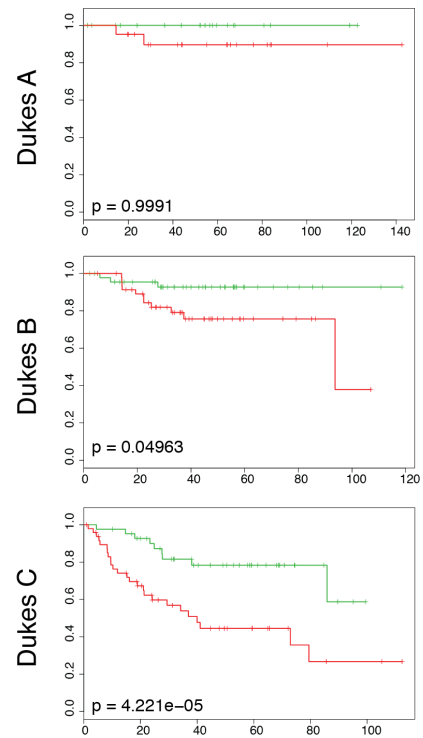
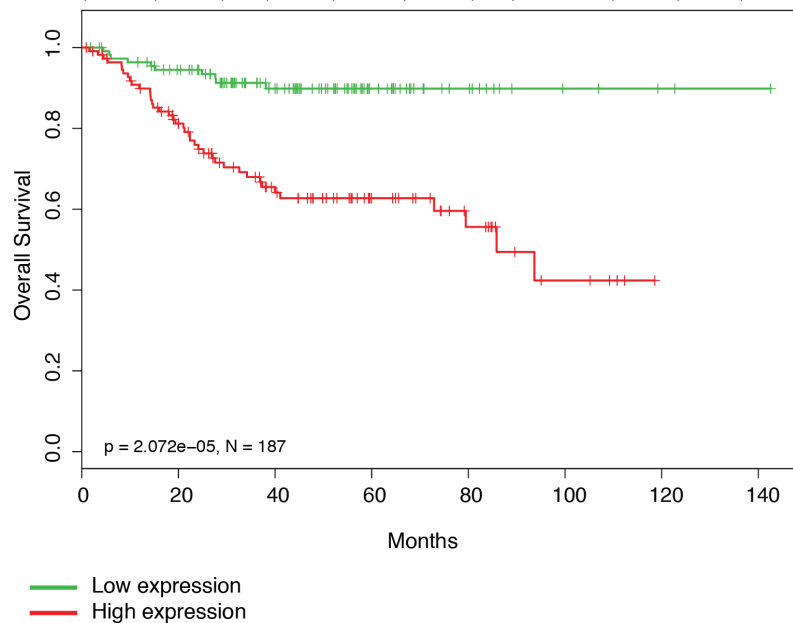
- A. Number of significant differentially expressed genes in cell line pairs.
- B. Gene Set Enrichment Analysis (GSEA) comparing SW480 and SW620 lines shows enrichment in genes programs for differentiation in SW480 cells.
- C. GSEA of SW480 dnLEF1 compared to SW480 Mock shows enrichment for gene programs related to invasion and metastasis. Significant enrichment for regulation of the canonical Wnt pathway in SW480 Mock serves as a positive control for this analysis.
- D. Comparing significantly upregulated genes from each Wnt-reduced cell line revealed 21 genes in common.
- E. Heatmap of the 21 common genes showing normalized count values for each replicate of each cell line. SW620 Cas9 and LRP6KO show the greatest heterogeneity in the expression of the 21 identified genes, due to the relatively small change to Wnt signaling activity with the knockout of LRP6
- F. Western blot validation of 3 genes, AXL, ARHGEF17/TEM4, and VIM shows significant upregulation of protein levels in Wnt-reduced cell lines.

Refining the invasive gene signature using patient survival data

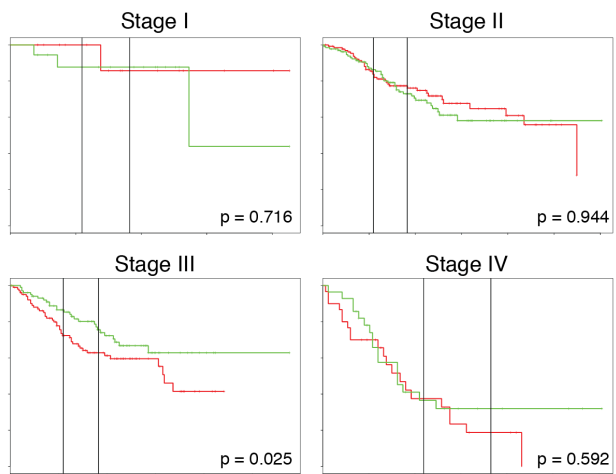
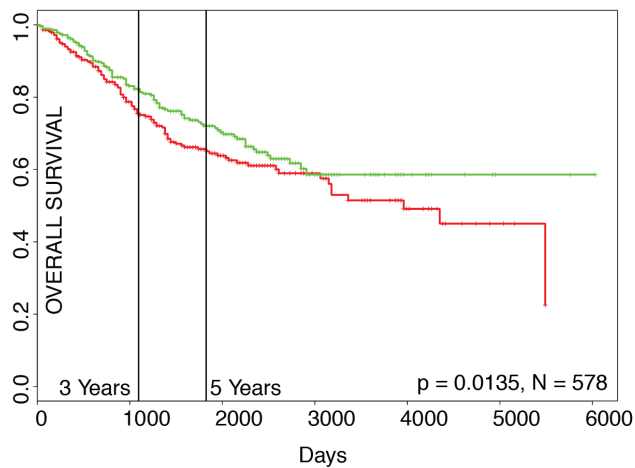
In order to determine if any of the 21 upregulated genes have clinical significance, we examined changes to patient survival with high expression of each gene in 15 colon cancer patient datasets. We observed tremendous heterogeneity between patient data sets, likely due to differences in sampling and proportions of patients at different stages of disease. Despite this, most of the 21 genes showed a significant decrease in patient survival with high gene expression in at least one dataset. To refine the signature, we focused on genes that were significantly correlated together with decreased survival in at least three datasets. The resulting 12-gene signature was used to look at patient survival in datasets in which staging information was available. In GSE14333 (Jorissen *et al*, 2009), we find that high expression of the gene signature significantly correlated with decreased patient survival when the patients are considered in aggregate. This dataset included tumor staging information using the Dukes classification system. When staging information is taken into consideration, high expression of the gene signature predicted worse prognoses only in Dukes B and Dukes C groups, not Dukes A (Figure 3.5A). In the Dukes classification system, Dukes A denotes tumors that have not yet invaded through the colon wall, Dukes B denotes invasion through the muscle layer but not yet into the lymphatic network, and Dukes C denotes invasion into the lymph nodes. Thus this data suggests that only in cases where a tumor has become invasive, high expression of our identified genes are correlated with poor patient outcome. However, as the Dukes system is no longer recommended for clinical practice, we looked to confirm our findings in a dataset using the current clinical staging system (AJCC TNM). In a larger patient dataset, GSE39582 (Marisa *et al*, 2013), we again find that when patients are considered in aggregate, high expression of our 12-gene signature significantly correlates with decreased patient survival (Figure 3.5B). When we separate

the patients by tumor stage, we find that a significant decrease in patient outcomes is observed at Stage III. Stage III is most similar to Dukes B & C, with local tumor invasion, but no distant metastases. This highlights the fact that these 12 genes are likely to play a role specifically in the early stages of the invasion-metastasis cascade. Nonetheless, high expression of these genes is predictive of poorer patient outcomes overall as shown in several other large studies (Figure 3.5D).

A. GSE14333 Disease-Free Survival:
 AXL, CYFIP2, PRDX5, TNC, EPHB6, CMTM3, HOXB8, VIM, ARHGEF17, FXYP6, FBLN1, SDPR



B. GSE39582 Overall Survival
 AXL, CYFIP2, PRDX5, TNC, EPHB6, CMTM3, HOXB8, VIM, ARHGEF17, FXYP6, FBLN1, SDPR



C. GSE 17536 GSE 17537 TCGA COADREAD (6/2016)

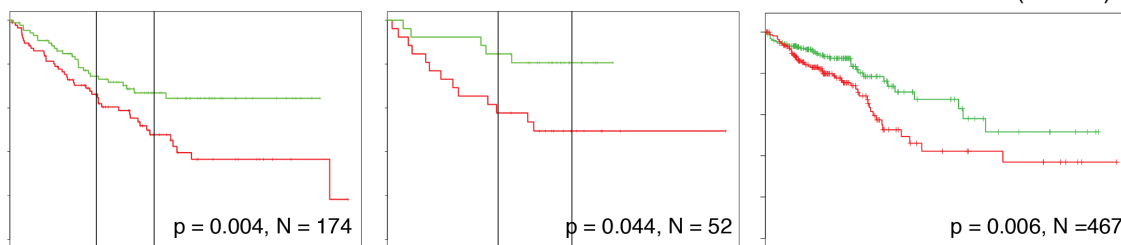


Figure 3.5. A 12-gene signature predicts poor patient outcomes in early invasive colon cancer.

A. GSE1433, Expression data from 290 primary colorectal cancers, shows significant difference in patient survival with high expression of the combined 12-gene signature. By dividing the patient samples by Dukes' classification, significant effects on patient survival are only observed in Dukes B and Dukes C.

B. GSE39582, Gene expression Classification of Colon Cancer defines six molecular subtypes with distinct clinical, molecular and survival characteristics, shows significant difference in patient survival with high expression of the combined 12-gene signature. Dividing patients by TNM stage revealed that the signature is only predictive of poor outcomes in Stage III tumors.

C. Other large-scale colon cancer studies in which high expression of the combined 12-gene signature is predictive of poor patient outcomes.

Identification of potential new targets in colon cancer

Of the 12 genes that we have identified as a signature for colon cancer invasion, several have been previously characterized to have roles in cell migration. In particular, Vimentin (*VIM*) has long been used as a marker for mesenchymal cells, and for cancer cells undergoing epithelial to mesenchymal transition (Hay, 2005; Mendez *et al*, 2010). *ARHGEF17* is not as well characterized as Vimentin, but appears to bind to Vimentin to play a role in cytoskeletal organization (Mitin *et al*, 2013). Two receptor proteins in our gene list, *AXL* and *EPHB6*, have been characterized as cell surface receptors that activate cell migration signaling (Axelrod & Pienta, 2014; Goyette *et al*, 2018; Matsuoka *et al*, 2005). Silencing of *EPHB6* was recently shown in to decrease the number of metastatic lesions in a mouse model of colon cancer (Mateo-Lozano *et al*, 2017), however, the investigators did not use an orthotopic model, and note a potential context-dependent or disease-dependent role for *EPHB6*, given is overexpression driving migration in other systems. Additionally, there are two genes encoding for secreted proteins, *TNC* and *FBLN1*. These proteins interact with the extracellular matrix and have been implicated with cell migration. In the case of *TNC*, it has been shown to interact with cancer-associated fibroblasts to induce expression of periostin and osteopontin, which collectively form a permissive niche at the leading edge of the tumor and at metastatic sites (Oskarsson *et al*, 2011; Kii *et al*, 2010). *FBLN1* binds fibronectin and is a positive regulator of cell migration (Pupa *et al*, 2002). *CYFIP2* has been characterized to be involved in fibronectin-mediated adhesion in immune cells, but the mechanism and whether it is relevant to other organ systems remains unclear (Michael *et al*, 2004). *HOXB8* is a transcription factor that has been characterized to drive the expression of *VEGF* and *NCAM*, two genes involved in tumor invasion (Ding *et al*, 2017; Kolkova, 2010). It has also been shown to be upregulated in a number of cancers including colon cancer. The remaining four members of the gene signature – *PRDX5*, *CMTM3*, *FXR1*, and *SDPR*, have not

been characterized to have a role in cell mobility, though they all may function in response to signals from the microenvironment. Of the genes that we have identified, a number of them have characterized inhibitors, though only *AXL* has inhibitors in clinical trials (Table 3.1). None of these inhibitors have been characterized for use in colon cancer, presenting a potential new avenue for investigation.

Table 3.1. Characterization of the twelve genes identified in Wnt signaling reduced cell lines

Name	Type	Localization	Known Interactions	Known functions	Drug Target	Compounds
AXL	Receptor Tyrosine Kinase	Membrane	Receptor for Gas6. Also associates with PI3K, SH2DA2, VAV downstream	Regulates cell survival, proliferation, migration, and differentiation	Yes	BGB324, TP-0903
CYFIP2	RNA binding	Nucleus	CYFIP1, WASF1/2/3, ABI1, NCKAP1L, NCK1, NCKAP1	Involved in p53-dependent induction of apoptosis.	No	
PRDX5	Peroxidase	Mitochondrial/Peroxisome	SUMO1, PRDX1/2, SPR, SPR, DSTN, TRAP1, NIT2, PARK7	Antioxidant. Catalyzes reduction of hydrogen peroxide	Yes	Barellet et al 2010
TNC	Secreted	Stroma	Ligand for integrins A8B1, A9B1, AVB3, AVB6, SRC, BCAN, VCAN, NCAN, ACAN	ECM protein, associated with migration. Downreg DKK1, metastatic niche component	No	
EPHB6	Receptor	Membrane	Binds ephrin B1 & ephrin B2, NCK2	Influences cell adhesion and migration	Indirect	General ephrin inhibitors
CMTM3	Cytokine	Membrane (Predicted)	BLNK	Cell cycle arrest	No	
HOXB8	DNA binding	Nuclear	EGR1, MEIS1/2, PBX1/2/3/4, RCHY1	Inhibits myeloid differentiation. Increased expression associated with CRC	No	
VIM	Filament	Cytoskeletal	AKT1, Caspases, DMD	Cell shape and integrity	Yes	Withaferin A, Epigallocatechin gallate
ARHGEF17/TEM4	GEF	Cytoskeletal	Binds to Vimentin, F-Actin	Associated with rectal cancer	No	
FXVD6	Ion channel	Membrane	Unknown	Ion transport, neuronal signaling	Yes	Gao et al 2014
FBLN1	Secreted	Stroma	Fibronectin, VCAN, PSG3	Secreted glycoprotein that becomes part of ECM. Implicated in cell transformation and tumor invasion.	No	
SDPR/CAVIN2	Cytoplasmic	Cytoskeletal/membrane bound	Phosphatidylserine, CAV1, PRKCA	Regulates caveolae morphology.	No	

Discussion

Here we report that interfering with β -catenin-dependent Wnt signaling in colon cancer cells increases their cell migration and invasion activities both *in vitro* and *in vivo*. We demonstrate this multiple ways by interfering with Wnt signal transduction at steps that lie either upstream or downstream of β -catenin. Wnt-signaling-inhibited cell lines show increased cell migration *in vitro*, a phenotype correlates well with *in vivo* phenotypes of increased invasiveness, including the formation of extracolonic tumors. We predict that increased invasiveness would eventually manifest as metastases were we able to carry out the experiments longer. However, the genetically manipulated cells are so invasive and aggressive at the primary site of injection that mice became moribund and therefore limit the timeline of the study. We compare significantly upregulated genes in each of our lines to identify twelve genes that are commonly upregulated when Wnt signaling is decreased, and show that high expression of these genes predicts poor patient outcome, most significantly in patients at the mid-stage of colon cancer, representing the early stages of invasion and metastasis. We suggest that therapies targeting one or more of these twelve genes may find clinical application in colon cancer patients presenting at the early stages of advanced disease.

Activating mutations to the Wnt signaling pathway have long been characterized as a hallmark of colon cancer, and multiple studies have pointed to the contribution of overactive Wnt signaling to metastatic disease. In patient samples, the leading edge of colon tumors stain strongly for nuclear β -catenin, a marker of active Wnt signaling (Jung *et al*, 2001). Additionally, Snail and Twist, two genes that play key roles in EMT are direct Wnt target genes (ten Berge *et al*, 2008). Many previous studies have shown that decreasing Wnt signaling in colon cancer, targeting varying components of the pathway, can significantly reduce tumor burden in subcutaneous xenograft mouse models, the most common mouse model for pre-clinical testing of

cancer therapeutics (Morin *et al*, 1996; Satoh *et al*, 2000; Tetsu & McCormick, 1999; Van de Wetering *et al*, 2002; Polakis, 2012). Indeed, studies by our group using the dnLEF1 construct here has shown the same result in subcutaneous xenografts (Pate *et al*, 2014; Lee *et al*, 2017). Because of this, many efforts and deep resources have been brought to bear on the goal of bringing small molecule inhibitors of Wnt signaling into clinical practice. However, so far, none of these compounds have been able to show measurable benefit in colon cancer treatment, and the trials of Wnt inhibitors have shifted their focus to other cancer types (Kahn, 2014). Recent evidence has shown that the genetic landscape of colon cancer is significantly more complex than previously appreciated; a meta-analysis of patient samples found that tumors with the lowest levels of Wnt signaling had the highest levels of stromal infiltration and the worst overall patient survival (Guinney *et al*, 2015). By contrast, tumors with the highest levels of Wnt signaling had the best overall patient survival. The data that we present here supports this notion, and suggests that decreasing Wnt signaling favors an invasive phenotype in colon cancer.

In this study, we focused our attention on SW480 and SW620, two colon cancer cell lines derived from the same patient; SW480 from the primary tumor and SW620 from a metastatic lymph node. These cell lines have been shown to have the same driver mutations, though SW620 cells have been shown to be more migratory in *in vitro* and *in vivo* assays. Notably, the baseline level of Wnt signaling is dramatically lower in SW620 compared with SW480 (Figure 3.2B). To mimic the application of a Wnt inhibitor, we further down-modulated the levels of Wnt signaling in these two cell lines using genetic manipulations. One manipulation targeted the actions of LEF/TCFs, the Wnt-specific transcription factors that recruit β -catenin to drive target gene expression. The second manipulation removed the Wnt ligand co-receptor LRP6 on the plasma membrane by CRISPR/Cas9 technology and in so doing we demonstrated this co-receptor to be essential for ligand-dependent activation of canonical Wnt signaling. We observe that dnLEF1

expression dramatically decreases Wnt signaling by 80-90%, as measured by luciferase activity and Wnt target gene expression, whereas deletion of LRP6 has a more moderate effect on Wnt- β -catenin activity (approximately 20%). Using these Wnt-reduced cell lines, we created orthotopic tumors by injecting cells into the wall of the caecum in immunocompromised mice. Compared to the parental lines, we observe an increase in pathological scores for edema (a marker of lymphatic invasion) and extracolonic tumors or submucosal invasion, indicating that the dnLEF1 and LRP6KO lines represent a more aggressive disease.

One avenue for drugging the Wnt signaling pathway has been inhibiting the Wnt ligand:receptor interaction. However, given the APC mutations prevalent in colon cancer, they have not been thoroughly tested in colon cancer. Saito-Diaz et al have recently shown that LRP6 has a modulatory effect on β -catenin stability even in the absence of a Wnt ligand (Saito-Diaz *et al*, 2018). Our data concurs with this finding, showing that total β -catenin protein is decreased in the SW620 LRP6KO cells, but not the dnLEF1 cells. Unexpectedly, we find that although SW480 cells express a detectable amount of LRP6 protein by Western blotting, it is either rapidly internalized or it is not localized to the cell surface because while we detect a robust signal when cells are permeabilized, we are unable to detect any significant amount of cell-surface LRP6 by live cell flow cytometry. Nonetheless, knockout of LRP6 in SW480 cells reduced Wnt signaling by approximately 20%, suggesting that the stability of β -catenin is the main mechanism by which LRP6 modulates Wnt signaling in colon cancer cells whether it is detected on the cell surface or not. In the orthotopic setting, SW620 LRP6KO cells generated tumors that formed larger extracolonic lesions than the parental line, similar to what we observe with the dnLEF1 tumors. In a scratch wound assay, we find that the dominant negative lines are significantly more motile after just 24 hours of culture, while the LRP6 knockout line only reaches significance after 48 hours, suggesting a less dramatic increase in migratory ability. Overall, this suggests a graded

change in Wnt signaling repression of cell migration wherein decreased Wnt signaling leads to an increased rate of cell migration.

Using Gene Set Enrichment Analysis, we find that SW480 dnLEF1 is enriched for genes associated with epithelial to mesenchymal transition and loss of cell adhesion. However, we do not see significant enrichment of the same gene programs in SW620 dnLEF1 or SW620 LRP6KO. This is likely due to the pre-existing invasive nature of the SW620 cells. However, when differentially expressed genes are compared in our three sequencing datasets, we find a concurrent upregulation of 21 genes, a number of which are previously characterized promoters of cell migration. We refined this gene list by cross-referencing expression levels of each individual gene with multiple colon cancer patient data sets, highlighting genes in which elevated expression levels were associated with significant decreases in patient survival. The resulting analysis highlighted a list of 12 genes. We find that high-level expression of these genes is significantly correlated with poorer patient outcomes in multiple patient data sets (Figure 5). Additionally, in data sets where pathological staging information was available, we find that the significance of this gene signature is specific to the early stages of tumor invasion in patients (Dukes B/C, AJCC TNM Stage III), suggesting that the signature may be clinically useful in predicting colon cancer progression to metastatic disease.

Eleven of the twelve genes identified have no previously characterized connection to Wnt signaling, though a number of them have been characterized to promote cell migration, either through changing gene expression or creating a more permissive extracellular matrix. Our findings suggest that Wnt signaling may indirectly repress these genes, and further research may reveal a specific role for Wnt signaling as an inhibitor of localized invasion in colon cancer. We suggest that this decrease in Wnt signaling is specific to the initiation event of the colon cancer invasion-metastasis cascade, and is necessary for the development of an invasion-promoting

tumor microenvironment. Further gene expression changes are likely necessary to induce a fully metastatic disease, as we did not reliably observe metastases in our orthotopically-injected mice using any of our Wnt signaling-reduced lines. However, using the classification scheme established by the Colorectal Cancer Subtyping Consortium (CRCSC) (Guinney *et al*, 2015; Linnekamp *et al*, 2018), we observed a shift from CMS2 (Wnt high, best patient prognosis) towards CMS4 (Wnt low, poorest prognosis) in the dnLEF1-expressing cell lines, though no significant changes are observed in comparing SW620 LRP6KO to the parental line, likely due to the relatively modest change in Wnt signaling. In particular, we observe upregulation of gene programs associated with matrix remodeling, TGF β signaling, wound response, and mesenchymal cell signature – all of which are upregulated in CMS4 cell lines relative to all other subtypes (Appendix C, Supplemental Figure 11). Our results are concurrent with the consortium findings; we conclude that a decrease in Wnt signaling is a contributing factor to more advanced colon cancer and poorer patient outcomes.

While the targeting of Wnt signaling in colon cancer may reduce tumor burden, our work shows that a potential, inadvertent side effect might be inducing surviving cancer cells to become invasive. Thus, as clinical trials continue to test the efficacy of newer, more specific Wnt inhibitors on Wnt-driven cancers, we suggest that in the context of colon cancer, treatment of patients with Wnt inhibitors should include concurrent treatment with drugs targeting one or more of the genes that we identified in this study. Small molecule inhibitors have already been developed against a number of these genes; however, only inhibitors targeting AXL have advanced to clinical trials. Though AXL has been observed to be significantly overexpressed in malignant cells, none of the clinical trials currently testing Axl inhibitors are focused on colon cancer (Axelrod, et al). We suggest that AXL inhibitors may be therapeutically useful in concert with other drug therapies, particularly Wnt or VEGF.

What remains unanswered from our study is the apparent “ β -catenin paradox” that our group and others have observed (Brabletz *et al*, 2001; Jung *et al*, 2001; Brabletz *et al*, 2005); that is, the heterogeneity of nuclear β -catenin in a colon tumor, and the high expression at the invading edge of colon cancer. We speculate that this may be due to a temporal function of tumor invasion; as high Wnt signaling promotes growth and proliferation, the decrease in Wnt signaling to promote invasion may be a transient state. The induction of tenascin C expression by the decrease of Wnt signaling is necessary to induce the establishment of a metastatic niche by the tumor stroma. These niche components, such as tenascin C and periostin, have been shown in other model systems to promote Wnt signaling (Malanchi *et al*, 2012; Morra & Moch, 2011; Oskarsson *et al*, 2011; Kii *et al*, 2010). A similar mechanism may exist at the leading edge of colon cancer metastases, but further studies are needed to validate this concept.

Progression to metastatic disease remains the most challenging aspect of colon cancer treatment, and the one in which patient outcomes remain poor overall. The finding that these advanced tumors harbor relatively lower levels of Wnt signaling compared to other colon cancer subtypes, points to a previously uncharacterized role of Wnt signaling in colon cancer. Our study demonstrates that decreasing Wnt signaling induces a more invasive phenotype, and identifies a number of contributors to this invasive state. We suggest that future studies of Wnt inhibitors for clinical use consider concurrent treatment with inhibitors of these identified targets, to both reduce tumor burden and prevent cancer invasion.

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CHAPTER 4

Discussion

The Wnt signaling pathway is one of the most highly conserved and fundamentally important regulatory systems in animal biology. Wnt signaling controls many critical aspects of embryonic development and tissue homeostasis, and its deregulation is tightly associated with a number of diseases, including developmental defects and cancer. As a result, it has been a target of pharmacological interest for several decades, though the path towards targeting Wnt signaling in clinical applications has been fraught with failures and complications, and no Wnt inhibitors are currently used in clinical practice. In a broad sense, this is due to the fact that the Wnt signaling pathway has been found to be far more complex than previously understood, having the ability to activate and regulate a diverse set of transcriptional gene programs in a context-dependent manner. This thesis explored two such Wnt-regulated gene programs and the contexts in which they are important in colon cancer - how heterogeneity in Wnt signaling affects tumor metabolism, and characterizing the role of Wnt signaling in tumor invasion.

Wnt signaling in colon cancer

In a majority of colon cancers, APC mutations allow for the Wnt signaling pathway to be activated in the absence of a Wnt ligand at the cell surface. Despite this constitutive activation, immunohistological staining of patient samples reveal that the level of Wnt signaling is highly heterogeneous within a tumor, featuring clusters of cells with elevated active Wnt signaling as well as clusters of cells with undetectable levels of Wnt signaling, even though all the cells have the same activating mutations. We find this to be the case even in subcutaneous xenograft models using clonally derived colon cancer cell lines, in which cells are genetically homogenous. This

suggests that additional regulation of Wnt signaling activity exists within the tumor, most likely through cell-to-cell communication. We explored the effects of Wnt heterogeneity on colon cancer in both studies presented here – in Chapter 2 addressing spatial heterogeneity, and in Chapter 3, temporal heterogeneity.

Wnt signaling and colon cancer metabolism

Compared to colon tumors from patients, the pattern of heterogeneity in Wnt signaling levels in SW480 cell line xenograft tumors revealed regularity in spatial distribution – an array of cell clusters with evidence of strong Wnt signaling. The pattern in patient tumors appears to be much more random, likely the result of signaling crosstalk from other pathways adding additional complexity to the pattern, as well as cell differentiation changing active gene programs within a cell. Our lab previously demonstrated that cellular metabolism is regulated by Wnt signaling, through direct regulation of metabolism genes pyruvate dehydrogenase kinase (*PDK1*) (Pate *et al*, 2014) and monocarboxylate transporter 1 (*SLC16A1*/MCT-1) (Sprowl-Tanio, 2016), both important in driving glycolysis. Staining xenograft tumors for their expression (MCT-1) and/or metabolic activity (PDK1) also revealed a pattern of regularly distributed cell clusters – a pattern that was highly concordant with that of Wnt signaling. Image analysis of these similar patterns using stains of successive tissue slices from xenograft tumors confirmed that the patterns are directly related. We describe a mathematical model in which Wnt signaling regulates the metabolic switch from aerobic glycolysis to oxidative phosphorylation. Our mathematical modeling suggested that Wnt signaling acts in a reaction-diffusion system to drive the formation of these patterns. Perturbing Wnt signaling in this system by expression of dnLEF/TCFs did indeed change the pattern, but it did not eliminate it fully. Quantitative analysis and systems modeling of the altered pattern revealed the unexpected finding that a decrease of Wnt signaling

is paired with an increase in expression of factors that can enhance Wnt ligand diffusion. Thus while overall levels of Wnt signaling are decreased, the increased range of the signal still potentially allows for the maintenance of Wnt and metabolic heterogeneity to some degree. Our model additionally predicted, and we validated *in vitro*, that the glycolytic and Wnt-high cell subpopulations should be most sensitive to drug perturbation. Targeting these specific subtypes lead to death of the overall system due to their critical roles in system maintenance. Other research groups have shown other mechanisms by which Wnt signaling can regulate metabolism (Esen *et al*, 2013), supporting our conclusion that metabolic targeting may be a new complementary therapeutic target. While metabolic patterning has previously been proposed as a mechanism for nutrient transport into hypoxic regions of tumors, to our knowledge, we are the first to show the establishment of this pattern as a result of Wnt signaling regulation.

One caveat to our study is that the system we modeled is a simplified view of the tumor and its microenvironment. Patient samples show heterogeneity in both Wnt and metabolism, but it is not manifest as a regular pattern like that in our xenograft tumors. Probably, the lack of a regularized pattern of metabolism is due to the much greater complexity of the tumor, its longer timeline of tumor development, an intact immune system that provides additional cell types and complex signaling as well as stromal infiltration. Also, our model allows for nutrients to diffuse into the system from all sides, whereas in patient tumors the majority of nutrient delivery is through the vasculature. Finally, signaling crosstalk with stromal cells is not considered in the model that we described. To address these concerns, we have recently used DropSeq to capture and analyze single cell transcriptomes of approximately 2000 SW480 Mock and 1000 SW480 dnLEF1 tumor cells. Preliminary analysis of this data has shown that the cell populations from both tumor types show heterogeneity at the transcriptome level, despite the genetic homogeneity of the injected cell line (G. Chen *et al.*, *in preparation*; data not shown). Bioinformatic analysis was

able to identify six sub-groups of SW480 Mock cells clustered on the basis of their transcriptome “signature”, and five sub-groups in the dnLEF1 tumor. In the Mock tumor cell population, we find a clear sub-group of cells defined by elevated expression of canonical Wnt target genes, evidence of strong Wnt signaling in these cells. The existence of this sub-group indicates that the Wnt heterogeneity we observed at the protein level in our study can be extended to Wnt heterogeneity at the transcriptome level. In contrast, we cannot easily discern a population enriched for glycolytic cells in the Mock tumor – suggesting that metabolism may be more dynamically regulated post-transcriptionally. Indeed, Esen et al reported that Wnt ligands direct an increase in the cytoplasmic levels of glycolytic enzymes via a signal transduction cascade in the cytoplasm and not involving β -catenin actions in the nucleus (Esen *et al*, 2013). Perhaps the reaction-diffusion system of Wnt ligand action that we modeled applies to this second mode of signaling as well. Additionally, we captured both human cells and mouse cells from the xenograft tumor, and scRNAseq analysis of both suggests that there is clear potential for signaling crosstalk between the mouse stroma and human tumor. By comparison, the SW480 dnLEF1 tumor is less heterogeneous with five sub-groups, although one cell population is still distinguished by a clear, but weaker expression of Wnt target genes, confirming that Wnt heterogeneity that we see at the protein level is evident in the transcriptome, even with a global decrease in Wnt signaling activity. These new findings are being incorporated into a new mathematical model that includes terms for a developing tumor vasculature and signaling interactions between cell tumor sub-groups. The goal is to use this new model to advance our understanding of tumor development and differentiation, and provide specific evidence for the roles of the microenvironment in tumor development over time.

Wnt signaling and tumor growth

The subcutaneous tumor model, while useful in studying tumor growth in an animal system, is poorly representative of the biology of the colon. We thus adopted an orthotopic tumor model, in which colon cancer cells were injected into the submucosal layer of the colon wall. In the subcutaneous xenograft model, we observed a significant decrease in tumor mass with expression of dnLEF1 in SW480 and SW620 lines – an outcome that was congruent with the observation of other groups – which is that decreased Wnt signaling leads to decreased tumor growth (Morin *et al*, 1996; Satoh *et al*, 2000; Van de Wetering *et al*, 2002; Polakis, 2012). However, when the cells were injected orthotopically, dnLEF1 tumors did not have a significant growth disadvantage – rather, the dnLEF1 tumors appeared to grow equally well and were actually more invasive than their parental cell lines, often invading through the muscle layer and establishing extracolonic tumors on the outside of the colon wall. While this finding appears to run counter to the established dogma that Wnt signaling drives tumor metastasis, recent evidence supports the notion that decreased Wnt signaling a hallmark of a more invasive colon tumor (see below). Our results also run counter to the general notion that interference with Wnt signaling decreases tumor growth. Our previous study showed that the reason subcutaneous tumors are smaller when Wnt signaling is reduced in the cancer cells, is that there is a decrease in glycolysis and a decrease in VEGF expression (Pate *et al*, 2014). Glycolysis and VEGF are important angiogenic signals; tumor development at a subcutaneous site depends heavily on them since this is, at least initially, a vascular-poor and nutrient-stressed environment. In contrast, orthotopic injection introduces colon cancer cells into the extremely well vascularized wall of a colon. Thus, all things being equal, the dnLEF1-expressing tumors are well perfused from the very beginning and thus appear to have no problem growing as well as the parental Mock tumors. This is quite consistent with the proliferative properties of dnLEF/TCF cells *in vitro* where an abundance of nutrients are supplied by a serum-rich media; in this setting we previously reported that there is

no difference in the proliferation rates of Mock-parental and dnLEF/TCF-expressing cell lines (Pate *et al*, 2014).

Wnt signaling and colon cancer cell invasion

There is a considerable history of studies concluding that elevated levels of β -catenin are found in the nuclei of cells at the periphery of colon tumors, suggesting a specific increase of Wnt signaling activity in these areas (Brabletz *et al*, 2005; Jung *et al*, 2001; Brabletz *et al*, 2001). Despite this increase, de Sousa E Melo *et al* found that cells at the tumor edge, as well as metastatic cancer cells are methylated at a number of Wnt target genes, including *ASCL2* and *LGR5*, indicating that nuclear β -catenin at the tumor boundary is not a reliable marker for Wnt target gene expression (de Sousa *et al*). Mirroring our findings with dnLEF1 expression, Varnat *et al* genetically modified colon cancer cells to express dominant negative TCF4 and observed an increase in metastatic potential when injected into mice (Varnat *et al*, 2010). Perhaps the most convincing argument that decreased Wnt signaling is correlated with advanced colon cancer is a recent large-scale effort to categorize colon cancer patients by molecular subtype. Meta-analysis of multiple patient datasets yielded a four-subtype classification system; patients with the highest levels of Wnt signaling activity (CMS2), had the best overall survival prognoses, while patients with the lowest levels of Wnt activity (CMS4), had the worst prognoses. Additionally, the CMS4 subtype was characterized by enhanced stromal infiltration and upregulation of epithelial to mesenchymal transition gene programs, in agreement with our phenotypic observations of dnLEF1 orthotopic tumors (Guinney *et al*, 2015; Dienstmann *et al*, 2017).

In addition to dnLEF1 expression, we perturbed Wnt signaling by knocking out the Wnt ligand co-receptor LRP6. Though colon cancer cell lines have constitutive Wnt activity (normally as a result of APC mutation downstream of the receptor complex), recent studies have shown

that Wnt signaling activity can still be modulated by ligand-dependent Wnt signaling (Voloshanenko *et al*, 2013; Seshagiri *et al*, 2012). In contrast to dnLEF1, which decreased endogenous Wnt signaling by 80-90%, LRP6 knockout decreased Wnt signaling by only 10-20%, suggesting a limited but still significant role for Wnt ligand-driven signaling. More recently however, emerging evidence suggests that LRP6 can modulate Wnt signaling in the absence of a Wnt ligand, through ligand-independent association with APC (Saito-Diaz *et al*, 2018). In agreement with this study, our data shows a decrease in β -catenin protein with the loss of LRP6, perhaps because the loss of LRP6 releases the destruction complex to target β -catenin for degradation, even in APC-mutant cells. Despite the more modest decrease in Wnt signaling, we find that in orthotopic tumors, SW620 LRP6KO cells are still more invasive than their parental counterpart, suggesting programs of cell invasion are highly susceptible to Wnt signaling modulation. *In vitro*, scratch wound assays reveal that the greater the change to Wnt signaling levels, the more motile the cells become, suggesting a rheostatic modulation of cell migration.

We compared the differentially expressed genes from each Wnt signaling-decreased cell line versus its associated parental line to identify a group of genes consistently upregulated when Wnt is decreased. The majority of these genes are also found to be significantly upregulated in Wnt-reduced colon cancer cell lines established by other labs (Appendix C Supplemental Figure 9 & data not shown), suggesting that these findings are not cell line specific, and may be more broadly applied. We identified twelve genes, whose elevated expression shows significant predictive power of poor patient outcomes. The gene signature was specifically predictive of poor patient outcomes at the locally invasive state, in which the tumor has invaded through the colon wall and possibly into the lymphatic network, but not yet to distant organ sites. This suggests that the activity of the genes that we have identified can be narrowly prescribed to the early events of

the invasion-metastasis cascade, and suggests that a dampening of Wnt signaling activity may be an initiating event in the cascade.

Considerations for Wnt-targeting therapies in colon cancer

While the orthotopic tumor model that we used in Chapter 3 is more representative of true tumor biology compared to *in vitro* or subcutaneous models, a possible caveat to this study is the fact that the cells were injected into immunocompromised mice, and thus not subjected to challenges from an adaptive immune response, which is known to interact with cancer cells. Additionally, SW480 and SW620 cell lines, while capable of recapitulating a patient's molecular subtype (Linnekamp *et al*, 2015), do not differentiate to the degree that patient tumors do, which adds a degree of complexity to the tumor microenvironment. As we described in Chapter 2, Wnt signaling activity within a tumor is spatially heterogeneous, and while we observe the same regular pattern in our orthotopic tumors (Appendix B9), we do not explore its potential role in Chapter 3. It is highly likely that inhibitor use will induce varying responses depending on the cell subpopulation; the invasive phenotype may emerge from a specific subpopulation of cells. Likewise, Chapter 3 unveiled a temporal component to Wnt signaling activity, as Wnt signaling decreases as tumors become more invasive. Our mathematical model in Chapter 2 simulates changes over time, but due to the limitations of the subcutaneous tumor model, we did not validate the simulations *in vivo*. Though in both studies we suggest targeting Wnt signaling therapeutically, we note that Wnt signaling heterogeneity is evident across space and over time. Drugging Wnt alone has potential adverse effects of spreading Wnt signaling at longer ranges or enabling tumor invasion. Thus, developing complementary therapies that consider the tumor as a system stand a greater likelihood of success.

The Wnt signaling pathway plays critical roles in normal organismal growth and development. In colon cancer, aberrant overactivation of the Wnt signaling pathway has been considered to be key in establishing, maintaining, and advancing the tumor. As such, targeting the Wnt signaling pathway has long been a goal in colon cancer therapy, but with little success. Here we have described two roles of Wnt signaling in colon cancer that have only recently begun to be understood and appreciated, and suggest Wnt-targeting therapies may find success in synergistic treatment regimens. In Chapter 2, we describe how Wnt signaling regulates spatial patterns of metabolism, driving clusters of cells to primarily utilize aerobic glycolysis for energy production. We find that glycolytic cells are necessary to sustain a tumor, and that targeting both Wnt and glycolysis at the same time causes enough stress on the system to induce tumor death. Though we validated this prediction *in vitro*, more work is needed to validate this hypothesis in mouse models and ultimately in patients. Chapter 3 discusses a repressive role Wnt signaling has on cancer invasion, in which decreasing Wnt signaling leads to increased cell invasion. As a result, we suggest that therapeutic targeting of Wnt signaling will need to be complemented with drugs targeting cell migration to counter this upregulated gene program. The diverse roles that Wnt plays in both normal and oncogenic cell signaling ultimately means that effective targeting of the Wnt signaling pathway in colon cancer will require a delicate balance between effectively treating tumors, limiting interference with normal tissue homeostasis, and limiting or countering interference with tumor suppressing functions Wnt may also play. Our studies add to a growing body of research that aims to better understand the molecular diversity in colon cancer as it relates to Wnt signaling, with the ultimate goal of improving patient outcomes as we are able to more precisely address their disease.

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APPENDIX A

Current Insights in Wnt Signaling Pathways

The Wnt signaling pathway is an evolutionarily conserved cell-to-cell communication system that is important for many different cell programs in normal growth and development. The hypo- or hyper-activation of the Wnt signaling cascade has been associated with human diseases such as cancer. Over the last thirty years, our understanding of the Wnt signaling pathways has become increasingly complex. Here, we attempt to summarize the current understanding of the broad spectrum of Wnt signaling pathways.

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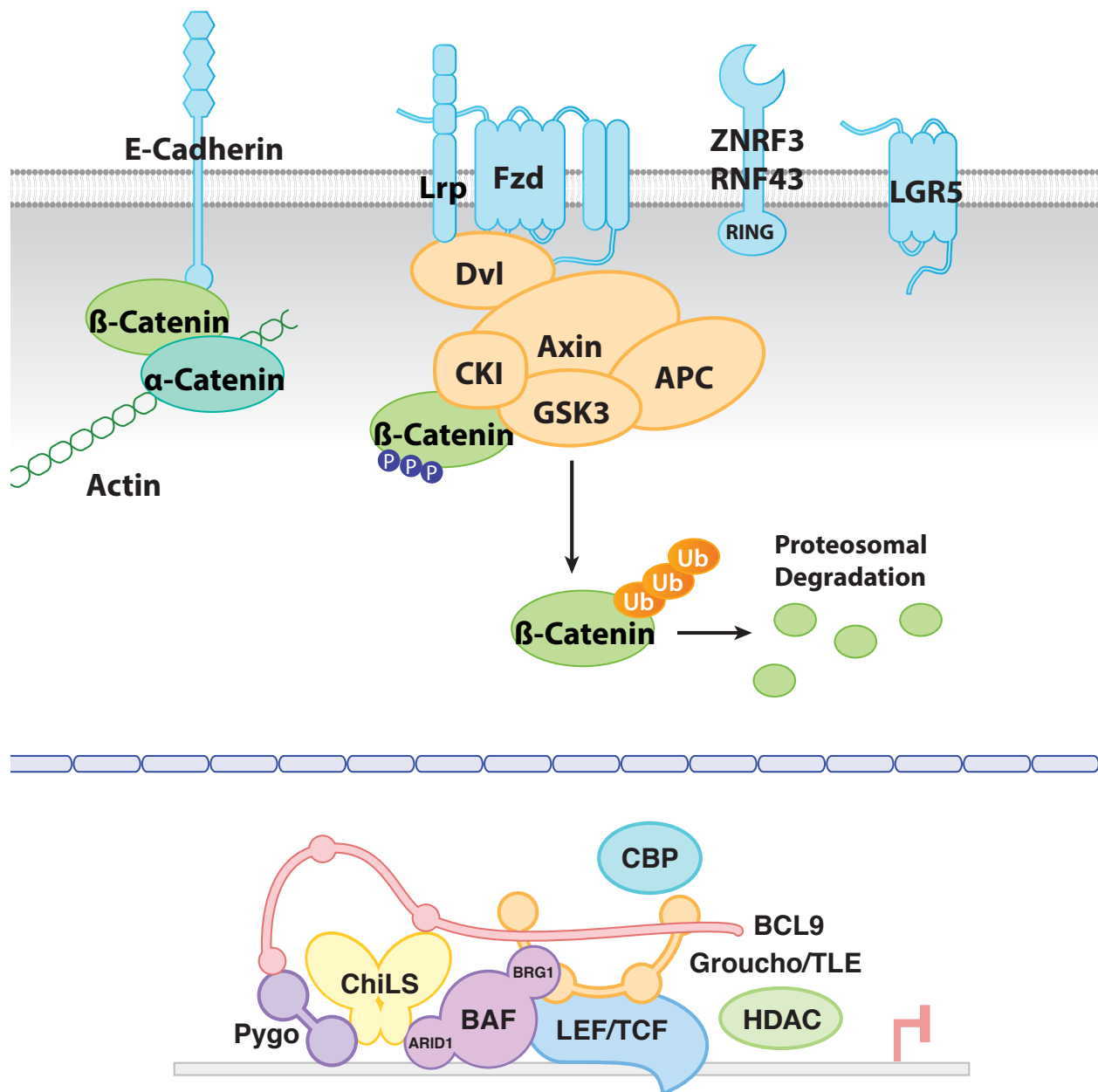


Figure A1.1. Canonical Wnt Signaling: OFF

(Gammons & Bienz, 2018; Saito-Diaz *et al*, 2018; Schuijers & Clevers, 2012)

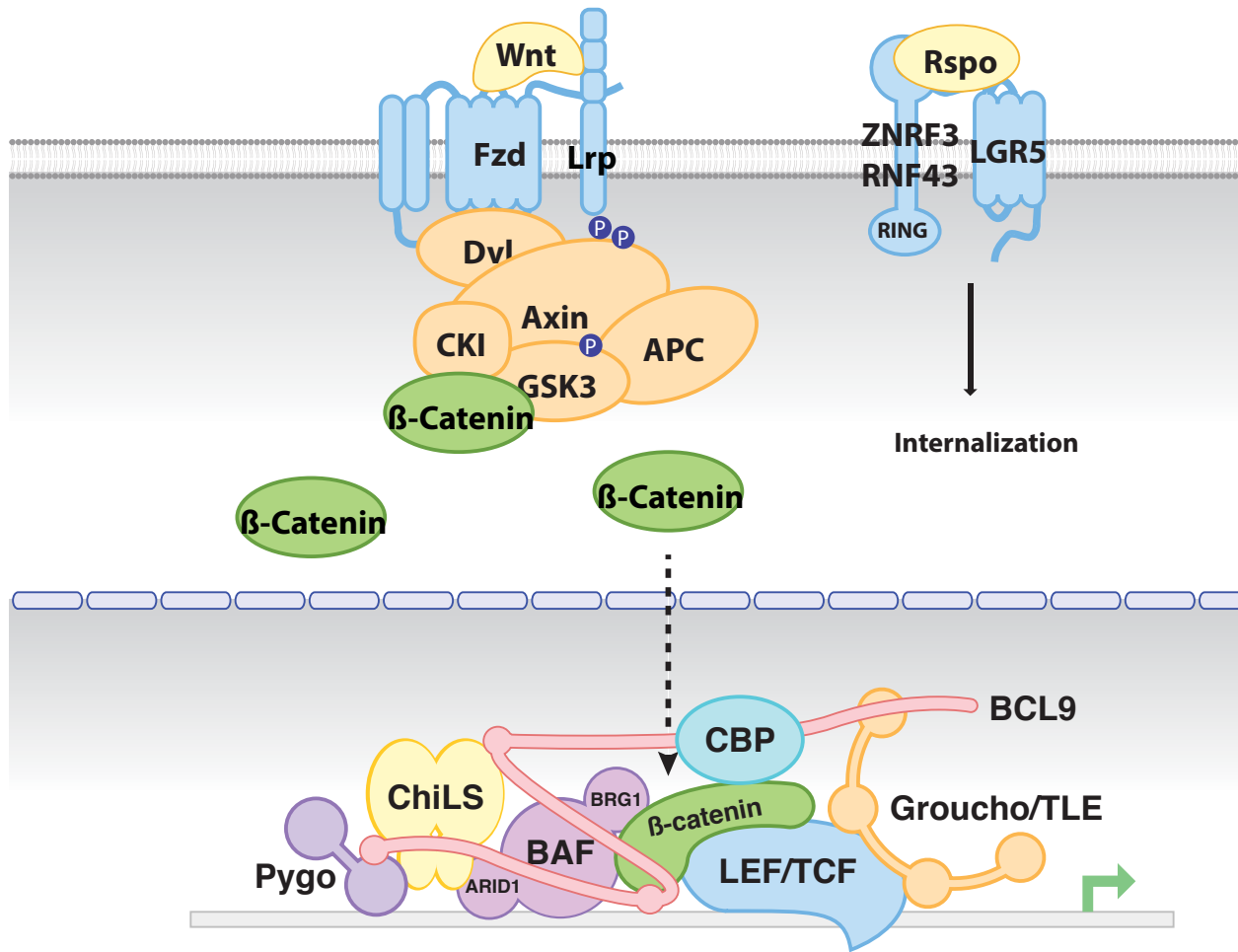


Figure A1.2. Canonical Wnt signaling: ON

(Niehrs, 2012; Schuijers & Clevers, 2012; Gammons & Bienz, 2018; Cruciat & Niehrs, 2013; Wu *et al*, 2013)

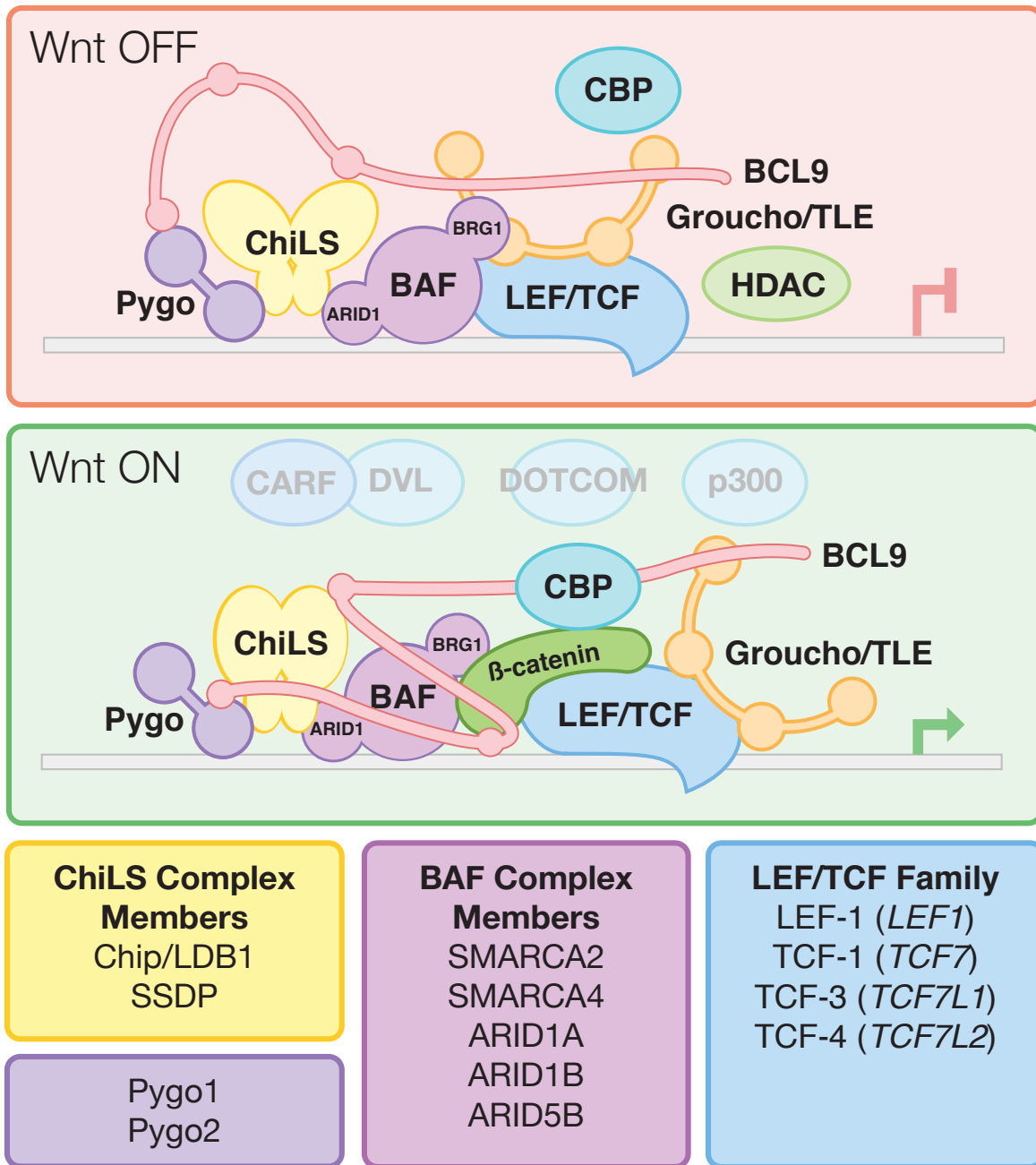


Figure A1.3. Canonical Wnt signaling enhanceosome
 (Lyou *et al*, 2017; van Tienen *et al*, 2017; Gammons & Bienz, 2018)

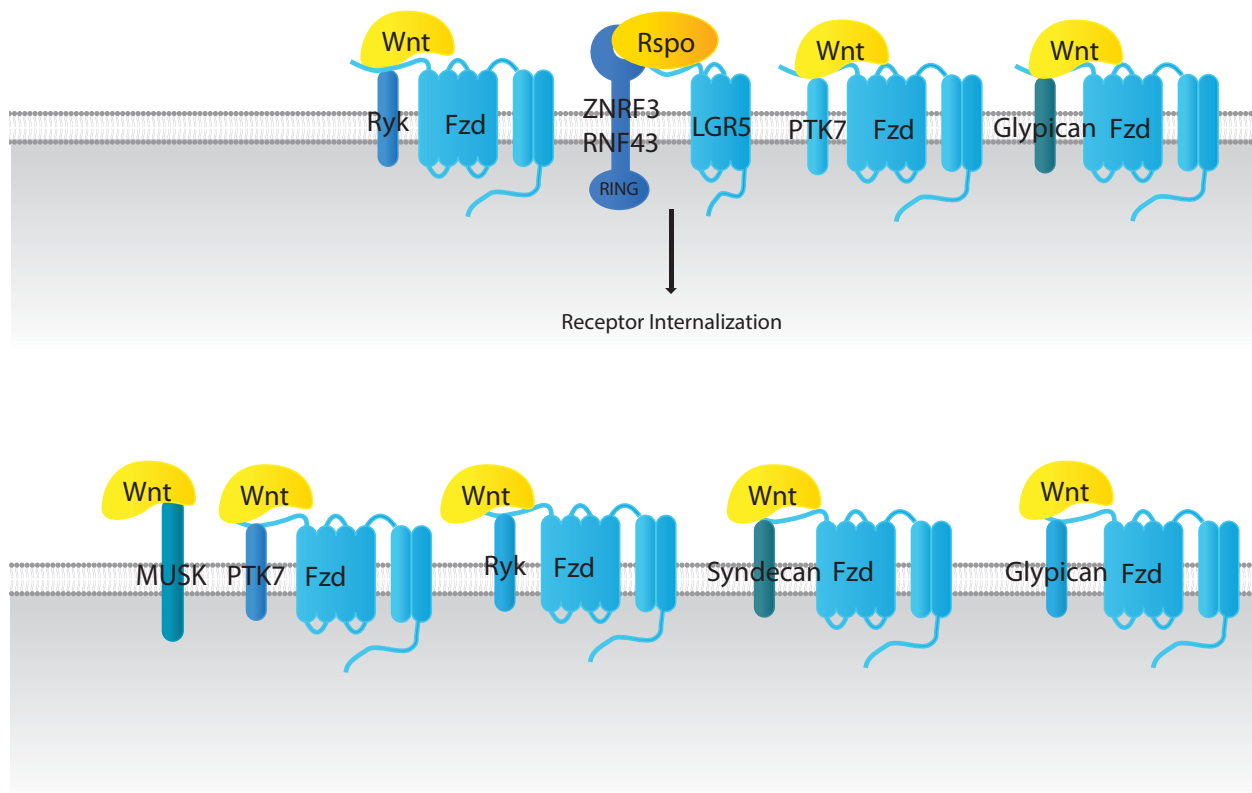


Figure A1.4. Additional Wnt receptors

(Cruciat & Niehrs, 2013; Niehrs, 2012; Schuijers & Clevers, 2012; Baron & Kneissel, 2013)

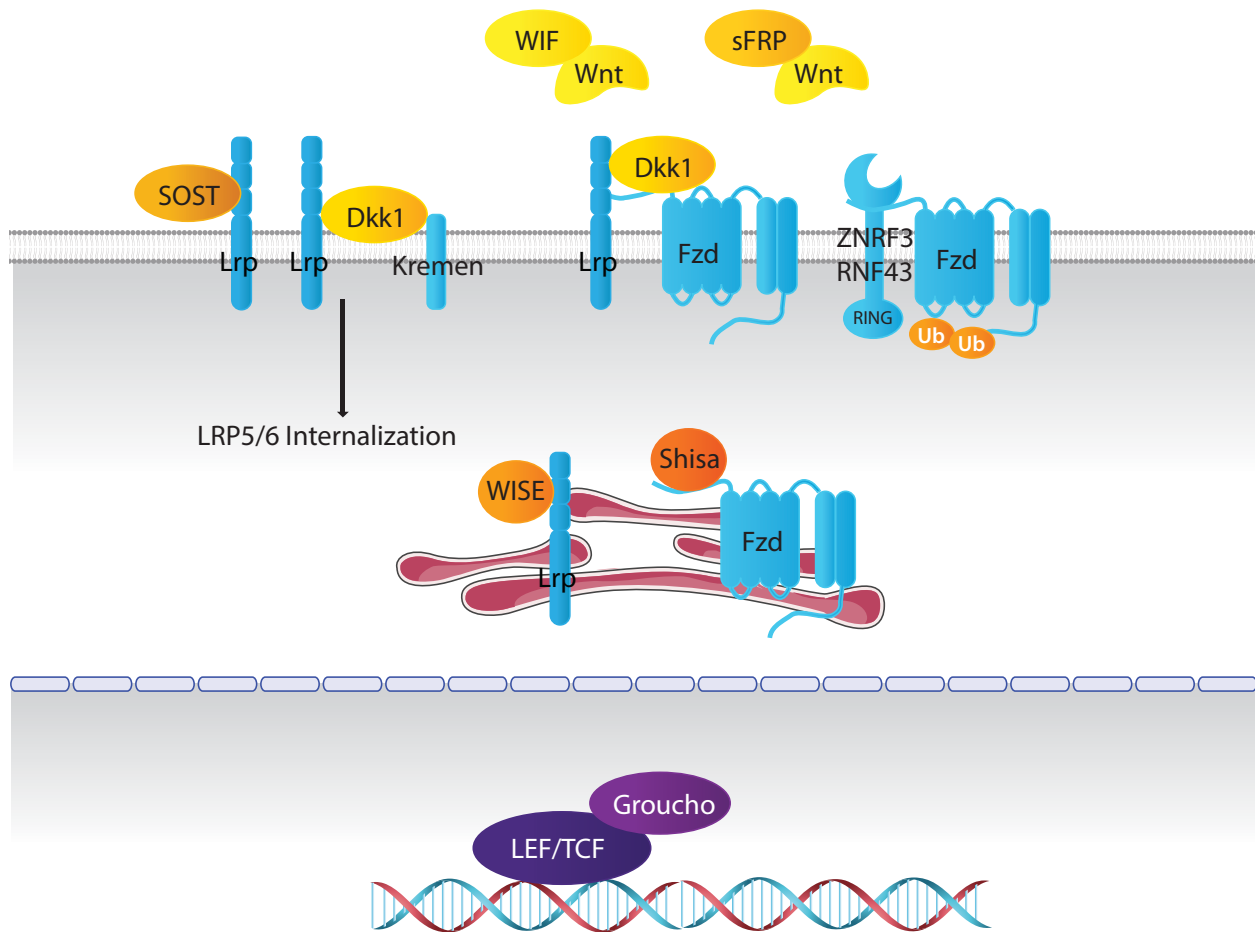


Figure A1.5. Wnt inhibitors

(Baron & Kneissel, 2013; Bovolenta *et al.*, 2014)

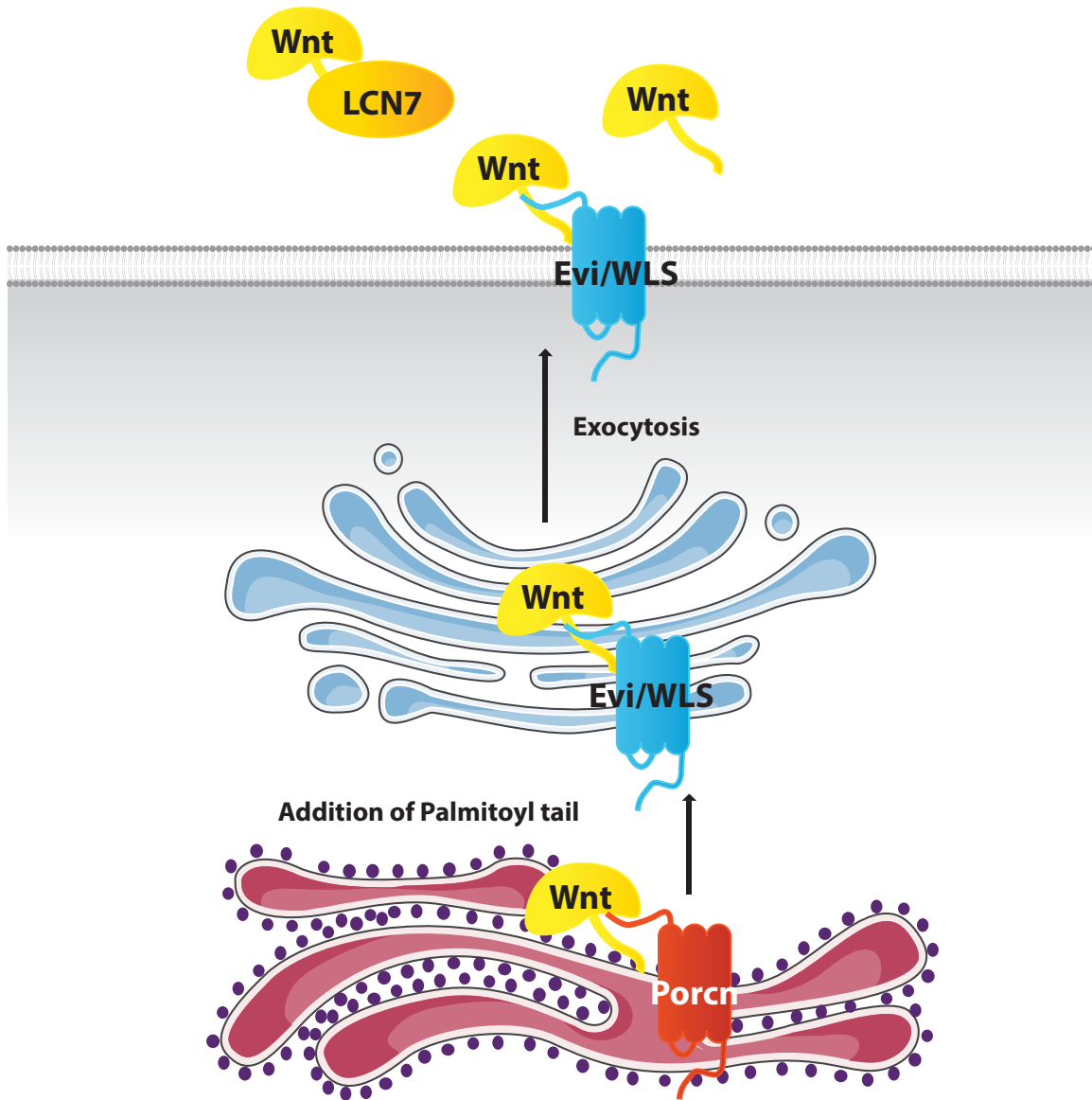


Figure A1.6. Wnt ligand secretion
(Mulligan & Fuerer, 2012; Maurice & Korswagen, 2014)

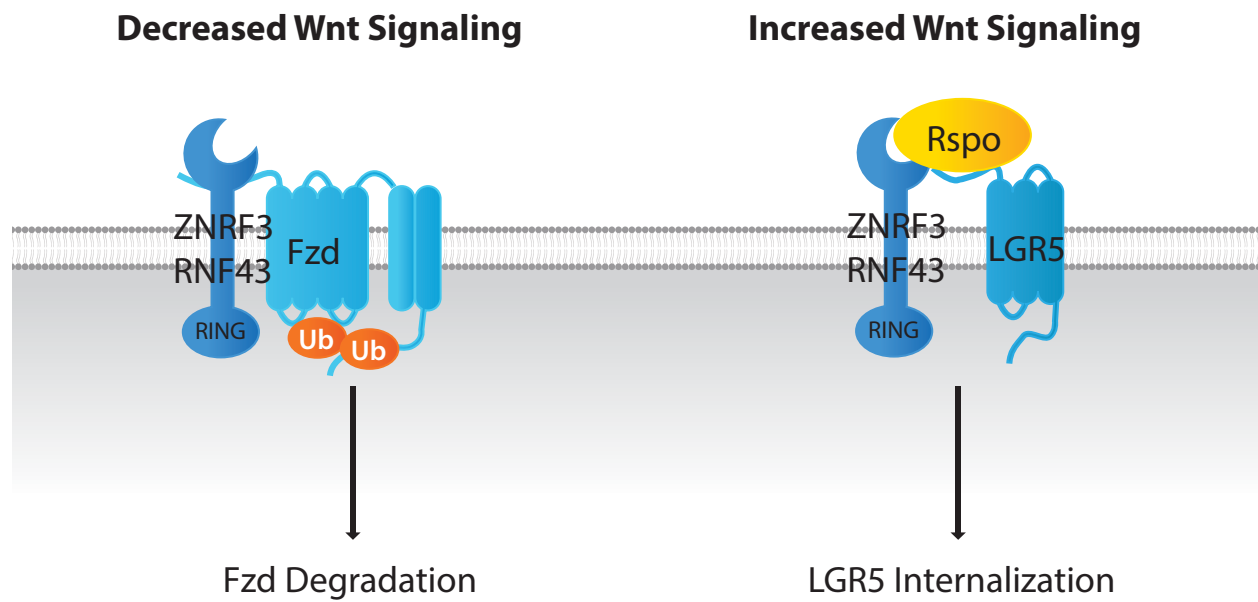


Figure A1.7. RNF43 in Wnt signaling
(Koo *et al.*, 2012; Hao *et al.*, 2012)

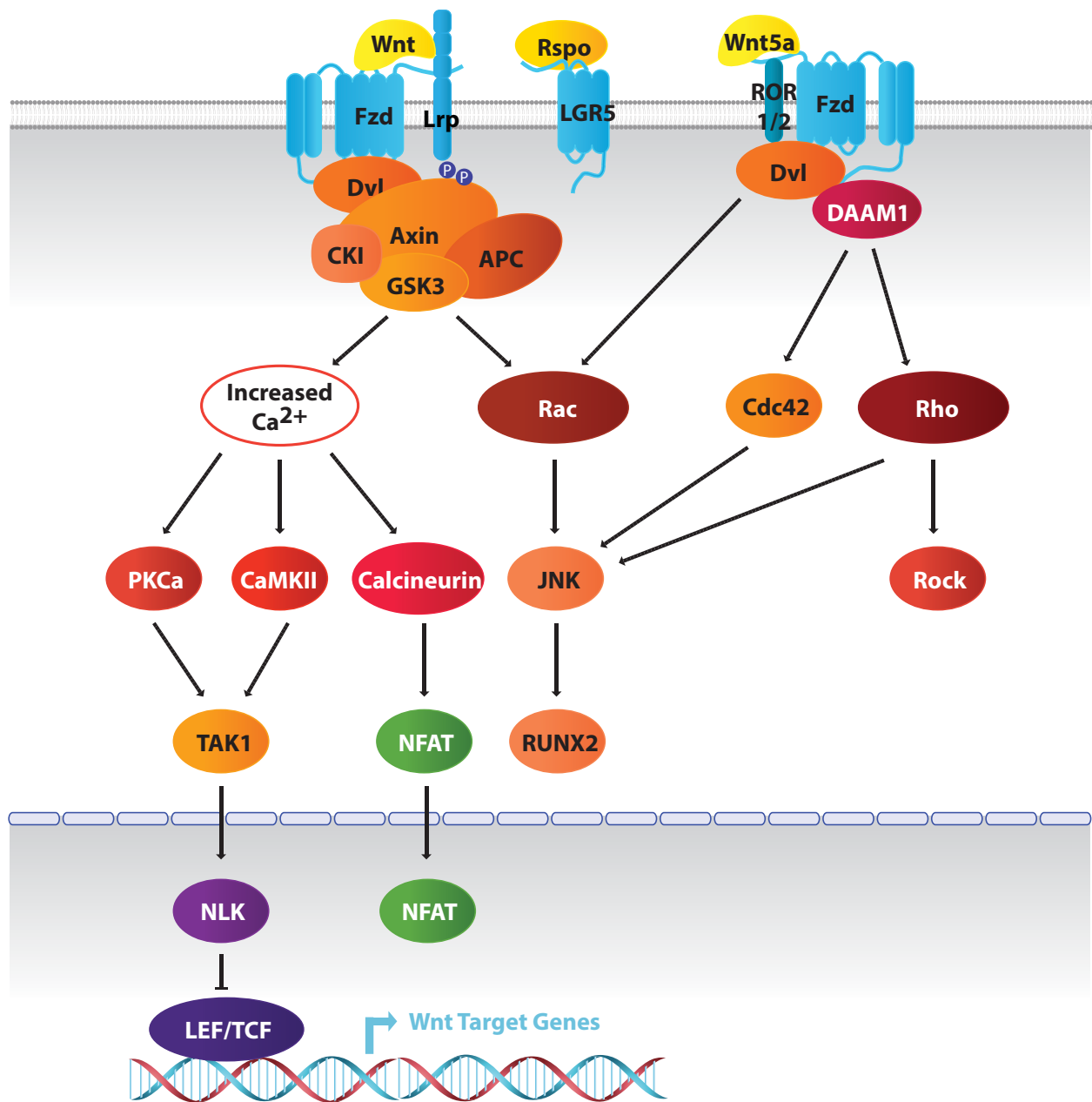


Figure A1.8. Noncanonical Wnt Signaling: Planar Cell Polarity pathway
 (Baron & Kneissel, 2013; Niehrs, 2012)

Kuhl, et al. Wnt Signaling in Dev & Disease, 2014
Chen, Ginty, Fan 2005
Torii, 2008
Hansen, 2009

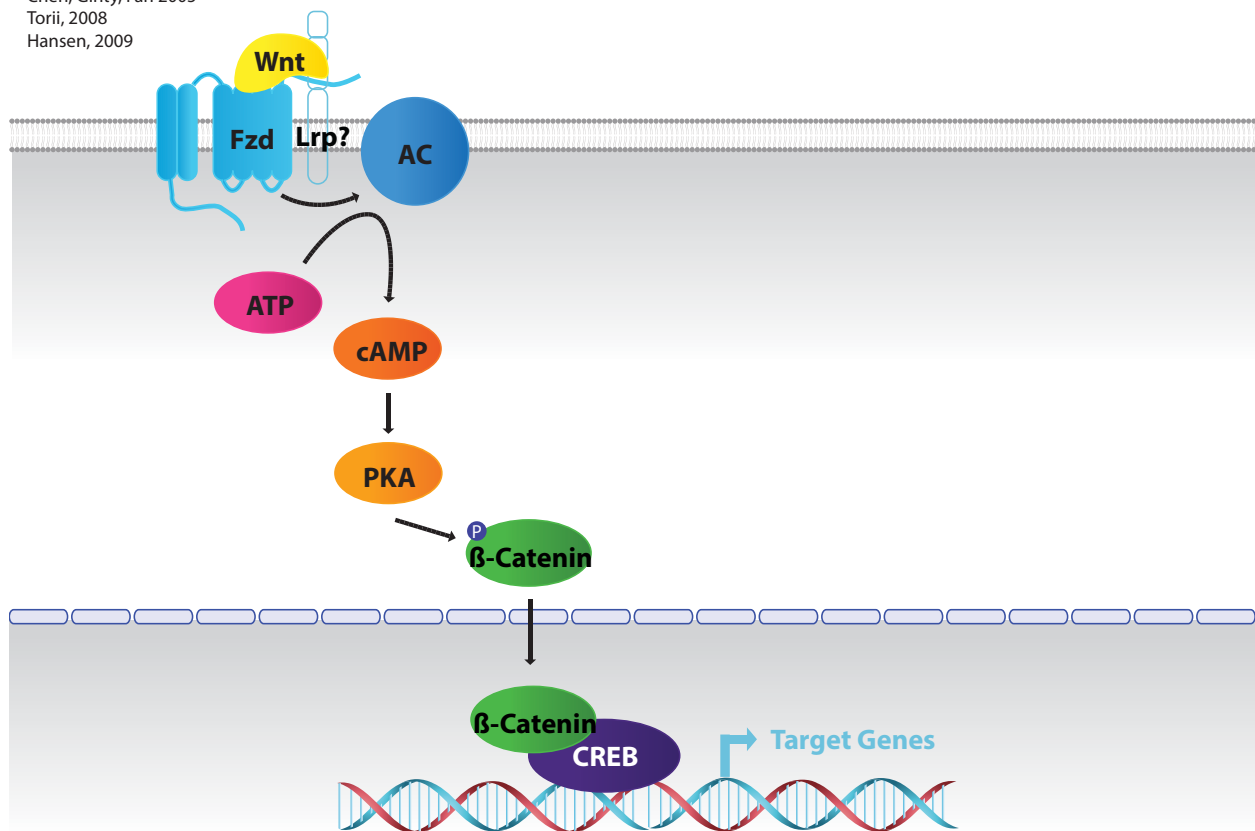


Figure A1.9. Wnt signaling and Cyclic AMP

(Kuhl & Kuhl, 2014; Chen *et al*, 2005; Hansen *et al*, 2009; Torii *et al*, 2008)

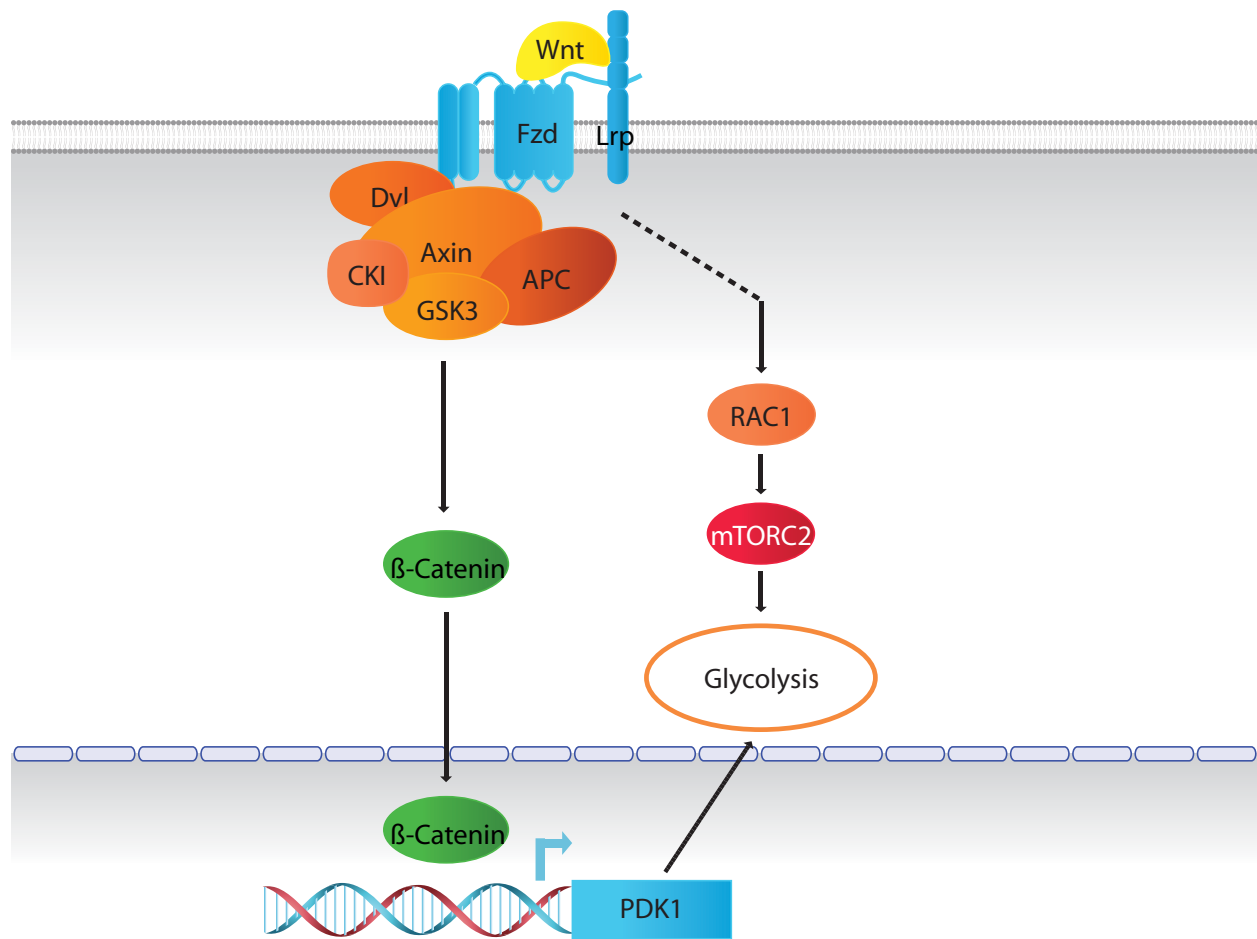


Figure A1.10. Wnt signaling regulation of metabolism
(Pate *et al*, 2014; Esen *et al*, 2013)

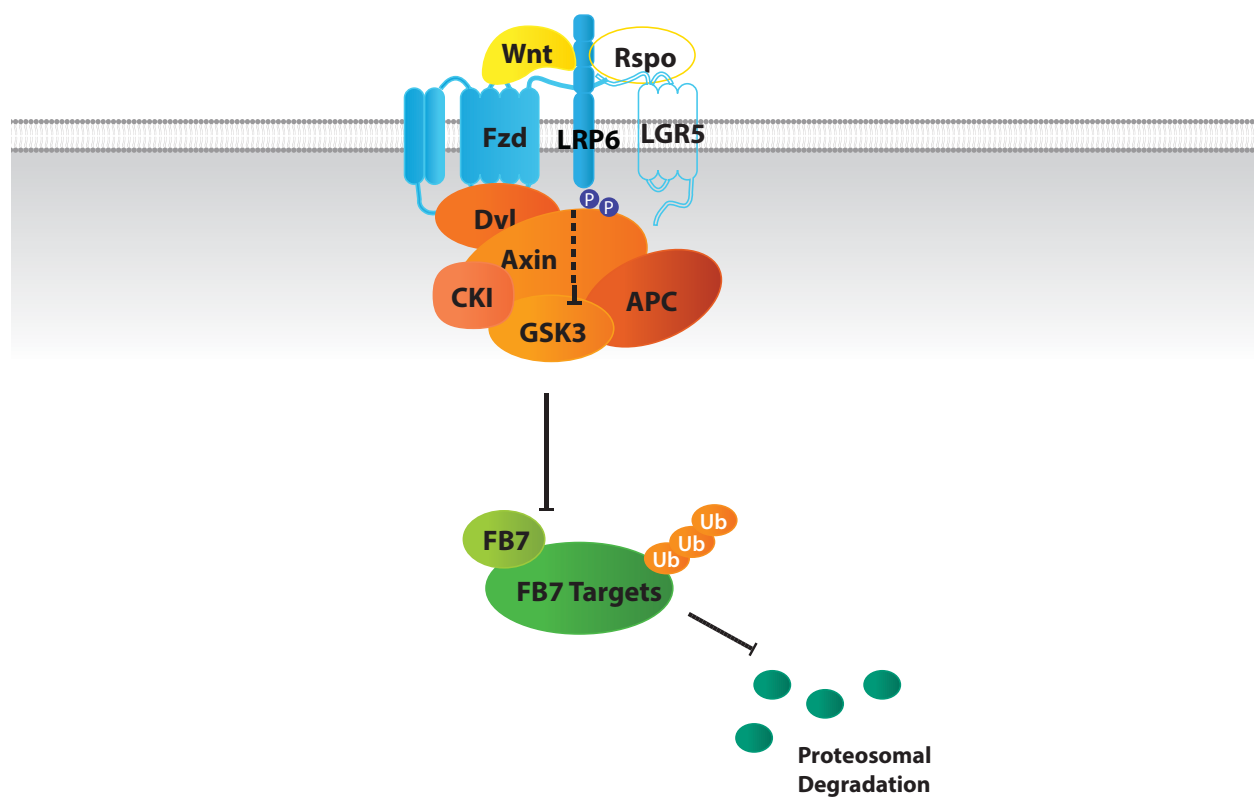


Figure A1.11. Wnt-STOP Pathway
(Acebron *et al.*, 2014)

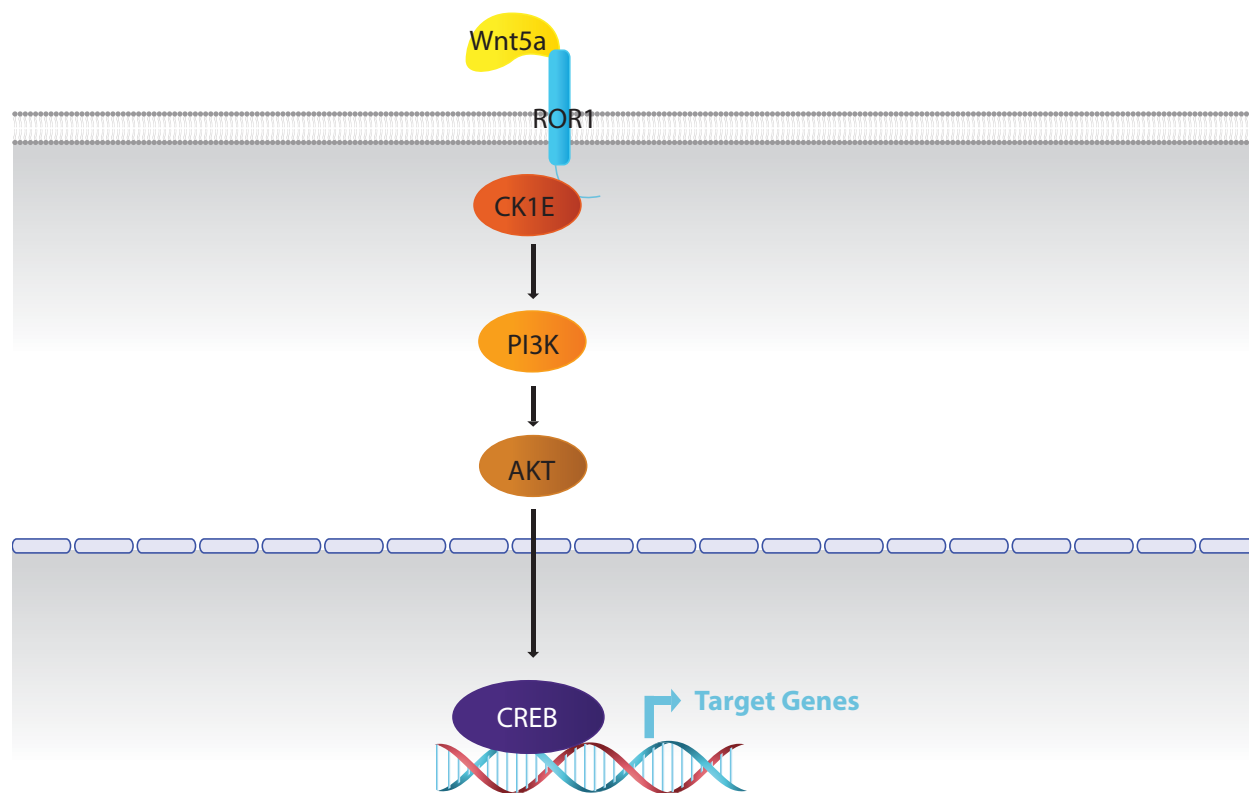


Figure A1.12. Wnt5a-ROR1
(Zhang *et al.*, 2012)

Table 1. Canonical and Non-canonical Wnt ligands

Canonical	Non-canonical
Wnt1	Wnt4
Wnt2	Wnt5a
Wnt3a	Wnt5b
Wnt3	Wnt6
Wnt7a	Wnt8a
Wnt7b	Wnt8b
Wnt9b	Wnt9a
Wnt10b	Wnt10a
	Wnt10b
	Wnt11
	Wnt16

(Najdi *et al*, 2012)

Based on luciferase activation activity; there is a potential for Wnt ligands to activate alternate pathways based upon context. See: (Alok *et al*, 2017; Nalesso *et al*, 2011)

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APPENDIX B

Supplemental Material for Chapter 2

Mathematical Modeling Links Wnt Signaling to Emergent Patterns of Metabolism in Colon Cancer

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B1 Image Processing

B1.1 Description of image processing of experimental data

Image processing for spot contours was done using MATLAB. Below is a detailed description of the processing including the built-in MATLAB functions that were used (appended by .m). Thresholds for the MATLAB functions are given in Appendix Table S3.

1. Read the image into MATLAB and output an $n \times n \times 3$ matrix of RGB values using `imread.m`.
2. Manually choose one of the R, G, or B matrices. Convert this matrix to black and white (0's and 1's) using `im2bw.m` (setting the threshold manually); convert by subtracting this matrix from 1 so that white (a value of 1) is part of a spot and black (a value of 0) is not part of a spot.
3. Apply a noise filter with `medfilt2.m` (setting the threshold manually) to remove as many of the smaller spots as possible (this function is a median filter used to reduce “salt and pepper” noise).
4. Fill in any holes using `imfill.m`. Set any values in the output matrix of `imfill` from 0.5 to 1.
5. Use `bwlabel.m` to label connected components.
6. Use `regionprops.m` to get centroid locations and areas.
7. Define an area limit (setting the threshold manually) to plot only those spots whose area is larger than this limit.
8. Set a threshold for distance between any two centroids (in our case, we used 35 pixels, or approximately 11 μm , which we estimate to be the diameter of a cell). If any two centroids are within this distance, group all of these spots together and take the convex hull using `convhull.m` to visualize the spot. The centroid of the combined spots, or cluster of cells, is computed by taking the weighted average of the centroids, where weights are based on areas. The area of the combined spots is computed as the sum of the original areas.

In the following sections, images are shown with both red and blue contours. Red contours were found using the steps 1-7 above. Blue contours (convex hulls) were found using step 8 above.

B1.2 Description of overlay analysis from image processing data (Figure EV1)

Spot contour overlay using image processing was done using MATLAB. Below is a detailed description of how the overlay was formed using built-in MATLAB functions that were used (appended by .m). Thresholds for the MATLAB functions are given in Appendix Table S

1. Apply steps 1 and 2 from A1.1 for both pPDH and LEF1 experimental images to obtain two separate matrices M_{pPDH} and M_{LEF1} .
2. Form matrix $\overline{M}$ by multiplying M_{pPDH} by M_{LEF1} entry by entry (not matrix multiplication). Matrix $\overline{M}$ now contains overlapping regions of pPDH and LEF1 where white (a value of 1) is part of an overlapping region and black (a value of 0) is a non-overlapping region.
3. Form matrix $\overline{M}_{pPDH} = M_{pPDH} - \overline{M}$ and $\overline{M}_{LEF1} = M_{LEF1} - \overline{M}$ (to remove overlapping regions). Now, $\overline{M}_{pPDH}$, $\overline{M}_{LEF1}$ and $\overline{M}$ are matrices that do not overlap where the value of 1 is located. Form matrix $\overline{S} = 1 * \overline{M}_{pPDH} + 2 * \overline{M}_{LEF1} + 3 * \overline{M}$, so that each a value of 0 assigned in non-spot region, a value of 1 is assigned in pPDH spots (minus the overlap), a value of 2 is assigned in LEF1 spots (minus the overlap), a value of 3 is assigned in the overlapping region.
4. Use `imagesc.m` to visualize overlay with matrix $\overline{S}$ using `colormap.m` (setting custom colors by manually selecting a 4 by 3 matrix where each row represents a color for the numbers 0, 1, 2, 3).

Percent coverage. The 4x (leftmost) images in Figs. 1A and 1B are both the same size and the images were assumed to be lined up as close as possible. In each image, a pixel inside a red contour was considered positively stained and a pixel outside a red contour was considered negatively stained. In the Mock pPDH image in Fig. 1A, there are 101,712 pixels inside the contours and there are 479,314 pixels in the tumor section, which yields a 21.2% coverage of pPDH spots. In the Mock LEF-1 image in Fig. 1B, there are 97,368 pixels inside the contours and there are 481,623 pixels in the tumor section, which yields a 20.2% coverage of LEF-1 spots. The number of pixels in the tumors in the images in Figs. 1A and 1B are slightly different because these images are serial sections. Because they are close, we can approximately pair the pixels in the images by assuming that each pixel location in one slice corresponds to the same pixel location in the other slice. The staining in each pixel pair can be described using a 2×2 contingency matrix. For example, when there is both pPDH and LEF1 staining in the pixel pair, the contingency matrix is given in Table S 1. The contingency matrices in the other cases (e.g., pPDH positive stain/LEF1 negative stain, etc) correspond to permutations of the location of the "1" in the matrix entries.

Appendix Table S 1: 2×2 contingency matrix showing LEF1 and pPDH staining in a pixel pair

	pPDH	no pPDH
LEF1	1	0
no LEF1	0	0

The number of pixels that overlap between the LEF-1 and pPDH images is 35,503 (blue area in Fig. EV1). Thus, the percent overlap of pPDH in LEF-1 spots is $35,503/97,368 = 36.4\%$ and the percent overlap of LEF-1 in pPDH spots is $35,503/101,712 = 34.9\%$. The area fraction of overlap between LEF-1 and pPDH spots in the tumor sections are $35,503/481,623 = 7.4\%$ and $35,503/479,314 = 7.4\%$, respectively.

Cochran-Mantel-Haenszel test. To test whether there is an association between the pPDH and the LEF1 spots, we used the Cochran-Mantel-Haenszel test (Cochran, 1954; Mantel & Haenszel, 1959). By collapsing the contingency matrices for all the pixels in the images, we obtain a single 2×2 contingency table that describes the numbers of pixel pairs with each type of staining. For Fig. 1B, this gives Table S 2

Appendix Table S 2: Collapsed 2×2 contingency matrix for all pixel pairs

	pPDH	no pPDH	Total
LEF1	35503	61865	97368
no LEF1	66209	318046	384255
Total	101712	379911	481623

The null hypothesis is that the pPDH and LEF1 spots are conditionally independent. Then, $z^2 = (n_{21} - n_{12})^2 / (n_{21} + n_{22})$, where n_{21} and n_{12} are the off-diagonal terms in the contingency matrix, is a χ^2 statistic with 1 degree of freedom. Calculating, we obtain $z^2 = 147$ from which we conclude $p < 0.0001$. This allows us to reject the null hypothesis and we conclude that pPDH and LEF1 spots are significantly associated with each other. The results obtained using Fig. 1A are similar.

B1.3 Image processing parameters and data

Appendix Table S 3: **Parameters and resulting number of spots in image processing of IHC stains**

Sample	RGB	im2bw	medfilt2	Minimum area	Number of spots
Mock pPDH 1	B	0.3	15	700	98
Mock pPDH 2	B	0.28	10	700	75
Mock pPDH 3	B	0.4	15	700	89
mock LEF1 1	G	0.55	10	800	69
mock LEF1 2	B	0.3	15	500	77
mock LEF1 3	B	0.39	15	500	92
mock LEF1 4	B	0.3	15	500	87
mock LEF1 5	B	0.3	15	500	100
mock β -catenin 1	G	0.62	10	200	301
mock β -catenin 2	G	0.62	10	200	315
mock β -catenin 3	G	0.62	10	200	272
dnLEF pPDH 1	B	0.5	18	1500	29
dnLEF pPDH 2	B	0.5	18	1500	35
dnLEF pPDH 3	B	0.5	18	1500	24
dnLEF pPDH 4	B	0.55	20	1500	37
dnLEF β -catenin	B	0.35	10	1000	74
dnTCF pPDH 1	B	0.35	17	1500	31
dnTCF pPDH 2	B	0.33	20	1500	33
dnTCF pPDH 3	B	0.28	20	1500	44
dnTCF β -catenin 1	B	0.45	15	2000	35
dnTCF β -catenin 2	B	0.45	15	2000	55
dnTCF β -catenin 3	B	0.45	15	2000	26

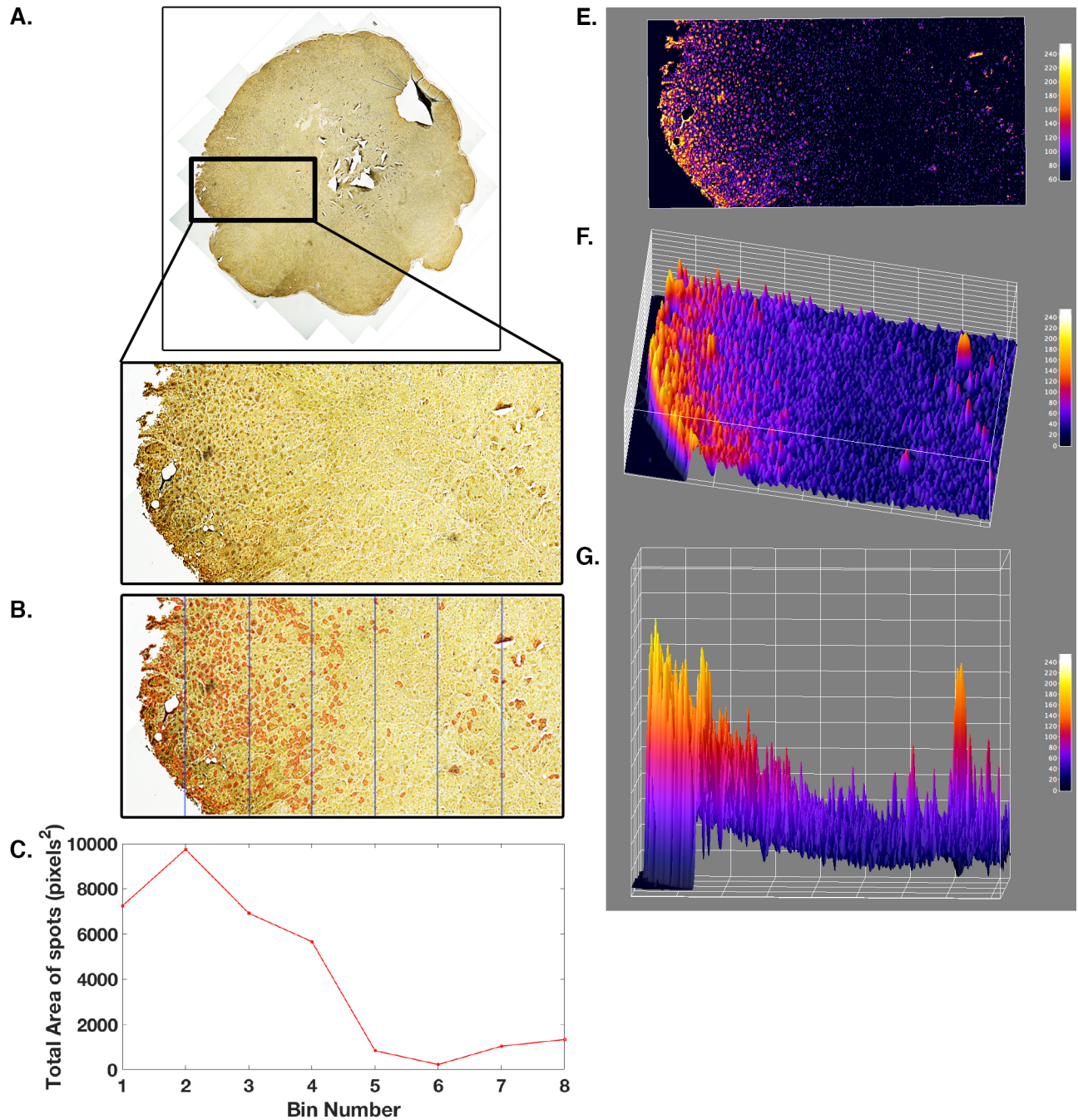
Appendix Table S 4: **Parameters and resulting number of spots in image processing of IHC stains for overlay analysis**

Sample	RGB	im2bw	medfilt2	Minimum area	Number of spots
Mock pPDH 1	B	0.325	10	140	131
Mock pPDH 2	B	0.39	10	140	106
Mock pPDH 3	B	0.36	10	140	153
Mock LEF1 1	B	0.3	10	100	173
Mock LEF1 2	B	0.4	10	100	166
Mock LEF1 3	B	0.4	10	100	191

Parameter values and resulting number of spots in image analysis of pPDH, LEF1, and β -catenin stains. The terms im2bw and medfilt2 refer to built-in Matlab tools. Minimum area refers to the smallest area (in terms of square pixels) that were outlined. Number of spots refers to number of resulting spots outlined from the image processing, after combining spots if their centroids are within some distance (see image processing description in previous section). Thresholds for image analysis were set by user input to capture strongest signals from the epithelial part of the tumors, avoiding mouse stroma and vessels.

B1.4 Gradient of phospho-PDH spot density in SW480 xenograft tumors

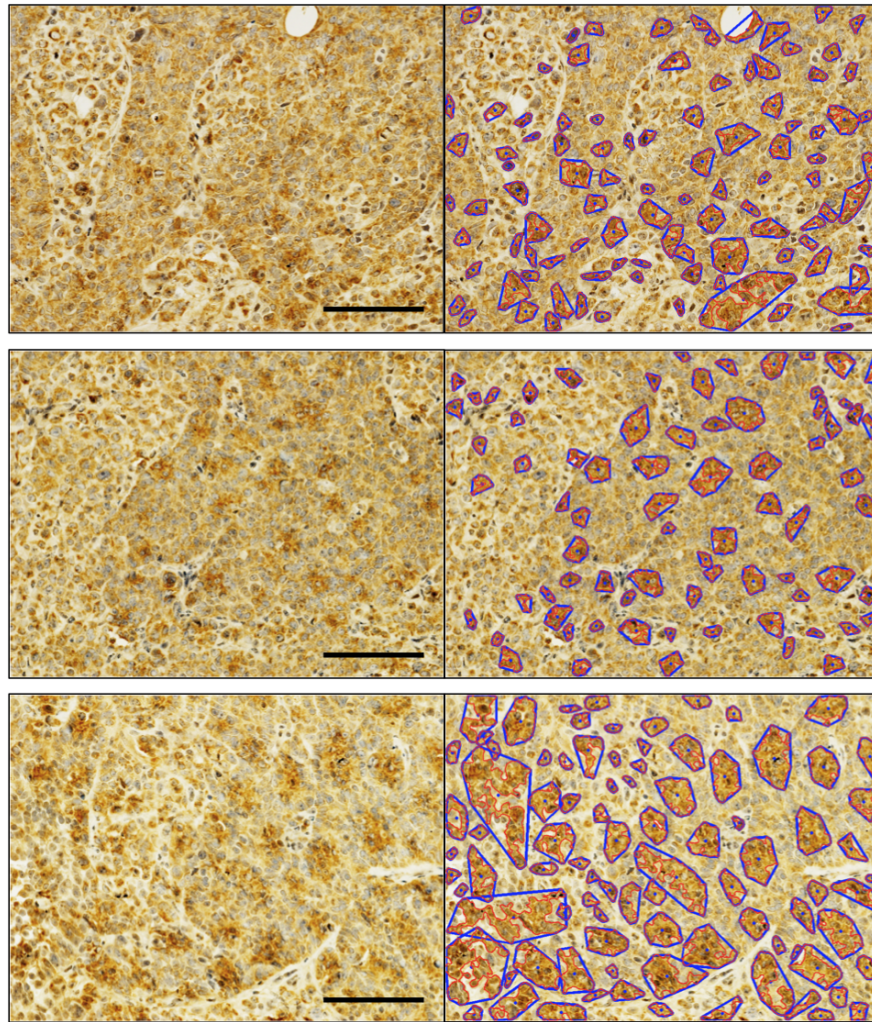
We quantified and visualized the changes in spot densities from the tumor periphery to the core using Matlab and ImageJ tools. To identify contours of the spots, we applied steps 1-7 as in A1.1. Several large artificial "spots" were formed on the periphery when only one parameter set was used for the entire image (due to changes in intensity from the periphery (left) to the core (right) in the tumor). Thus, we separately analyzed the two largest "spots" near the periphery and formed contours of the spots using different parameters. We replaced the large "spots" with the corresponding new contours. As indicated in figure 1, we divided the image into 8 bins from left to right. For each bin we computed the total area of the spots. If the bin divides a spot into multiple pieces, the areas of each individual piece contained within the bin were added and the total areas of the spots in each bin were plotted in (C). To visualize the gradient in spot density and intensity, we used the ImageJ plugin "Interactive 3D Surface Plot" to create topographic maps from the green channel of tumor images (D-G). The spikes at the right are from tears in the tissue.



Appendix Figure S 1: Gradient of phospho-PDH spot density in SW480 xenograft tumors (A) A section of the pPDH-stained SW480 xenograft tumors was selected for analysis. (B) We divided the image into 8 bins (1 is leftmost and 8 is rightmost). For each bin we computed the total area of the spots. If the bin divides a spot into multiple pieces, the areas of each individual piece contained within the bin were added. (C) The areas of the spots in each bin are plotted and show that the areas of the spots generally decreases towards the core of the tumor. (D-F) To further visualize the gradient in spot density and intensity, the ImageJ plugin "Interactive 3D Surface Plot" was used to create topographic maps from the green channel of tumor images. (D) Top view, showing ability to detect spots using ImageJ. (F) Isometric view (G) Side view, showing intensity of spotting decreases towards the core of the tumor. The spikes at the right are from tears in the tissue.

B1.5 SW480 Mock xenograft tumor phospho-PDH immunohistochemistry

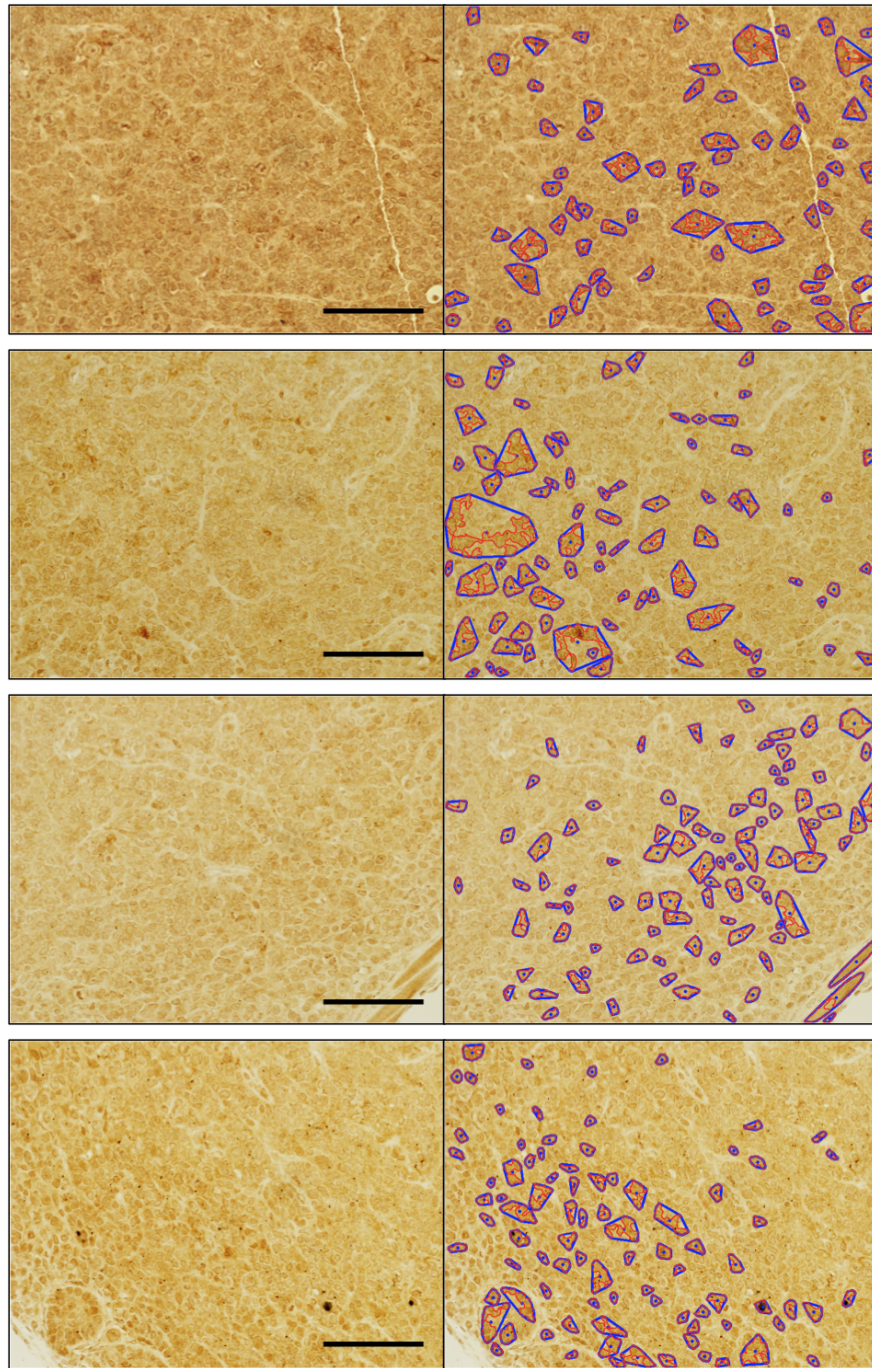
The figures that follow are stains that were processed and whose data (area of each spot and distance to nearest neighbor) appear in the image analysis scatter plots in the main text (Figure 1C).



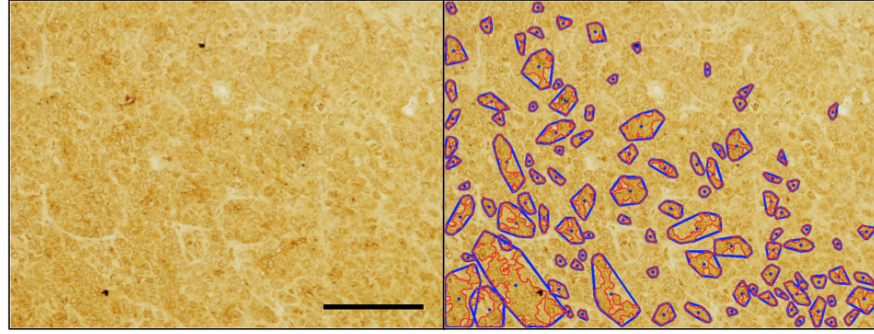
Appendix Figure S 2: Mock phospho-PDH contours. Scalebars indicate $100\mu\text{m}$, with each images' dimensions approximately $440 \times 330\mu\text{m}^2$. The left column shows stained images. The right column shows stained images with red contour outlines drawn around each spot and blue convex hull outlines which group red spots together if their centroids are within 35 pixels (approximately $11\mu\text{m}$) of each other (see image processing methods, A1.1).

B1.6 SW480 Mock xenograft tumor LEF1 immunohistochemistry

The figures that follow are stains that were processed and whose data (area of each spot and distance to nearest neighbor) appear in the image analysis scatter plots in the main text (Figure 1C).



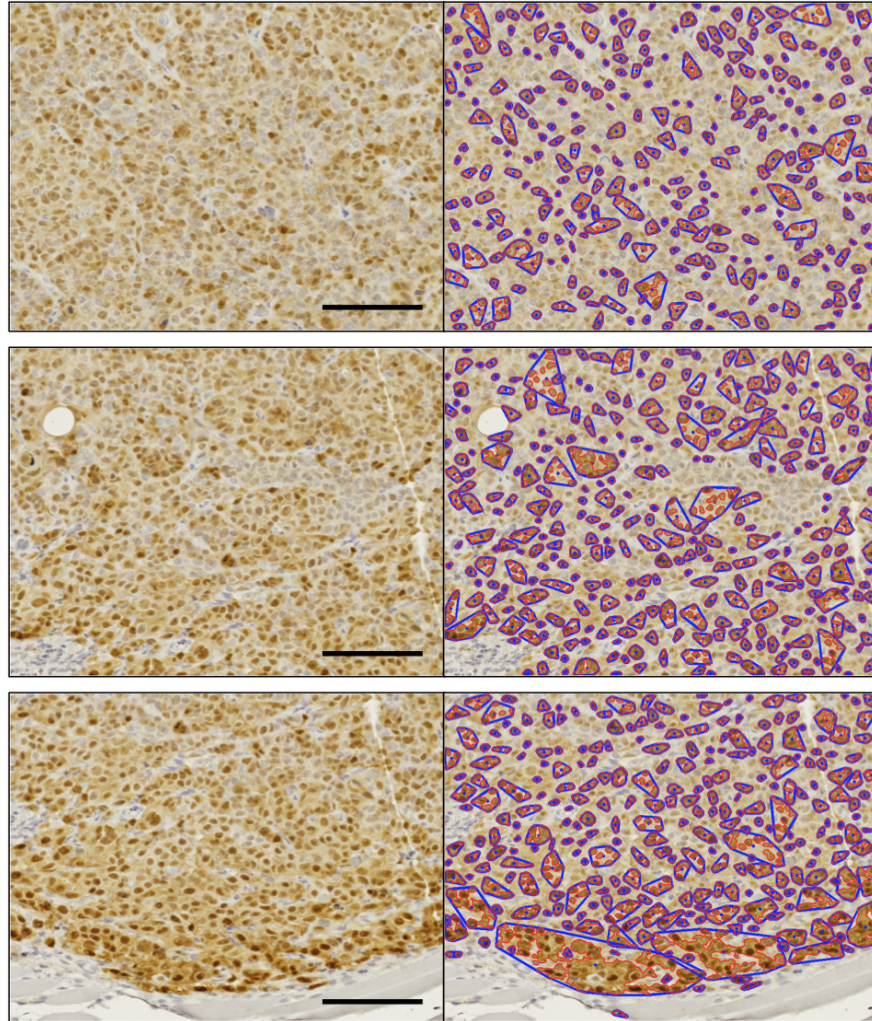
Appendix Figure S 3: Mock LEF1 contours. Scalebars indicate $100\mu\text{m}$, with each images' dimensions approximately $440 \times 330\mu\text{m}^2$. The left column shows stained images. The right column shows stained images with red contour outlines drawn around each spot and blue convex hull outlines which group red spots together if their centroids are within 35 pixels (approximately $11\mu\text{m}$) of each other (see image processing methods, A1.1).



Appendix Figure S 4: Mock LEF1 contours (continued). Scalebars indicate $100\mu\text{m}$, with each images' dimensions approximately $440 \times 330\mu\text{m}^2$. The left column shows stained images. The right column shows stained images with red contour outlines drawn around each spot and blue convex hull outlines which group red spots together if their centroids are within 35 pixels (approximately $11\mu\text{m}$) of each other (see image processing methods, A1.1).

B1.7 SW480 Mock xenograft tumor β -catenin immunohistochemistry

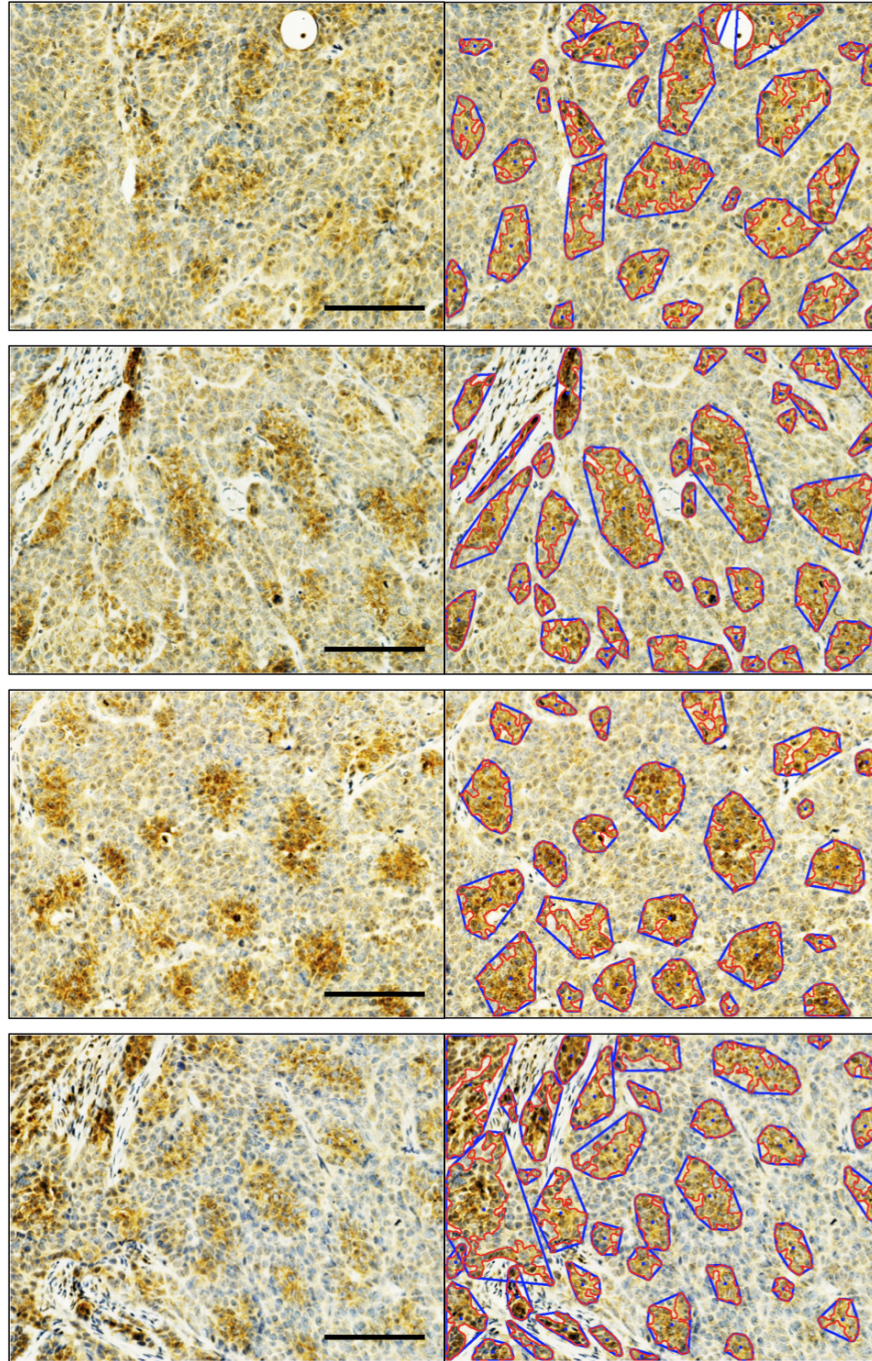
The figures that follow are stains that were processed and whose data (area of each spot and distance to nearest neighbor) appear in the image analysis scatter plots in the main text.



Appendix Figure S 5: Mock β -catenin contours. Scalebars indicate $100\mu\text{m}$, with each images' dimensions approximately $440 \times 330\mu\text{m}^2$. The left column shows stained images. The right column shows stained images with red contour outlines drawn around each spot and blue convex hull outlines which group red spots together if their centroids are within 35 pixels (approximately $11\mu\text{m}$) of each other (see image processing methods, A1.1).

B1.8 SW480 dnLEF xenograft tumor phospho-PDH immunohistochemistry

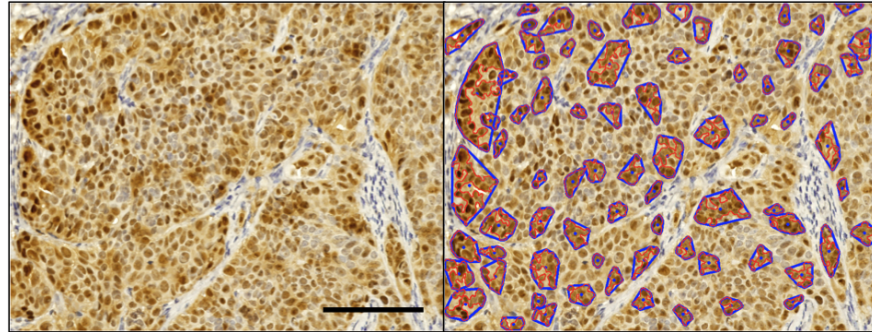
The figures that follow are stains that were processed and whose data (area of each spot and distance to nearest neighbor) appear in the image analysis scatter plots in the main text (Figure 3D).



Appendix Figure S 6: dnLEF phospho-PDH contours. Scalebars indicate $100\mu\text{m}$, with each images' dimensions approximately $440 \times 330\mu\text{m}^2$. The left column shows stained images. The right column shows stained images with red contour outlines drawn around each spot and blue convex hull outlines which group red spots together if their centroids are within 35 pixels (approximately $11\mu\text{m}$) of each other (see image processing methods, A1.1).

B1.9 SW480 dnLEF xenograft tumor β -catenin immunohistochemistry

The figures that follow are stains that were processed and whose data (area of each spot and distance to nearest neighbor) appear in the image analysis scatter plots in the main text (Figure 3E).

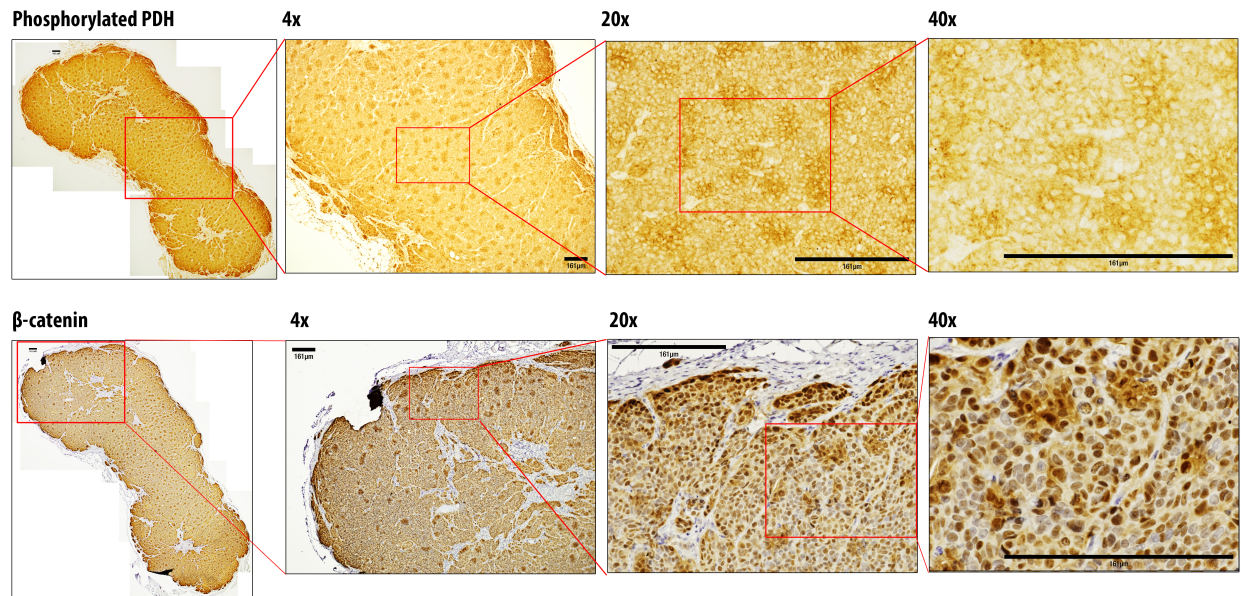


Appendix Figure S 7: dnLEF1 β -catenin contours. Scalebars indicate $100\mu\text{m}$, with each images' dimensions approximately $440 \times 330\mu\text{m}^2$. The left panel shows stained images. The right panel shows stained images with red contour outlines drawn around each spot and blue convex hull outlines that group red spots together if their centroids are within 35 pixels (approximately $11\mu\text{m}$) of each other (see image processing methods, A1.1). Spots highlighted here have high β -catenin expression in the cytoplasm and not just the nucleus.

B1.10 Heterogeneity in SW480 dnTCF xenograft tumors

Heterogeneity in metabolism and Wnt activity was also observed in dnTCF tumors. There is no counterstain for nuclei in these stains.

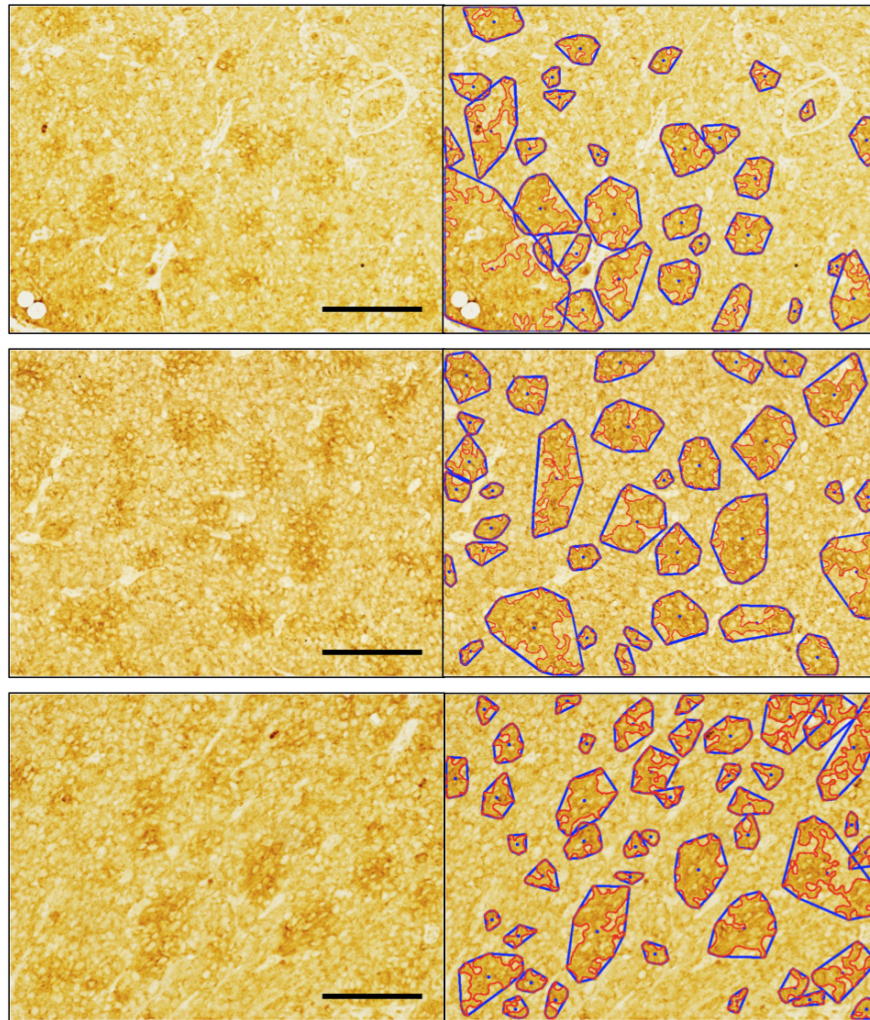
SW480 dnTCF xenograft tumor



Appendix Figure S 8: Phospho-PDH and β -catenin immunohistochemical detection of expression in a dnTCF tumor. SW480 cells were lentivirally transduced to express dominant negative TCF1, which interferes with Wnt signaling activation of target genes. Transduced cells were injected subcutaneously into immunocompromised mice. Tumor sections were stained for phosphorylated PDH and beta-catenin. Scalebars are 161 μ m.

B1.11 SW480 dnTCF phospho-PDH immunohistochemistry

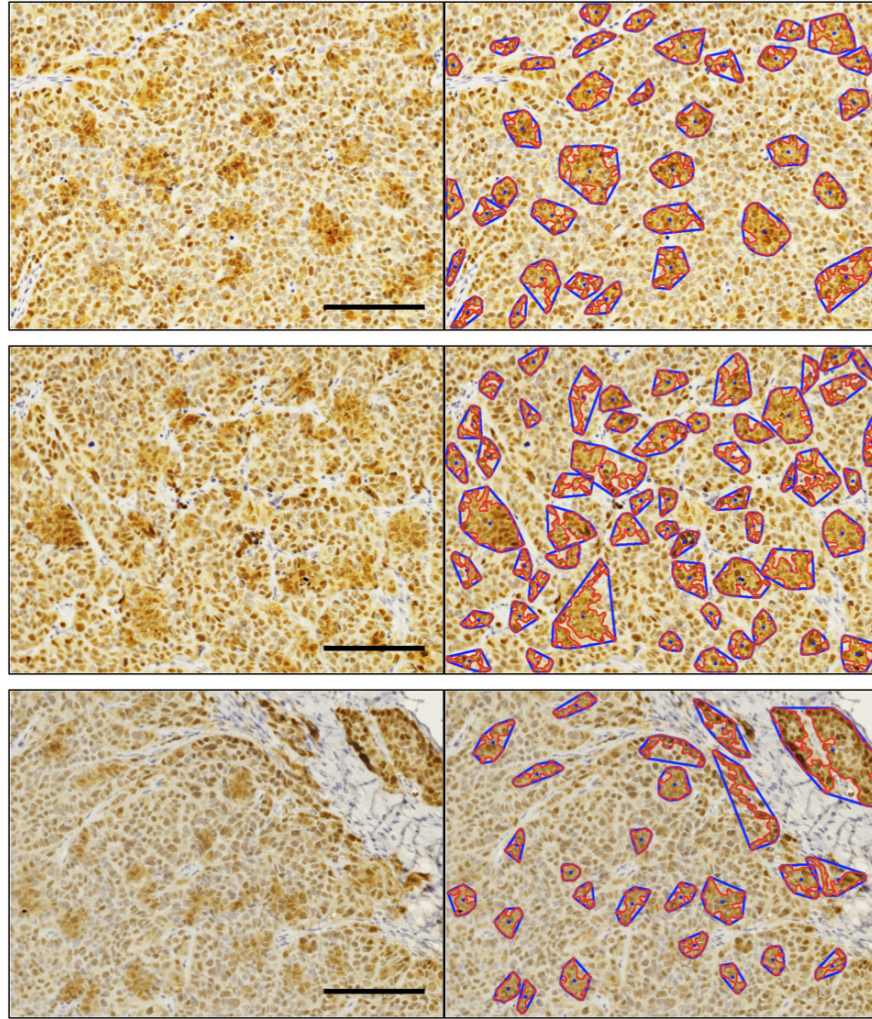
The figures that follow are stains that were processed and whose data (area of each spot and distance to nearest neighbor) appear in the image analysis scatter plots in the main text (Figure 3D).



Appendix Figure S 9: dnTCF phospho-PDH contours. Scalebars indicate $100\mu\text{m}$, with each images' dimensions approximately $440 \times 330\mu\text{m}^2$. The left column shows stained images. The right column shows stained images with red contour outlines drawn around each spot and blue convex hull outlines which group red spots together if their centroids are within 35 pixels (approximately $11\mu\text{m}$) of each other (see image processing methods, A1.1).

B1.12 SW480 dnTCF β -catenin immunohistochemistry

The figures that follow are stains that were processed and whose data (area of each spot and distance to nearest neighbor) appear in the image analysis scatter plots in the main text (Figure 3E).



Appendix Figure S 10: dnTCF β -catenin contours. Scalebars indicate $100\mu\text{m}$, with each images' dimensions approximately $440 \times 330\mu\text{m}^2$. The left column shows stained images. The right column shows stained images with red contour outlines drawn around each spot and blue convex hull outlines which group red spots together if their centroids are within 35 pixels (approximately $11\mu\text{m}$) of each other (see image processing methods, A1.1).

B1.13 Significance in differences between mock and dominant negative tumors in metabolic patterning

The p -values in the tables below were computed using the image processing data after combining spots whose centroids were more than 35 pixels apart (approximately 11 μm ; see image processing description in previous section). Wilcoxon's rank-sum test was done using Matlab's `ranksum.m` function, which tests the statistical significance in the difference of the medians of two datasets, regardless of their distribution.

Appendix Table S 5: p -values for area of spot

	mock pPDH	mock LEF1	mock β -catenin	dnLEF pPDH	dnTCF pPDH	dnLEF β -catenin	dnTCF β -catenin
mock pPDH		3.91E-17	1.25E-68	6.87E-30	9.82E-21	0.0111	1.69E-20
mock LEF1			1.47E-43	2.40E-51	1.21E-41	5.03E-15	5.81E-45
mock β -catenin				1.93E-68	7.85E-59	1.96E-32	2.62E-64
dnLEF pPDH					0.0292	6.69E-16	6.79E-06
dnTCF pPDH						1.09E-10	0.1525
dnLEF β -catenin							9.54E-11

Appendix Table S 6: p -values for distance to nearest neighbor

	mock pPDH	mock LEF1	mock β -catenin	dnLEF pPDH	dnTCF pPDH	dnLEF β -catenin	dnTCF β -catenin
mock pPDH		8.15E-08	3.15E-100	3.31E-40	1.41E-23	1.40E-03	3.29E-21
mock LEF1			3.19E-84	7.74E-50	5.35E-33	1.01E-09	7.59E-32
mock β -catenin				7.81E-73	1.44E-62	2.33E-39	8.21E-67
dnLEF pPDH					2.70E-05	2.32E-19	2.48E-08
dnTCF pPDH						9.03E-10	0.24
dnLEF β -catenin							1.29E-07

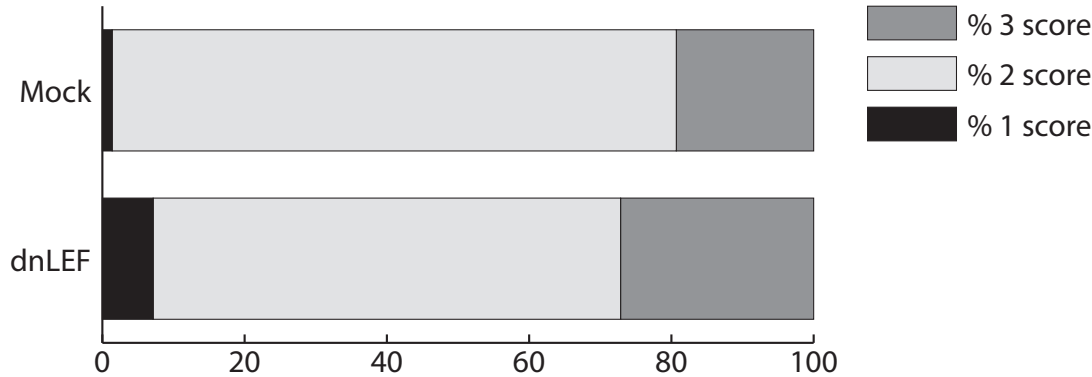
B1.14 Description of image processing of simulation results

Simulations results were processed in the following way, after numerical solutions were obtained:

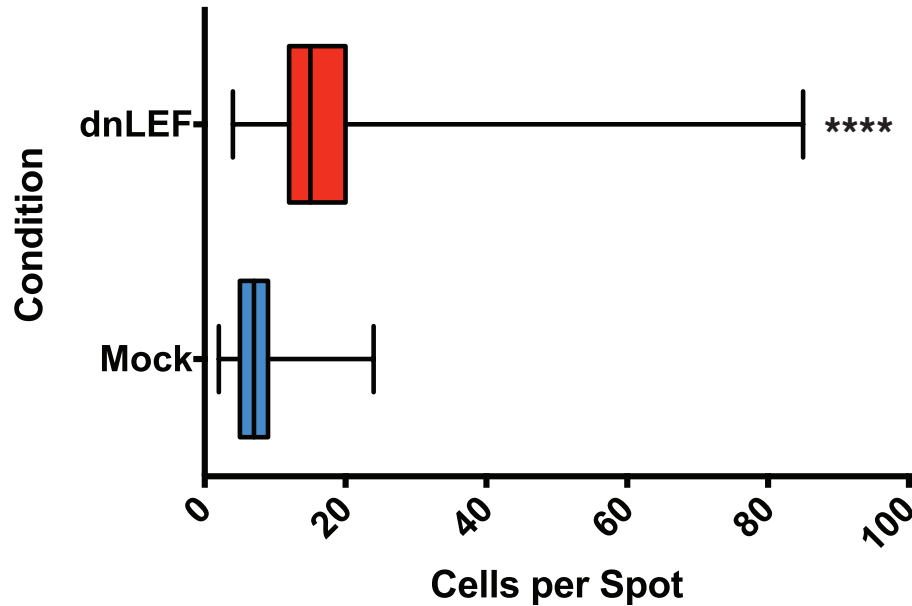
The P_g cell matrix was converted to 0's and 1's by setting matrix entries to 1 if greater than or equal to 0.99 and 0 otherwise. Areas and centroids were computed using Matlab's `regionprops.m` function. Distances to nearest neighbor (centroid-to-centroid) were then calculated. These distances are based on the simulation length scale, which we converted to a dimensional length scale using the following: 1 computational unit = $37.5043 \mu\text{m}$. This length scale was chosen so that there was good agreement between experimental and simulation averages. Computations for the W matrix were similar, except the cutoff used was 8 (i.e., if $W \geq 8$, set the entry to 1, and 0 otherwise). The averages are plotted and labeled as simulation averages in the main text (P_g spots represent pPDH spots and W spots represent β -catenin spots).

B1.15 Quantification of IHC staining intensity and cells per spot in SW480 Mock and dnLEF pPDH staining

A. Proportion of spots by staining intensity



B. Cells per spot



Appendix Figure S 11: Quantification of pPDH spots in SW480 Mock and dnLEF xenograft tumors for cells per tumor and intensity of staining. Three images each of SW480 Mock and dnLEF pPDH staining were provided to blinded researchers who were asked to identify the number of spots within each image and rate the staining intensity of each spot on a scale of 0 to 3, where 3 is the highest intensity. A. The proportion of spot scores for each tumor type. B. Cells per spot per tumor type.

B2 Nondimensionalization

The full set of dimensionalized equations are given by the following, where $\bar{P}$ is the characteristic cell population:

$$\frac{\partial P_o}{\partial t} = D_o \nabla^2 P_o + \frac{1}{\tau_o} N \left(1 - \frac{P_o + P_g + P_d}{\bar{P}} \right) P_o + \frac{1}{\tau_{go}} \chi_W(W) P_g \quad (1a)$$

$$- \frac{1}{\tau_{og}} \chi_W^*(W) \chi_N^*(N) P_o - \mu_o \chi_N(N) P_o \quad (1b)$$

$$\frac{\partial P_g}{\partial t} = D_g \nabla^2 P_g + \frac{1}{\tau_g} \frac{W}{\alpha_W + W} N \left(1 - \frac{P_o + P_g + P_d}{\bar{P}} \right) P_g - \frac{1}{\tau_{go}} \chi_W(W) P_g \quad (1c)$$

$$+ \frac{1}{\tau_{og}} \chi_W^*(W) \chi_N^*(N) P_o - \mu_g \chi_N(N) P_g \quad (1d)$$

$$\frac{\partial P_d}{\partial t} = D_d \nabla^2 P_d + \mu_o \chi_N(N) P_o + \mu_g \chi_N(N) P_g - \mu_d P_d \quad (1e)$$

$$\frac{\partial W}{\partial t} = D_W \nabla^2 W + \frac{1}{a + b W_I} \kappa_W N W^2 P_g + S_W (P_o + P_g) - \mu_W W \quad (1f)$$

$$\frac{\partial W_I}{\partial t} = D_{W_I} \nabla^2 W_I + \kappa_{W_I} N W^2 (P_o + P_g) - \mu_{W_I} W_I \quad (1g)$$

$$\frac{\partial N}{\partial t} = D_N \nabla^2 N - \nu_{NG} N P_g - \nu_{NO} N P_o - \mu_N N + N_s \quad (1h)$$

and

$$\chi_W(W) = \frac{1}{2} [1 - \tanh(\gamma_W(W - W^*))]$$

$$\chi_W^*(W) = \frac{1}{2} [1 + \tanh(\gamma_W(W - W^*))]$$

$$\chi_N(N) = \frac{1}{2} [1 - \tanh(\gamma_N(N - N_d))]$$

$$\chi_N^*(N) = \frac{1}{2} [1 + \tanh(\gamma_N(N - N_g^*))]$$

Introducing the nondimensional quantities below, where overbars indicate characteristic values, we have

$$P'_o = \frac{P_o}{\bar{P}}, P'_g = \frac{P_g}{\bar{P}}, P'_d = \frac{P_d}{\bar{P}}, W' = \frac{W}{\bar{W}}, W'_I = \frac{W_I}{\bar{W}_I}, N' = \frac{N}{\bar{N}}.$$

We define nondimensionalized time as $t' = \frac{t}{T}$ and nondimensionalized length as $x' = \frac{x}{\ell}$, where $T = \frac{\tau_o}{\bar{N}}$ and

$$\ell = \sqrt{\frac{D_{W_I} \tau_o}{\bar{N}}}.$$

We also define the following nondimensional parameters:

$$\begin{aligned}
D'_o &= \frac{\tau_o D_o}{\bar{N} \ell^2} \\
\frac{1}{\tau'_{go}} &= \frac{\tau_o}{\bar{N} \tau_{go}} \\
\frac{1}{\tau'_{og}} &= \frac{\tau_o}{\bar{N} \tau_{og}} \\
\mu'_o &= \frac{\tau_o \mu_o}{\bar{N}} \\
D'_g &= \frac{\tau_o D_g}{\bar{N} \ell^2} \\
\frac{1}{\tau'_g} &= \frac{\tau_o}{\tau_g} \\
\mu'_g &= \frac{\tau_o \mu_g}{\bar{N}} \\
a' &= a \\
b' &= b \bar{W}_I \\
D'_W &= \frac{\tau_o D_W}{\bar{N} \ell^2} \\
\mu'_W &= \frac{\tau_o \mu_W}{\bar{N}} \\
\kappa'_W &= \tau_o \bar{P} \kappa_W \bar{W} \\
S'_W &= \frac{\tau_o S_W \bar{P}}{\bar{N} \bar{W}} \\
\kappa_{W_I} &= \frac{\bar{W}_I}{\tau_o \bar{P} \bar{W}^2} \\
\mu'_{W_I} &= \frac{\tau_o \mu_{W_I}}{\bar{N}} \\
W'^* &= \frac{W^*}{\bar{W}} \\
\gamma'_W &= \gamma_W \bar{W} \\
N'_d &= \frac{N_d}{\bar{N}} \\
N'^*_g &= \frac{N^*_g}{\bar{N}} \\
\gamma'_N &= \gamma_N \bar{N}
\end{aligned}$$

We redefine the switch functions in the following way:

$$\begin{aligned}
\chi'_W(W') &= \frac{1}{2} [1 - \tanh(\gamma'_W(W' - W'^*))] \\
\chi'^*_W(W') &= \frac{1}{2} [1 + \tanh(\gamma'_W(W' - W'^*))] \\
\chi'_N(N') &= \frac{1}{2} [1 - \tanh(\gamma'_N(N' - N'^*))] \\
\chi'^*_N(N') &= \frac{1}{2} [1 + \tanh(\gamma'_N(N' - N'^*))]
\end{aligned}$$

Using these nondimensional quantities, and dropping the prime notation, the nondimensional equations are

$$\frac{\partial P_o}{\partial t} = D_o \nabla^2 P_o + N (1 - P_o - P_g - P_d) P_o + \frac{1}{\tau_{go}} \chi_W(W) P_g \quad (2a)$$

$$- \frac{1}{\tau_{og}} \chi_W^*(W) \chi_N^*(N) P_o - \mu_o \chi_N(N) P_o \quad (2b)$$

$$\frac{\partial P_g}{\partial t} = D_g \nabla^2 P_g + \frac{1}{\tau_g} \frac{W}{\alpha_W + W} N (1 - P_o - P_g - P_d) P_g - \frac{1}{\tau_{go}} \chi_W(W) P_g \quad (2c)$$

$$+ \frac{1}{\tau_{og}} \chi_W^*(W) \chi_N^*(N) P_o - \mu_g \chi_N(N) P_g \quad (2d)$$

$$\frac{\partial P_d}{\partial t} = D_d \nabla^2 P_d + \mu_o \chi_N(N) P_o + \mu_g \chi_N(N) P_g - \mu_d P_d \quad (2e)$$

$$\frac{\partial W}{\partial t} = D_W \nabla^2 W + \frac{1}{a + b W_I} \kappa_W N W^2 P_g + S_W (P_o + P_g) - \mu_W W \quad (2f)$$

$$\frac{\partial W_I}{\partial t} = \nabla^2 W_I + N W^2 (P_o + P_g) - \mu_{W_I} W_I \quad (2g)$$

$$\frac{\partial N}{\partial t} = D_N \nabla^2 N - \nu_{NG} N P_g - \nu_{NO} N P_o - \mu_N N + N_s \quad (2h)$$

B3 Wnt-PDK-Lactate-HIF1 α Cross-Feeding Model

The model presented in this paper was developed to have minimal complexity. Because other signaling pathways and growth or inhibition factors are known to be involved in cancer growth and metabolism, we considered an extension to the Wnt signaling model to include these additional effects.

B3.1 Augmenting the Wnt Signaling Model

We added more detail to the Wnt signaling model by including equations for PDK, lactate, and HIF. The equations for P_o , P_g , P_d , W , W_I , and N (Equations 3b through 3k) are identical to those in the Wnt signaling model in the main text, except that the metabolic switch between OXPHOS and glycolysis is regulated by PDK activity rather than Wnt levels. If PDK is high, then the cells are more likely to switch to glycolysis, and if PDK is low, the cells are more likely to switch to OXPHOS. This larger model was built with the assumptions that Wnt and the hypoxia transcription factor HIF1 α promote PDK expression and activity (?), (?), (?), PDK activity promotes lactate production through upregulation of glycolysis (?), and lactate increases HIF production (?).

The equation for P , PDK activity, is given by equation (3h). The first term on the right of the equality is random motion; the second term represents nonlinear upregulation by Wnt, since Wnt signaling upregulates PDK (?); the third term represents upregulation by HIF1 α (?), (?); the fourth term stands for upregulation through the cells, with sufficient nutrient; and the last term is a decay term.

Equation (3i) is the equation for lactate, which is assumed to diffuse long-range; is upregulated nonlinearly by PDK through the P_g cells, since PDK drives glycolysis (?); and can decay and be uptaken by P_o cells, a form of metabolic symbiosis or cross feeding.

The dynamics for HIF are given by Equation (3j). HIF is assumed to diffuse, to be upregulated nonlinearly by lactate, and to decay. The nonlinear upregulation by lactate comes from the assumption that lactate stabilizes HIF (?). The last two terms in the HIF equation are production terms. There is a small rate (d_H) at which HIF is produced everywhere; and there is a large rate (d_{Hni}) at which HIF is produced when nutrient is low, which represents the stabilization of HIF in hypoxic environments.

$$\frac{\partial P_o}{\partial t} = \underbrace{D_o \nabla^2 P_o}_{\text{random motion}} + \underbrace{\frac{1}{\tau_o} \left(N + \frac{L}{L_s} \right) (1 - P_o - P_g - P_d) P_o}_{\text{proliferation}} - \underbrace{\mu_o \chi_N(N) P_o}_{\text{death}} \quad (3a)$$

$$+ \underbrace{\frac{1}{\tau_{go}} \chi_P(P) P_g}_{\text{switch to OXPHOS}} - \underbrace{\frac{1}{\tau_{og}} \chi_P^*(P) \chi_N^*(N) P_o}_{\text{switch from OXPHOS}} \quad (3b)$$

$$\frac{\partial P_g}{\partial t} = \underbrace{D_g \nabla^2 P_g}_{\text{random motion}} + \underbrace{\frac{1}{\tau_g} \frac{W}{\alpha_W + W} N (1 - P_o - P_g - P_d) P_g}_{\text{proliferation}} - \underbrace{\mu_g \chi_N(N) P_g}_{\text{death}} \quad (3c)$$

$$- \underbrace{\frac{1}{\tau_{go}} \chi_P(P) P_g}_{\text{switch from glycolysis}} + \underbrace{\frac{1}{\tau_{og}} \chi_P^*(P) \chi_N^*(N) P_o}_{\text{switch to glycolysis}} \quad (3d)$$

$$\frac{\partial P_d}{\partial t} = \underbrace{D_d \nabla^2 P_d}_{\text{random motion}} + \underbrace{\mu_o \chi_N(N) P_o}_{\text{dead } P_o \text{ cells}} + \underbrace{\mu_g \chi_N(N) P_g}_{\text{dead } P_g \text{ cells}} - \underbrace{\mu_d P_d}_{\text{decay}} \quad (3e)$$

$$\frac{\partial W}{\partial t} = \underbrace{D_W \nabla^2 W}_{\text{random motion}} + \underbrace{\frac{1}{a + b W_I} \kappa_W N W^2 P_g}_{\text{inhibition upregulation}} + \underbrace{S_W (P_o + P_g)}_{\text{upregulation}} - \underbrace{\mu_W W}_{\text{downregulation}} \quad (3f)$$

$$\frac{\partial W_I}{\partial t} = \underbrace{D_{W_I} \nabla^2 W_I}_{\text{random motion}} + \underbrace{\kappa_{W_I} N W^2 (P_o + P_g)}_{\text{upregulation}} - \underbrace{\mu_{W_I} W_I}_{\text{downregulation}} \quad (3g)$$

$$\frac{\partial P}{\partial t} = \underbrace{D_P \nabla^2 P}_{\text{random motion}} + \underbrace{\nu_{PW} \frac{W^2}{\alpha_{PW} + W} N}_{\text{upregulation}} + \underbrace{\nu_{PH} \frac{H}{\alpha_{PH} + H} N}_{\text{upregulation}} + \underbrace{\nu_P N (P_o + P_g)}_{\text{upregulation}} - \underbrace{\mu_P P}_{\text{downregulation}} \quad (3h)$$

$$\frac{\partial L}{\partial t} = \underbrace{D_L \nabla^2 L}_{\text{random motion}} + \underbrace{\nu_{LP} P_g \frac{P^2}{\alpha_P + P} N}_{\text{production}} - \underbrace{\mu_L L}_{\text{decay}} - \underbrace{\nu_{LO} L P_o}_{\text{uptake by } P_o \text{ cells}} \quad (3i)$$

$$\frac{\partial H}{\partial t} = \underbrace{D_H \nabla^2 H}_{\text{random motion}} + \underbrace{\nu_{HL} \frac{L^2}{\alpha_L + L}}_{\text{lactate-induced production}} - \underbrace{\mu_H H}_{\text{decay}} + \underbrace{d_H}_{\text{bulk production}} + \underbrace{d_{HN_i} (1 - N)}_{\text{hypoxia-induced production}} \quad (3j)$$

$$\frac{\partial N}{\partial t} = \underbrace{D_N \nabla^2 N}_{\text{random motion}} - \underbrace{\nu_{NG} N P_g}_{\text{uptake by } P_g \text{ cells}} - \underbrace{\nu_{NO} N P_o}_{\text{uptake by } P_o \text{ cells}} - \underbrace{\mu_N N}_{\text{decay}} + \underbrace{N_s}_{\text{bulk source}} \quad (3k)$$

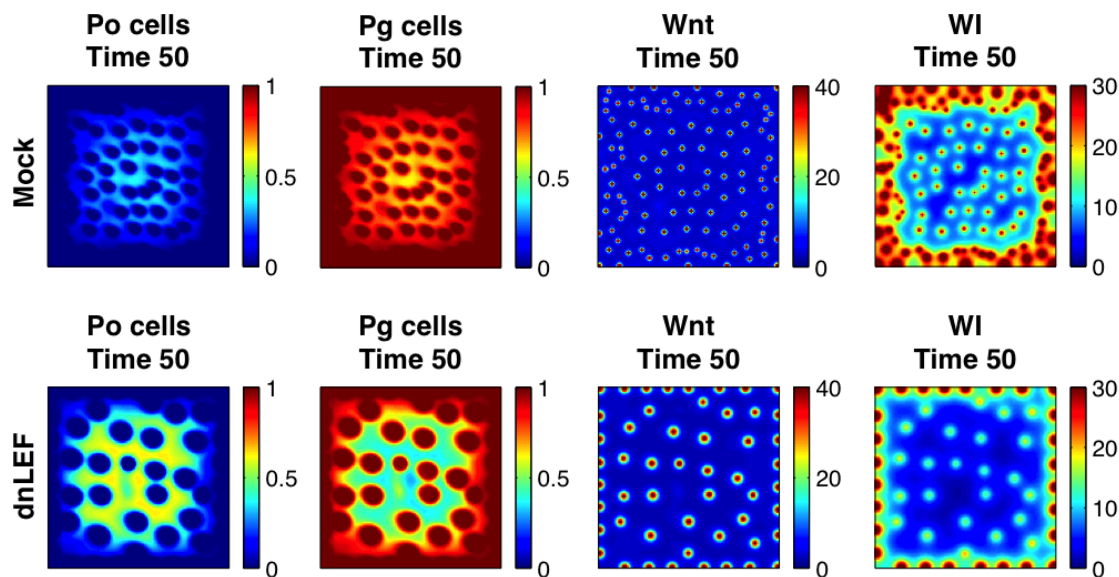
B3.2 Results

The parameters used in the results shown here are given by Table 7. The system evolves over time and its final output at time 50 is shown in Figures 12-13. The numerical results are similar to those in Figure 2 and 3, with a striking spotted pattern in glycolysis and OXPHOS. Near the boundary, there is a very high level of glycolysis, with localized spots of high glycolysis surrounded by relatively lower levels. We also see this spotted pattern in PDK activity, lactate, and HIF. When the mock and dnLEF tumors were stained for HIF, we saw a spotted pattern in both, with the dnLEF tumor exhibiting larger and fewer spots (see Supplemental Figure 14).

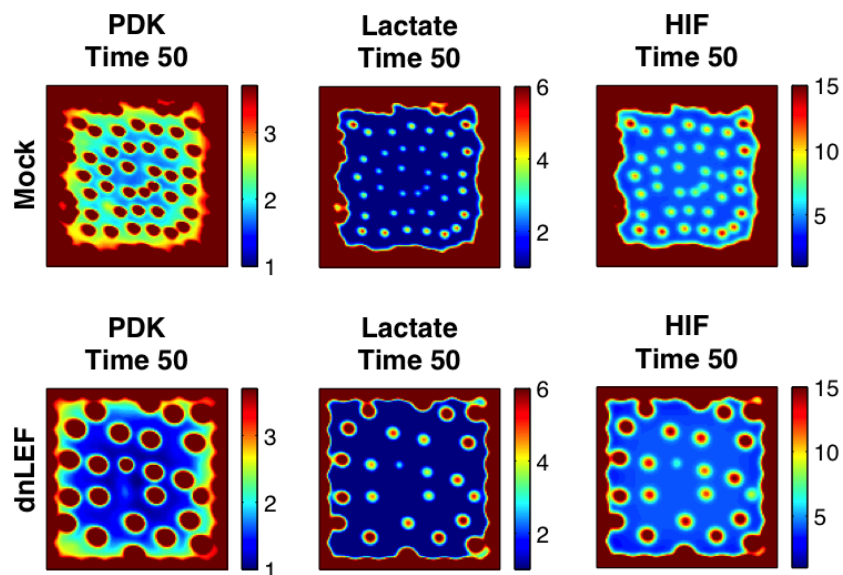
Appendix Table S 7: **Wnt-PDK-Lactate-HIF1 α** Model Parameters

Parameter	Description	Mock value	dnLEF value
D_o	Diffusion coefficient of oxidative cells	0.01	0.01
D_g	Diffusion coefficient of glycolytic cells	0.01	0.01
D_W	Diffusion coefficient of Wnt	0.01	0.035
D_{W_I}	Diffusion coefficient of Wnt inhibitor	1	1.5
D_P	Diffusion coefficient of PDK activity	0.01	0.01
D_L	Diffusion coefficient of lactate	100	100
D_H	Diffusion coefficient of HIF	0.01	0.01
D_N	Diffusion coefficient of nutrient	100	100
τ_o	Oxidative cell proliferation time	1	1
τ_g	Glycolytic cell proliferation time	1	1
τ_{og}	Switch time from OXPHOS to glycolysis	1/24	1/24
τ_{go}	Switch time from glycolysis to OXPHOS	1	1
α_W	Constant for Michaelis-Menten dynamics	1	1
κ_W	Rate of nonlinear Wnt production	5	5
κ_{W_I}	Rate of Wnt inhibitor production	1	1
μ_o	Decay rate of P_o cells	1	1
μ_g	Decay rate of P_g cells	1	1
μ_d	Decay rate of P_d cells	1	1
μ_W	Decay rate of Wnt	3	3
μ_{W_I}	Decay rate of Wnt inhibitor	5	5
μ_P	Decay rate of PDK activity	1	1
μ_L	Decay rate of lactate	1000	1000
μ_H	Decay rate of HIF	1	1
μ_N	Decay rate of nutrient	1	1
ν_{PW}	Rate of PDK upregulation through Wnt	2	2
ν_{PH}	Rate of PDK upregulation through HIF	0.5	0.5
ν_{LP}	Rate of lactate upregulation through PDK activity	3000	3000
ν_{HL}	Rate of HIF stabilization through lactate	3	3
ν_P	PDK activity upregulation through cells	1	1
ν_{LO}	Rate of lactate uptake by P_o cells	10^5	10^5
d_H	Constitutive HIF production	0.5	0.5
d_{HNI}	Rate of HIF stabilization due to low nutrient	5	5
S_W	Rate of Wnt production through cells	7.5	5.5
a	Constant of inhibition	10^{-8}	10^{-8}
b	Constant of inhibition by W_I	1	1
γ_P	Sensitivity level of PDK switch functions	1	1
γ_N	Sensitivity level of nutrient switch function	100	100
ν_{NG}	Uptake of nutrient by P_g cells	100	100
ν_{NO}	Uptake of nutrient by P_o cells	100	100
N_s	Parameter for nutrient source	30	30
L_s	Parameter for characteristic value of L	1	1
P^*	PDK activity level at which 50% of cells switch metabolism	3	3
N_d	Nutrient level below which cells will die	0.07	0.07
N_g^*	Nutrient level below which P_o cells cannot switch to glycolysis	0.1	0.1
α_N	Value of scaling function when $\int P_g = 0$	0.025	0.025
S_x	Horizontal length of spatial domain	12	12
S_y	Vertical length of spatial domain	12	12

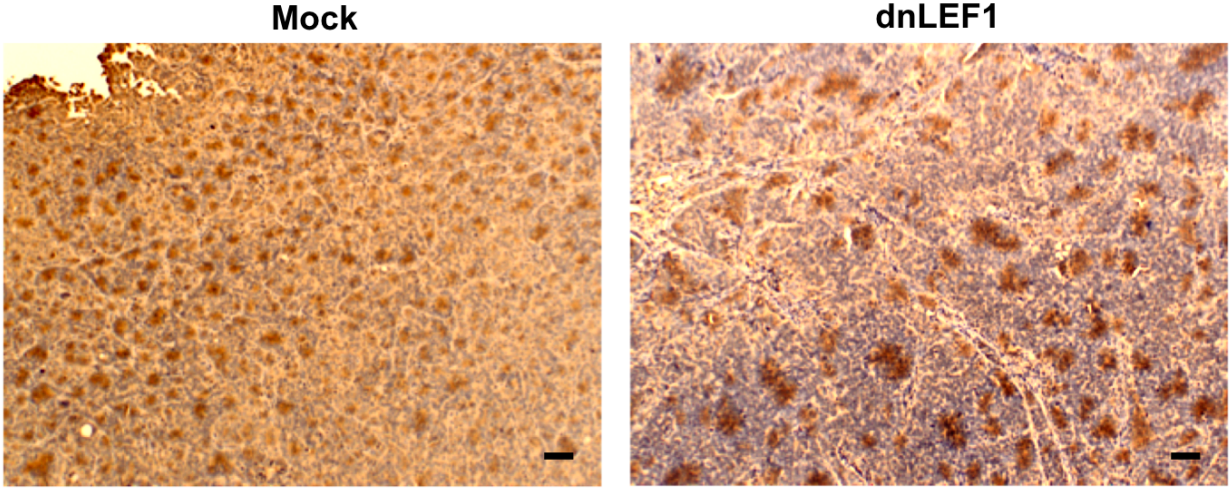
Model parameters for mock and dnLEF/dnTCF simulations



Appendix Figure S 12: Numerical results for mock and dnLEF P_o , P_g , W , and W_I in Wnt-PDK-lactate-HIF1 α model.



Appendix Figure S 13: Numerical results for mock and dnLEF PDK, lactate, and HIF in Wnt-PDK-lactate-HIF1 α model.



Appendix Figure S 14: Stains for HIF1 α in mock and dnLEF tumors. Scalebars indicate 100 μ m

B3.3 Discussion

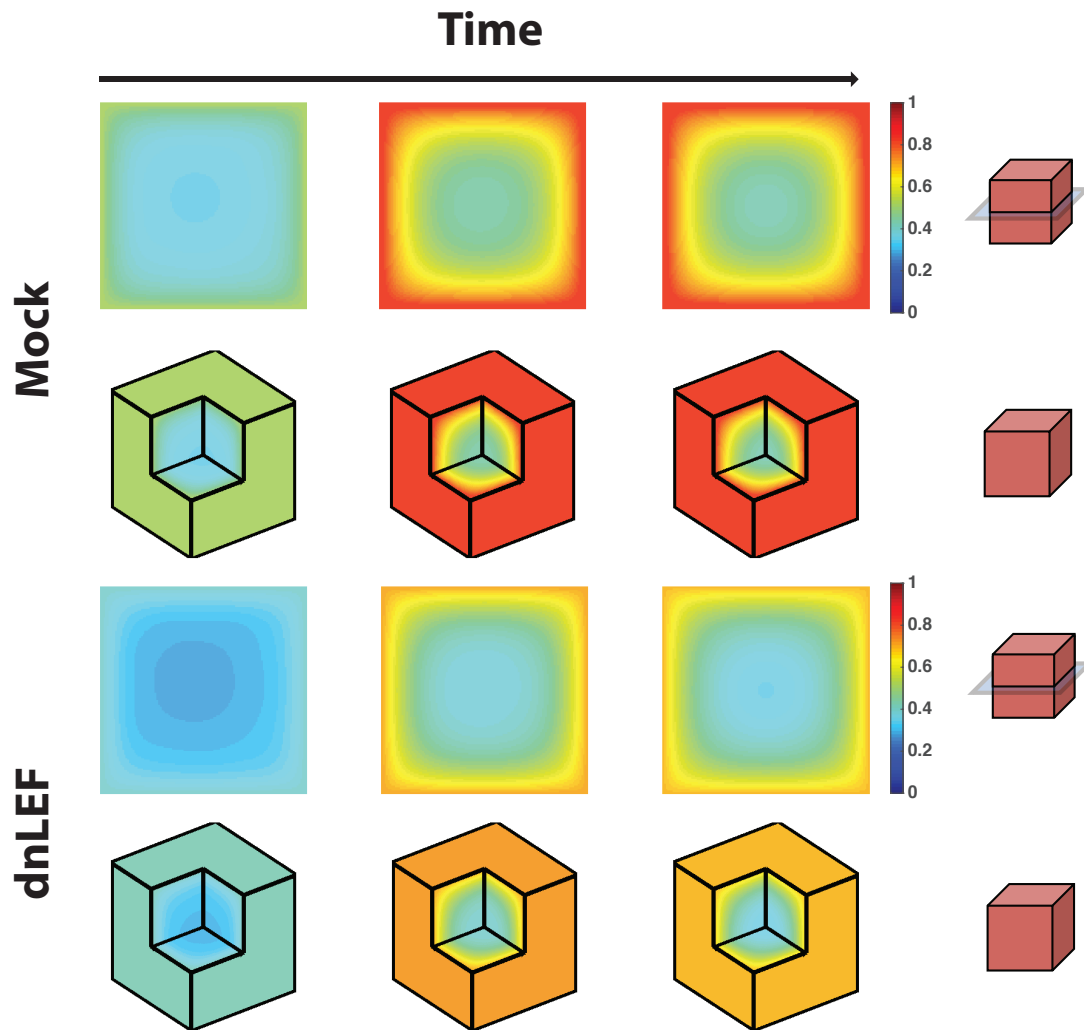
The results from this more detailed model give us good qualitative agreement with our IHC data when we compare the numerical results for PDK activity with our phospho-PDH stains. We see the larger, fewer spots and lighter background in the dominant negative LEF PDK results in comparison to mock. It was not possible to stain for lactate, but experimental data show that the cells and HIF1 α follow the same spotted pattern, and the same change in the pattern from mock to dnLEF/dnTCF, and we therefore conclude that this model is sufficient to recapitulate qualitatively our experimental observations.

Importantly, this more detailed model gives similar qualitative results to the simpler model presented in the main text when the experimental results are compared to numerical results for PDK activity. Since PDK drove switching in the cells, we saw patterns in metabolism as well. The simpler model retains the most important elements of the larger model while producing similar results. In the Wnt signaling model discussed in the main text, the positive feedback between Wnt and PDK (high PDK implies more P_g cells; higher levels of P_g cells imply more Wnt activity; more Wnt activity means increased PDK) has been distilled so that Wnt activity level is the effective switch rather than PDK. Because PDK was directing the switch in metabolism, we can use P_g and P_o as the effective patterned state to compare to the xenograft stains. Thus, the equations for PDK activity, HIF1 α concentration, and lactate concentration can be removed from the system, with Wnt activity driving the switch rather than PDK, so that the remaining equations contain only most important elements of the model.

B3.4 Numerical method

Numerical simulations were performed in MATLAB, using a forward difference method for each time derivative. P_o , P_g , W , W_I , P , L , and H equations were solved implicitly in centered diffusion terms. The nutrient equation was solved implicitly in uptake, decay, and centered diffusion terms. No-flux boundary conditions were used for all fields except for the nutrient boundary which used a Dirichlet boundary condition. The initial condition for the P_g cell population was a random distribution near the boundary of the domain. W and W_I initial conditions were a random distribution in the same locations where initial P_g cells were located. Initial N was 1 everywhere in the domain; results did not change qualitatively if N was solved as a quasi-steady state equation. Initial conditions for P_o , P_d , P , L , and H terms were 0.

B4 Nutrient diffusion simulation



Appendix Figure S 15: Nutrient diffusion simulation for Wnt signaling model. Nutrients diffuse into the system from all boundaries. The rate of diffusion and total concentration of nutrient is influenced by Wnt signaling and metabolic program switching.

B5 Parameter exploration

This table lists the set of parameters we investigated in the Wnt signaling model in order to test our model for robustness. The parameters that are missing from this table were explored in the main part of the paper, so their effects are already evaluated and are not included here. We varied each parameter one by one, varying between the minimum and maximum values listed in the table, and kept all other values the same as the mock values in the table of parameter values in the main text, to ensure that a pattern would emerge that was qualitatively consistent with our mock stains. Next, we made the same changes from mock to dnLEF as presented in the paper (increased the diffusion coefficients for W and W_I and decreased S_W) to see if we still obtained qualitatively similar answers as the dnLEF/dnTCF experiments. In this way we determine whether our model is robust to a large range of values, so that the results we obtain from the parameters presented in the main part of this paper can be considered characteristic of our system.

Appendix Table S 8: **Parameter sensitivity study**

Parameter	Mock value	dnLEF value	Minimum	Maximum	Comment
κ_W	5	5	3	50	$\kappa_W = 2$: no pattern in dnLEF
κ_{W_I}	1	1	0.1	1.25	$\kappa_{W_I} = 1.5$: no pattern in dnLEF
a	10^{-8}	10^{-8}	10^{-16}	0.75	$a = 1$: no pattern in dnLEF
b	1	1	0.1	1.75	$b = 2$: no pattern in dnLEF
τ_{og}	1/24	1/24	1/85	1/20	$\tau_{og} = 1/18$: no pattern in dnLEF; $\tau_{og} = 1/90$: almost all P_g ; not enough spots in mock
μ_o	1	1	0	40	$\mu_o = 50$: no pattern in dnLEF
μ_g	1	1	0	100	Did not try larger than 100
μ_d	1	1	0	100	Did not try larger values
α_W	1	1	10^{-8}	1.5	Numerical error if $\alpha_W = 0$; no pattern in dnLEF if $\alpha_W = 2$
μ_W	2	2	2	2	$\mu_W = 1$, dnLEF: pattern in Wnt but none in P_g (100% P_g); $\mu_W = 1.5$, dnLEF: slight pattern in P_g (almost 100% P_g); $\mu_W = 1.75$, mock: slight pattern in P_g (almost 100% P_g , but fewer spots than in dnLEF); $\mu_W = 2.25$, dnLEF: all terms $\rightarrow 0$
μ_{W_I}	3	3	3	20	$\mu_{W_I} = 2.75$, dnLEF: no pattern; $\mu_{W_I} = 20$, mock: almost 100% P_g , 3 “spots”
ν_{NG}	10	10	0	25	$\nu_{NG} = 30$: no pattern in dnLEF
ν_{NO}	10	10	0	20	$\nu_{NO} = 25$: no pattern in dnLEF
μ_N	0.1	0.1	0	1	$\mu_N = 1.5$: no pattern in dnLEF
N_s	2	2	0	10	$N_s = 10$: nutrient essentially constant throughout domain in dnLEF and mock
L_s	65.9	57.7	1/100	100	Small values of L_s result in the pattern forming more quickly

Robustness to parameter changes: The minimum and maximum values listed in this table indicate the range of tested values for which we still see the same characteristic phenotype changes in our model. This means that from mock to dnLEF, we observe fewer, larger spots farther apart from each other.

B6 Diffusive stability analysis

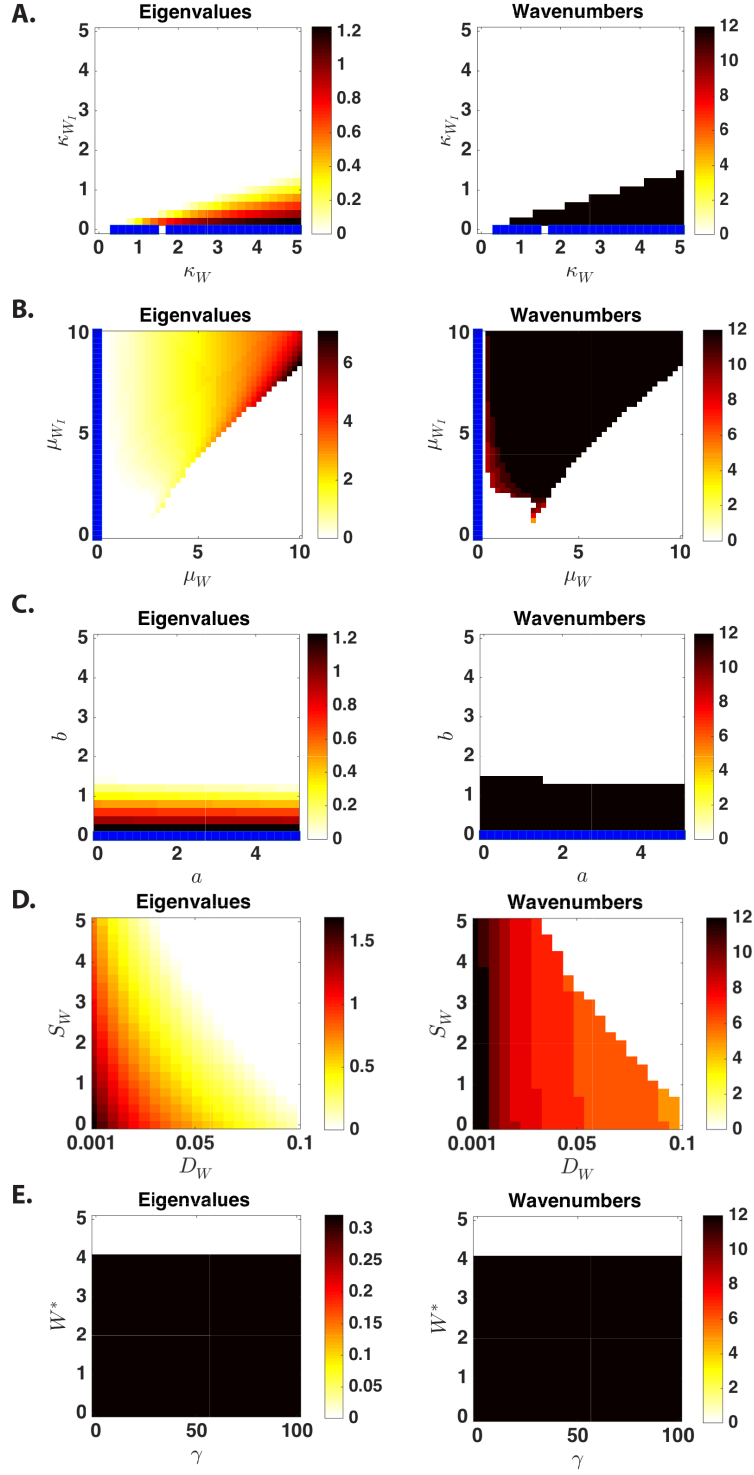
We chose our parameters first by looking only at a modified version of the two equation Gierer-Meinhardt activator-inhibitor system, given by

$$\begin{aligned}\frac{\partial a}{\partial t} &= D_a \nabla^2 a + \kappa_a \frac{a^2}{h} - \mu_a a + \rho_a \\ \frac{\partial h}{\partial t} &= D_h \nabla^2 h + \kappa_h a^2 - \mu_h h + \rho_h\end{aligned}$$

where a is the activator that creates its own inhibitor h (modified so that the rates for nonlinear production of a and h can be different). Classic diffusive instability analysis was done on this smaller system to find parameters that expect to give us patterns. After finding and fixing suitable parameters for these equations, we performed diffusive stability analysis on the larger system by solving the equations without diffusion using MATLAB's ode45 solver and 100 time steps, fixing other values and varying two parameters at a time. If a steady state was not found, then that set of parameters was assumed to give us oscillations and so the linear stability analysis could not be performed with those parameters. If a steady state was found, we linearized around that steady state and we determined whether it was stable or not by looking at the real part of each eigenvalue. If all real parts were negative, we include diffusion and if any these new eigenvalues had a positive real part, then that set of parameters was expected to give us a pattern.

In the figures that follow, areas that are white are associated with parameters where no patterns are expected to form. Areas that are neither white nor blue show the eigenvalue and wavenumber associated with the pattern that is predicted to emerge. Any area that is blue is a region in which the solution of the ordinary differential equation (ODE) versions of the equations is oscillatory rather than steady, hence the equations could not be linearized about a steady state and so the analysis could not be performed. That is not to say, however, that patterns could not form with those parameter values.

We plot eigenvalues, which quantify the instability of an initial perturbation, and wavenumbers, which predict the dominant number of spikes or peaks in the entire system. Note that each wavenumber graph is plotted with a fixed colorscale between white (0) and black (12); wavenumbers above 12 were not tested. If a set of parameters has a nonzero eigenvalue, it is expected that a perturbation grows in time, hence a pattern should form in early times. The higher the eigenvalue, the higher the instability and hence more likelihood for a pattern to emerge and remain over the long term. Hence, a nonzero eigenvalue that is very small may yield a pattern only in very early times in the simulation. Moreover, a nonzero eigenvalue and wavenumber may mean a pattern in Wnt and inhibitor, but this may not necessarily translate to a pattern in the cells.



Appendix Figure S 16: Diffusive instability analysis A. Diffusive instability analysis varying κ_W and κ_{W_I} . B. Analysis varying μ_W and μ_{W_I} . C. Analysis varying a and b . D. Analysis varying D_W and S_W . E. Analysis varying γ and W^* .

B7 Simulation of in vitro tumor growth with DCA and XAV939 treatment

B7.1 Boundary Conditions

N is set to Dirichlet constant 0.25. P_g, P_o, P_d, W, W_I, L is Neumann.

B7.2 Equations

$$\frac{\partial P_o}{\partial t} = \underbrace{D_o \nabla^2 P_o}_{\text{random motion}} + \underbrace{\frac{1}{\tau_o} \left(N + \frac{L}{L_s} \right) (1 - P_o - P_g - P_d) P_o}_{\text{proliferation}} - \underbrace{\mu_o \chi_{NL}(N, L) P_o}_{\text{death}} \quad (4)$$

$$+ \underbrace{\frac{1}{\tau_{go}} \chi_W(W) P_g}_{\text{switch to OXPHOS}} - \underbrace{\frac{1}{\tau_{og}} \chi_W^*(W) \chi_N^*(N) P_o}_{\text{switch from OXPHOS}} \quad (5)$$

$$\frac{\partial P_g}{\partial t} = \underbrace{D_g \nabla^2 P_g}_{\text{random motion}} + \underbrace{\frac{1}{\tau_g} \frac{W}{\alpha_W + W} N (1 - P_o - P_g - P_d) P_g}_{\text{proliferation}} - \underbrace{\mu_g \chi_N(N) P_g}_{\text{death}} \quad (6)$$

$$- \underbrace{\frac{1}{\tau_{go}} \chi_W(W) P_g}_{\text{switch from glycolysis}} + \underbrace{\frac{1}{\tau_{og}} \chi_W^*(W) \chi_N^*(N) P_o}_{\text{switch to glycolysis}} \quad (7)$$

$$\frac{\partial P_d}{\partial t} = \underbrace{D_d \nabla^2 P_d}_{\text{random motion}} + \underbrace{\mu_o \chi_{NL}(N, L) P_o}_{\text{dead } P_o \text{ cells}} + \underbrace{\mu_g \chi_N(N) P_g}_{\text{dead } P_g \text{ cells}} - \underbrace{\mu_d P_d}_{\text{decay}} \quad (8)$$

$$\frac{\partial W}{\partial t} = \underbrace{D_W \nabla^2 W}_{\text{random motion}} + \underbrace{\frac{1}{a + b W_I} \kappa_W N W^2 P_g}_{\text{inhibition upregulation}} + \underbrace{S_W (P_o + P_g)}_{\text{upregulation}} - \underbrace{\mu_W W}_{\text{downregulation}} \quad (9)$$

$$\frac{\partial W_I}{\partial t} = \underbrace{D_{W_I} \nabla^2 W_I}_{\text{random motion}} + \underbrace{\kappa_{W_I} N W^2 (P_o + P_g)}_{\text{upregulation}} - \underbrace{\mu_{W_I} W_I}_{\text{downregulation}} \quad (10)$$

$$\frac{\partial N}{\partial t} = \underbrace{D_N \nabla^2 N}_{\text{random motion}} - \underbrace{\nu_{NG} N P_g}_{\text{uptake by } P_g \text{ cells}} - \underbrace{\nu_{NO} N P_o}_{\text{uptake by } P_o \text{ cells}} - \underbrace{\mu_N N}_{\text{decay}} \quad (11)$$

$$\frac{\partial L}{\partial t} = \underbrace{D_L \nabla^2 L}_{\text{random motion}} - \underbrace{\nu_{LO} L P_o}_{\text{uptake by } P_o \text{ cells}} + \underbrace{N_s P_g}_{\text{bulk source by } P_g} \quad (12)$$

$$(13)$$

Bulk source term:

$$N_s = \overline{N_s}$$

Switch functions:

$$\chi_w(W) = \frac{1}{2} (1 - \tanh(\gamma_w(W - W^*)))$$

$$\chi_w^*(W) = \frac{1}{2} (1 + \tanh(\gamma_w(W - W^*)))$$

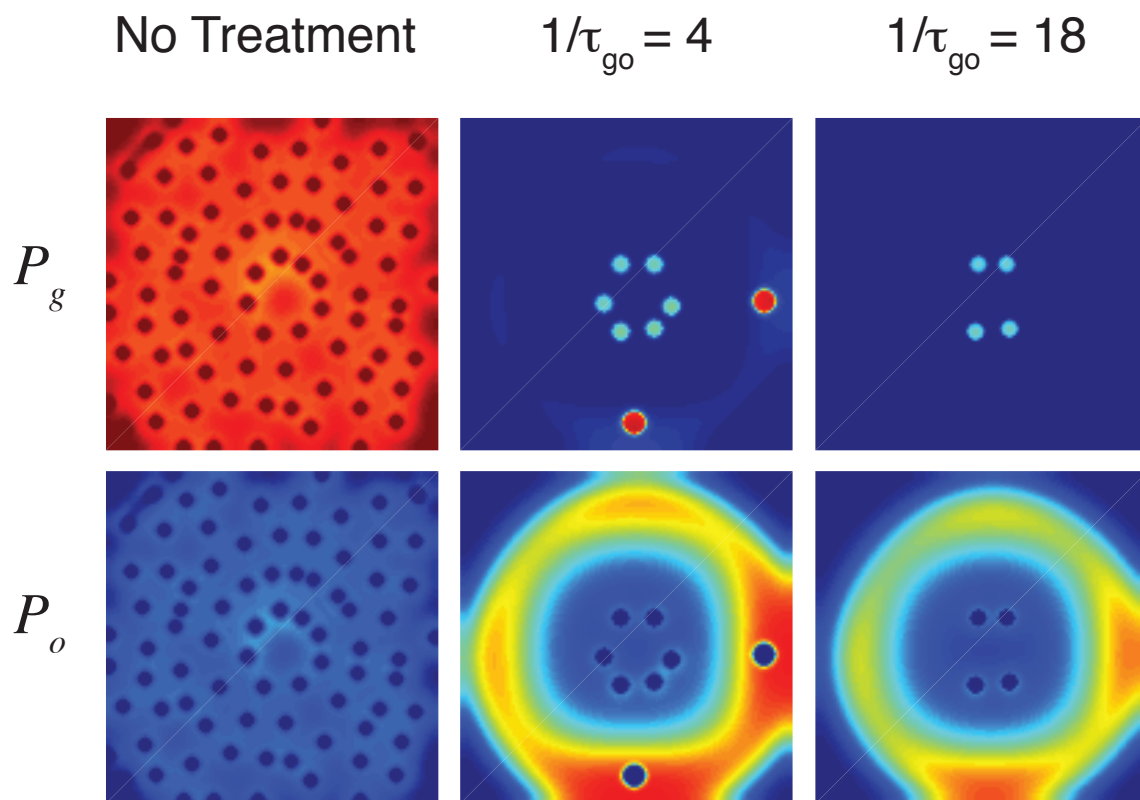
$$\chi_{NL}(N, L) = \frac{1}{2} \left(1 - \tanh \left(\gamma_N \left((rN + (1-r) \frac{\gamma_L L}{1 + \gamma_L L}) - N^* \right) \right) \right)$$

$$\chi_N^*(N) = \frac{1}{2} (1 + \tanh(\gamma_N(N - N_g^*)))$$

B7.3 Parameter values for in vitro model

Parameter	Description	Mock value	dnLEF value
D_o	Diffusion coefficient of oxidative cells	0.01	0.01
D_g	Diffusion coefficient of glycolytic cells	0.01	0.01
D_d	Diffusion coefficient of dead cells	0	0
D_w	Diffusion coefficient of Wnt	0.004	0.008
D_{wI}	Diffusion coefficient of Wnt inhibitor	1	1.5
D_N	Diffusion coefficient of Nutrient	100	100
D_L	Diffusion coefficient of L	100	100
τ_o	Oxidative cell proliferation time	1	1
τ_g	Glycolytic cell proliferation time	1	1
τ_{og}	Switch time from OXPHOS to glycolysis	$\frac{1}{24}$	$\frac{1}{24}$
τ_{go}	Switch time from glycolysis to OXPHOS	1	1
α_w	Constant for Michaelis-Menten dynamics	1	1
κ_w	Rate of nonlinear Wnt production	5	5
κ_{wI}	Rate of nonlinear Wnt inhibitor production	1	1
μ_o	Decay rate of P_o cells	1	1
μ_g	Decay rate of P_g cells	1	1
μ_d	Decay rate of P_d cells	1	1
μ_w	Decay rate of Wnt	2	2
μ_{wI}	Decay rate of Wnt inhibitor	3	3
μ_{wI}	Decay rate of nutrient	0.1	0.1
S_w	Rate of Wnt production through cells	7.5	6.5
a	Constant of inhibition	10^{-8}	10^{-8}
b	Constant of inhibition by W_I	1	1
γ_w	Sensitivity level of Wnt switch functions	1	1
γ_N	Sensitivity level of nutrient switch functions	100	100
γ_L	Parameter for scaling L for P_o death	1	1
ν_{NG}	Uptake of nutrient by P_g cells	10	10
ν_{NO}	Uptake of nutrient by P_o cells	10	10
ν_{LO}	Uptake of L by P_o cells	10	10
$\overline{N_s}$	Parameter for nutrient source	2	2
W^*	Wnt level at which 50% of cells switch metabolism	5	5
N_d	Nutrient level below which cells will die	0.07	0.07
N_g^*	Nutrient level below which P_o cells cannot switch to glycolysis	0.1	0.1
β_N	Nutrient boundary condition parameter	0.25	0.25
S_x	Horizontal length of spatial domain	12	12
S_y	Vertical length of spatial domain	12	12
r	Parameter for scaling N for P_o death	0.375	0.375

B7.4 Simulations of DCA and XAV939 treatment on in vitro tumors



Appendix Figure S 17: Using the in vitro simulation equations described above, we modeled the same drug concentrations as the in vivo simulations presented in Figure 6. The tumor size over increasing drug concentrations for these simulations are described in Figure 7D.

B8 Synergy is evident in simulations with combined DCA and XAV939 treatment

We can measure the synergistic effect of treatment using the Bliss Independence model (Foucquier and Guedj, 2015). The Bliss combination index is defined by $BCI = \frac{E_A + E_B - E_{AB}}{E_{AB}}$, where the effectiveness of treatment by drug A is E_A and the effectiveness of treatment by drug B is E_B . The Bliss model assumes that the drugs A and B act independently of each other. Effectiveness for each drug is defined by the ability to reduce tumor size as in the following:

$$E_A = 1 - (\text{surviving tumor fraction after applying drug A})$$

$$E_B = 1 - (\text{surviving tumor fraction after applying drug B})$$

$$E_{AB} = 1 - (\text{surviving tumor fraction after applying drug A and drug B in combination}).$$

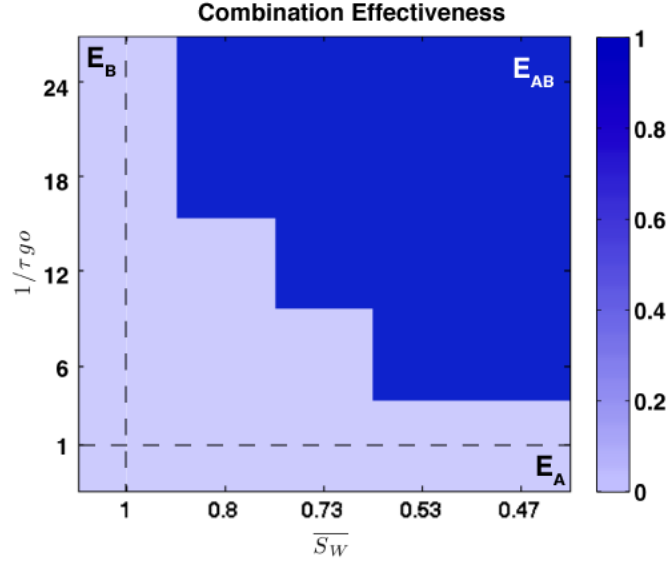
A BCI of less than 1 indicates the synergy of drug action, while a score closer to 1 indicates the treatments act additively rather than synergistically. A BCI greater than 1 implies that the drugs interfere with each other. In the in vivo simulation and in vitro experimental model of tumor spheroid growth, $E_{XAV939} = E_{DCA} = 0$ (see figure below), which implies that $BCI=0$ in these cases.

In vivo:

Neither DCA alone nor XAV939 alone are successful in eradicating any of the tumor cells by the end of treatment (unitless time 50) unless the treatment doses are relatively high (e.g., $\bar{S}_W \lesssim 0.4$ or $1/\tau \gtrsim 30$). Thus, $E_{XAV939} = E_{DCA} = 0 = BCI$. In figure 18 below, we plot E_{AB} with $A = XAV939$ and $B = DCA$. The areas with dark blue indicate the regions where the combination effectiveness $E_{AB} = 1$ and the Bliss model predicts that there is synergy (this would also be true if we used the Highest Single Agent or Response Additivity models to assess synergy).

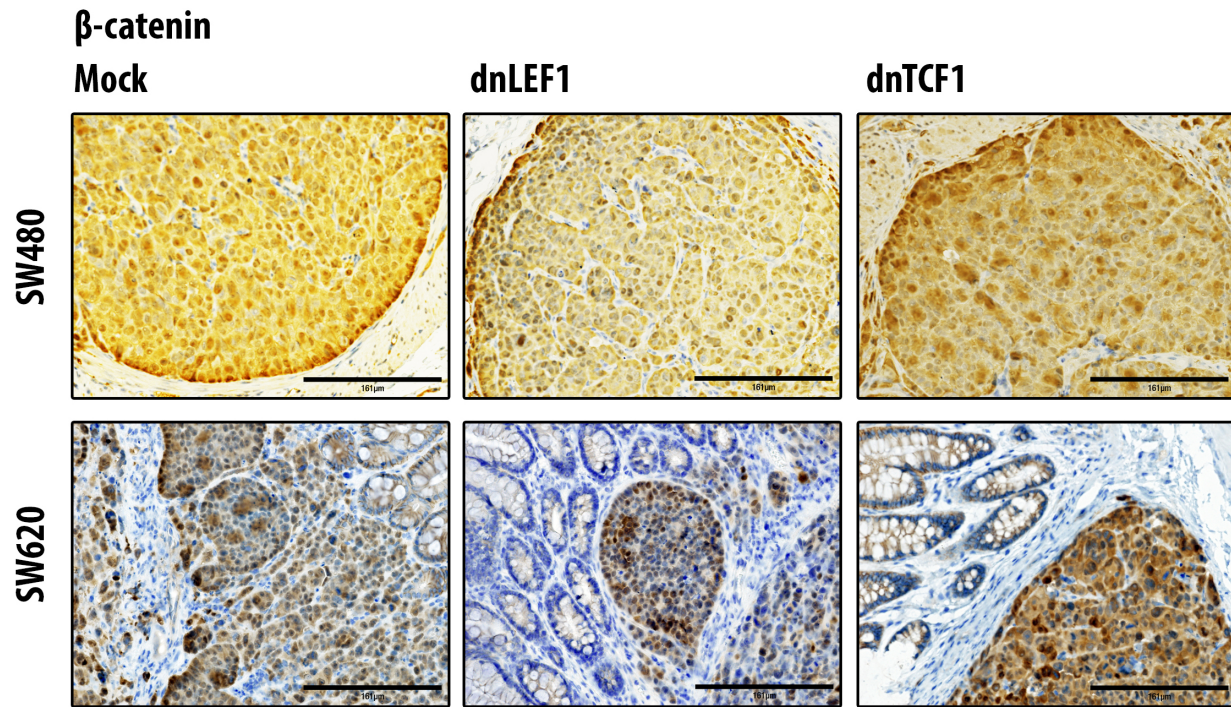
In vitro:

In the in vitro model simulations, we assessed synergy using $\bar{S}_W = 0.8$ (the same conditions for the red curve in figure 7D in the main text) and $\frac{1}{\tau_{go}} = 4$. We obtain $E_{XAV949} = 0.0156$, $E_{DCA} = 0.1855$, and $E_{XAV949+DCA} = 0.5725$. Thus $BCI = 0.3462$. The BCI depends on the drug concentrations, however. For example, taking $\bar{S}_W = 0.8$ and $\frac{1}{\tau_{go}} = 18$, we find $E_{XAV949} = .0156$ as before but now $E_{DCA} = 0.6679$, and $E_{XAV949+DCA} = 0.68$, which yields $BCI = 0.9898$. Thus, the BCI indicates that DCA and XAV939 combined treatments are more synergistic at smaller concentrations of DCA (the effect of DCA reaches a saturation point - see figure 7D in the main text).



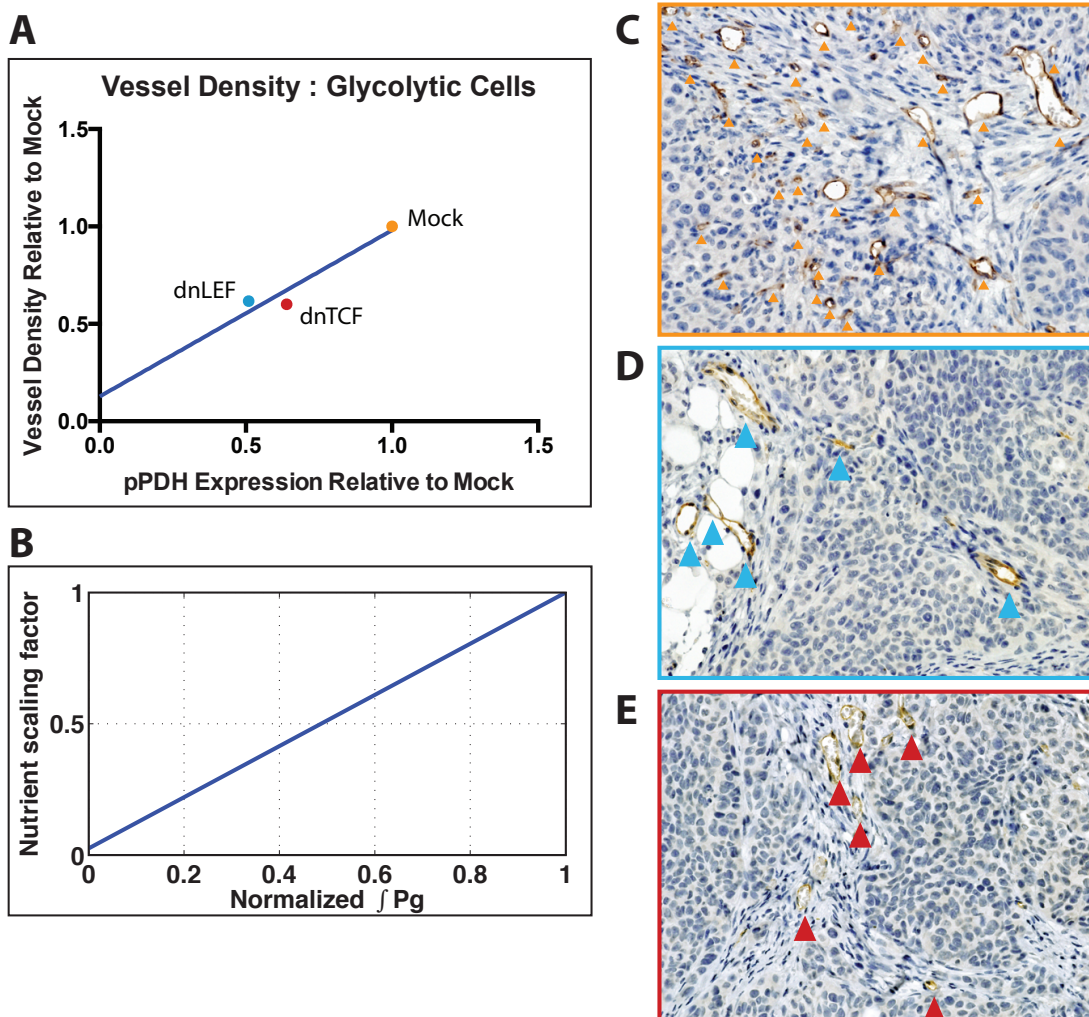
Appendix Figure S 18: Effectiveness of combination therapy. Here we plot the proportion of total tumor that is eradicated by DCA (vertical axis, $1/\tau_{go}$) and XAV939 (horizontal axis, $\overline{S_W}$) treatment, where 0 (light blue) means none of the tumor cells are killed by treatment, and 1 (dark blue) means 100% of the tumor cells have died. Pure DCA treatment (bottom row, $1/\tau_{go} = 1$) or pure XAV939 treatment (left column, $\overline{S_W} = 1$) are not at all effective in killing the tumor. Away from the bottom row and left column (as indicated by the dotted lines), the plot shows E_{AB} with $A = \text{XAV949}$ and $B = \text{DCA}$. The upper right area of the plot, which is a combination of low levels of DCA and XAV939 treatment together, shows that the tumor is completely killed, and hence a synergy between the two drugs.

B9 Heterogeneity in orthotopic tumors



Appendix Figure S 19: β -catenin stains in SW480 and SW620 orthotopic tumors. Patterns of Wnt signaling heterogeneity are present in orthotopic tumors of SW480 and SW620 cells. Cell lines were lentivirally transduced to express dominant negative LEF1 or TCF1 and the transduced cells were injected into the submucosal layer of the colon wall. Tumors were harvested after three weeks and stained for beta-catenin. Scalebars are 161 μ m. Decreasing Wnt signaling in SW480 tumors leads to changes in hypoxic patterning. SW480 cells lentivirally transduced to express either an empty vector or dominant negative LEF1 were subcutaneously injected into immunocompromised mice. Tumor sections were stained for HIF1 α .

B10 Vessel density versus pPDH expression in relative to Mock tumor



Appendix Figure S 20: We quantified the vasculature of the tumors by staining for CD31, an endothelial cell marker. We graphed the vessel density per field and the level of pPDH expression on the x-axis, both values normalized to Mock tumors. Mock tumors demonstrated significantly higher pPDH levels and vasculature than dnLEF or dnTCF tumors. Our mathematical model was capable of replicating the best fit line from the biological data.

APPENDIX C

Supplemental Materials for Chapter 3: Decreasing Wnt signaling activity primes colon cancer cells for invasion and metastasis

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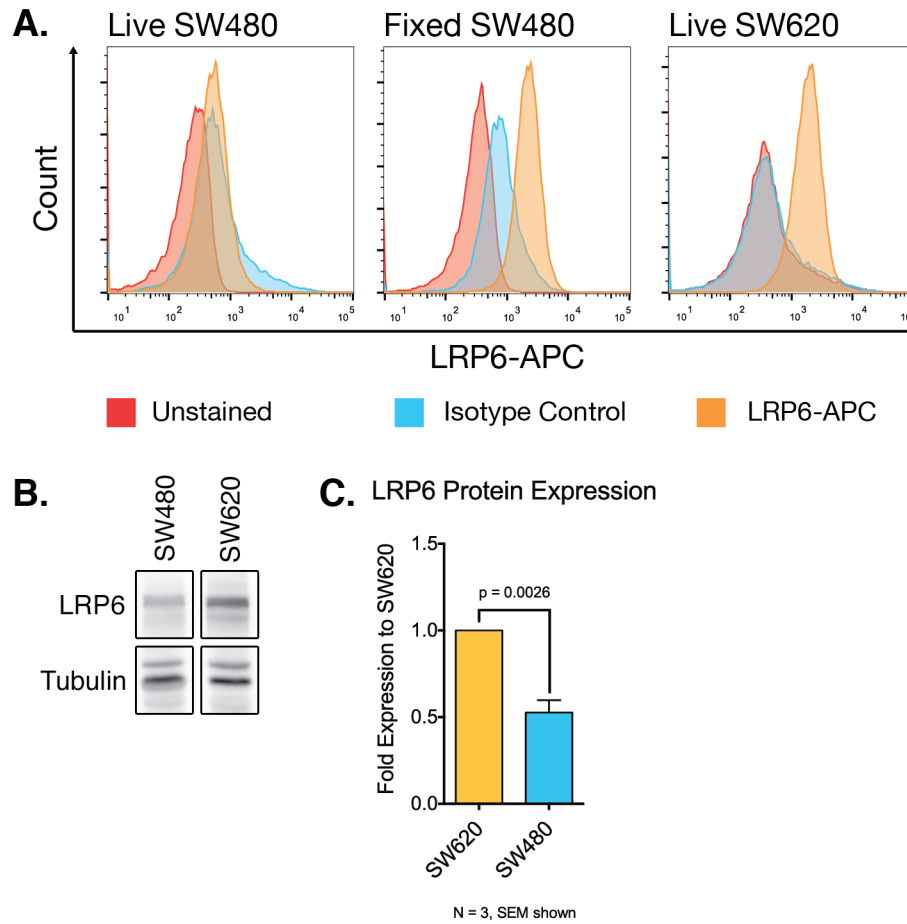
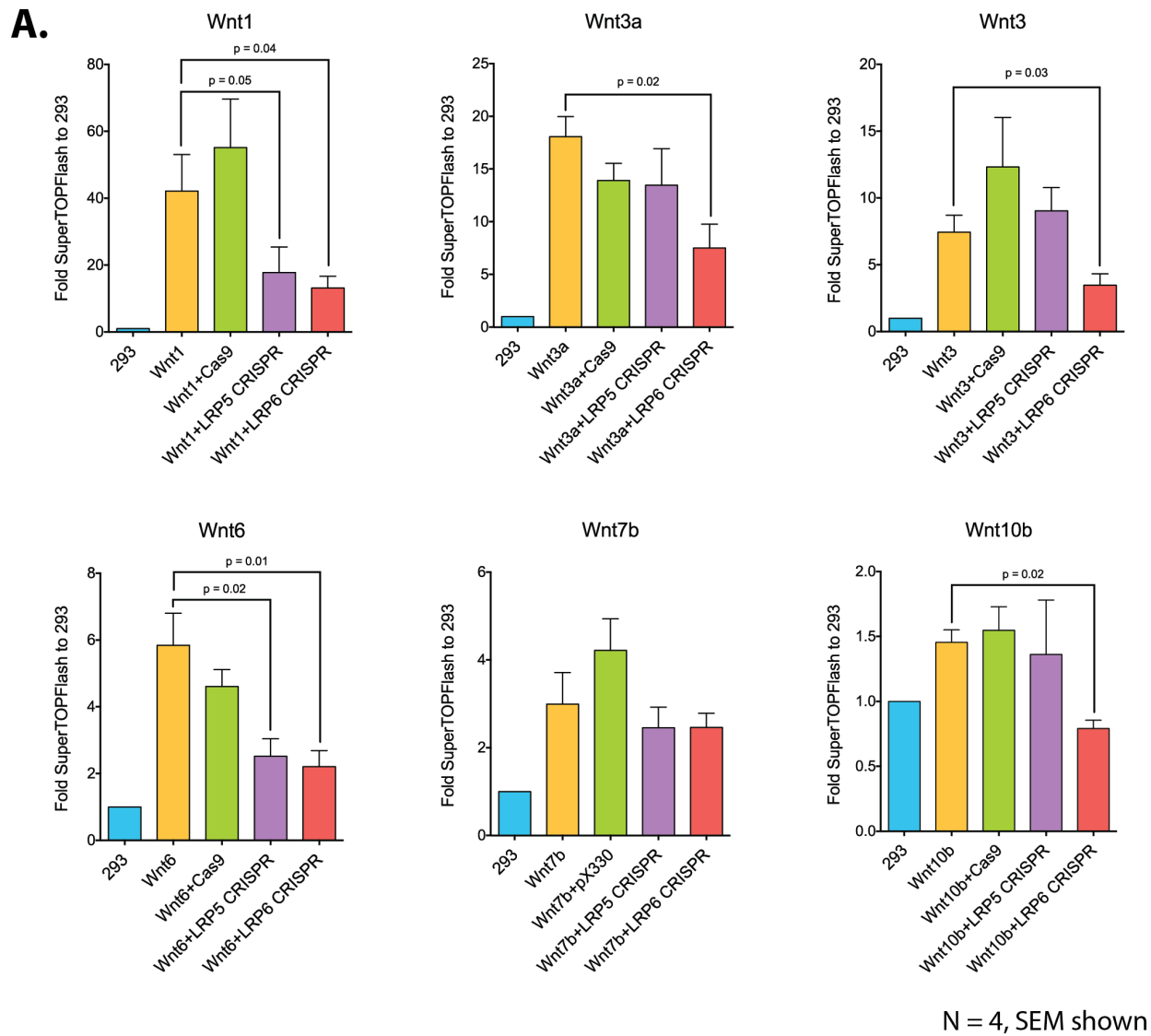


Figure A3.1. LRP6 is not expressed on the cell membrane of SW480 cells

A. Flow cytometry of SW480 and SW620 cells reveals that LRP6 is not detectable in SW480 cells, but a signal is clearly detected after cells were fixed and permeabilized, indicating LRP6 protein is present but not localized to the cell membrane. B-C. Western blot of whole cell lysates from SW480 and SW620 cells probed for LRP6 show that SW620 cells express significantly more LRP6 protein than SW480 cells.



B.

	Wnt1	Wnt3a	Wnt3	Wnt6	Wnt7b	Wnt10b
LRP5	Required for signaling			Required for signaling		
LRP6	Required for signaling	Required for signaling	Required for signaling	Required for signaling		Required for signaling

Required for signaling

Figure A3.2. Wnt ligands require LRP6 to activate Wnt signaling.

HEK293 cells were transiently transfected with a Wnt ligand and a LRP5/6 CRISPR construct. Of the ligands tested, all except Wnt7b showed a significant decrease in Wnt signaling activity with the loss of LRP6. A number of Wnt ligands also showed sensitivity to loss of LRP5, however, no Wnt ligand was solely active through LRP5. Additionally, loss of LRP5 or LRP6 decreased Wnt activity by more than 50%, suggesting that HEK293 cells were not simply switching reliance to the other LRP co-receptor. We find that these ligands require both LRP5 and LRP6 to activate signaling. B. Summary of Wnt ligand requirements for LRP5 and LRP6.

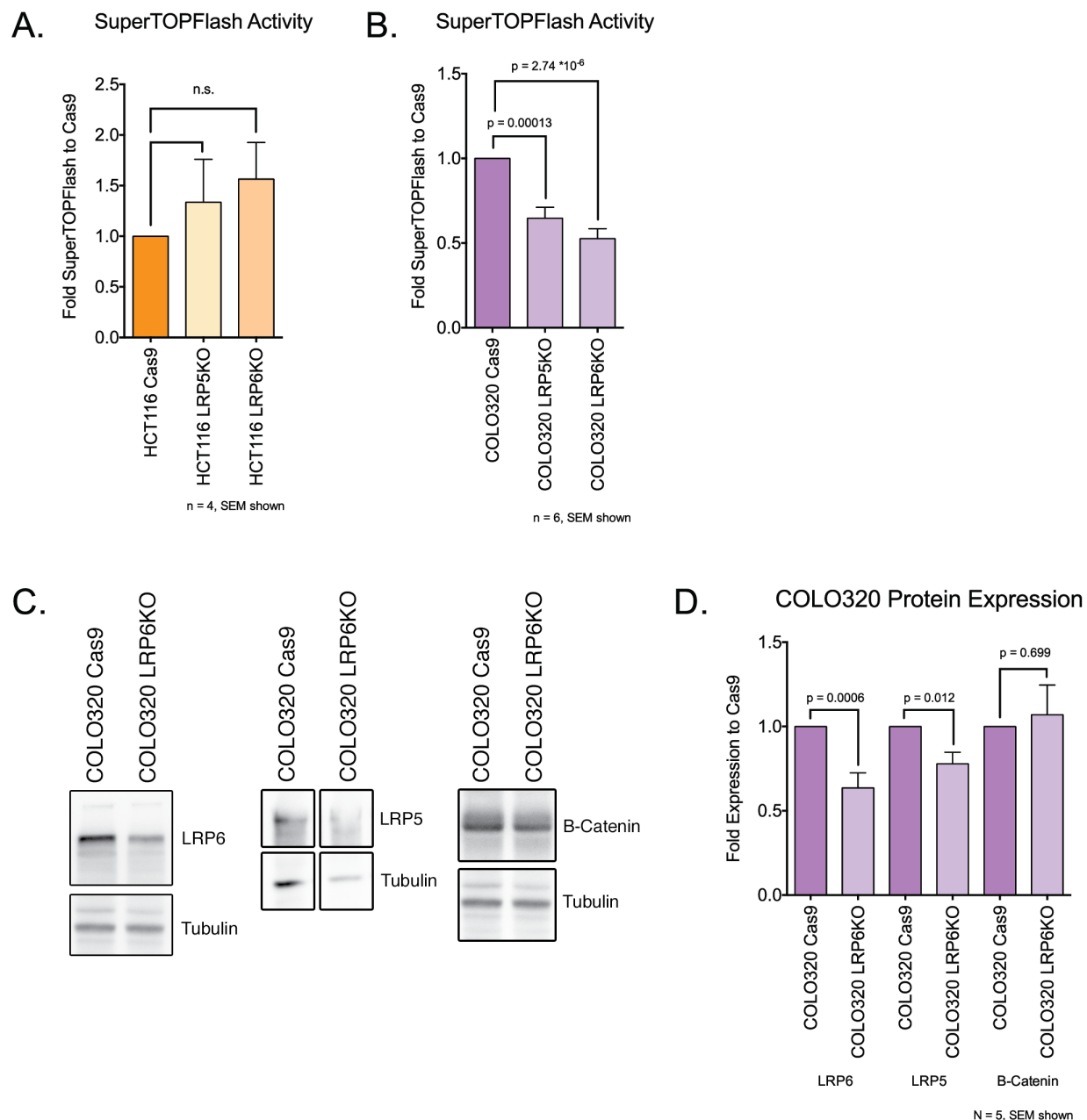


Figure A3.3. Loss of LRP6 reduces Wnt signaling in COLO320, but not HCT116 cells

A. HCT116 cells do not show a significant change to Wnt signaling activity in response to loss of LRP5 or LRP6. HCT116 cells have a mutation in b-catenin that allows the cells to evade the destruction complex in order to activate Wnt target genes. This suggests that in HCT116 cells, Wnt ligands may play a negligible role in modulating signaling activity. B. COLO320 cells show a significant decrease in Wnt signaling activity with the loss of LRP5 or LRP6. C-D. Western blots and quantitation of COLO320 Cas9 and LRP6KO cells shows a decrease in total LRP6 protein as expected. Interestingly, a loss of LRP5 is also observed with the knockout of LRP6. This is also observed in SW620 cells (See Figure A3.4). Unlike the SW620 cells, we do not find a significant decrease in b-catenin in the COLO320 LRP6KO cells.

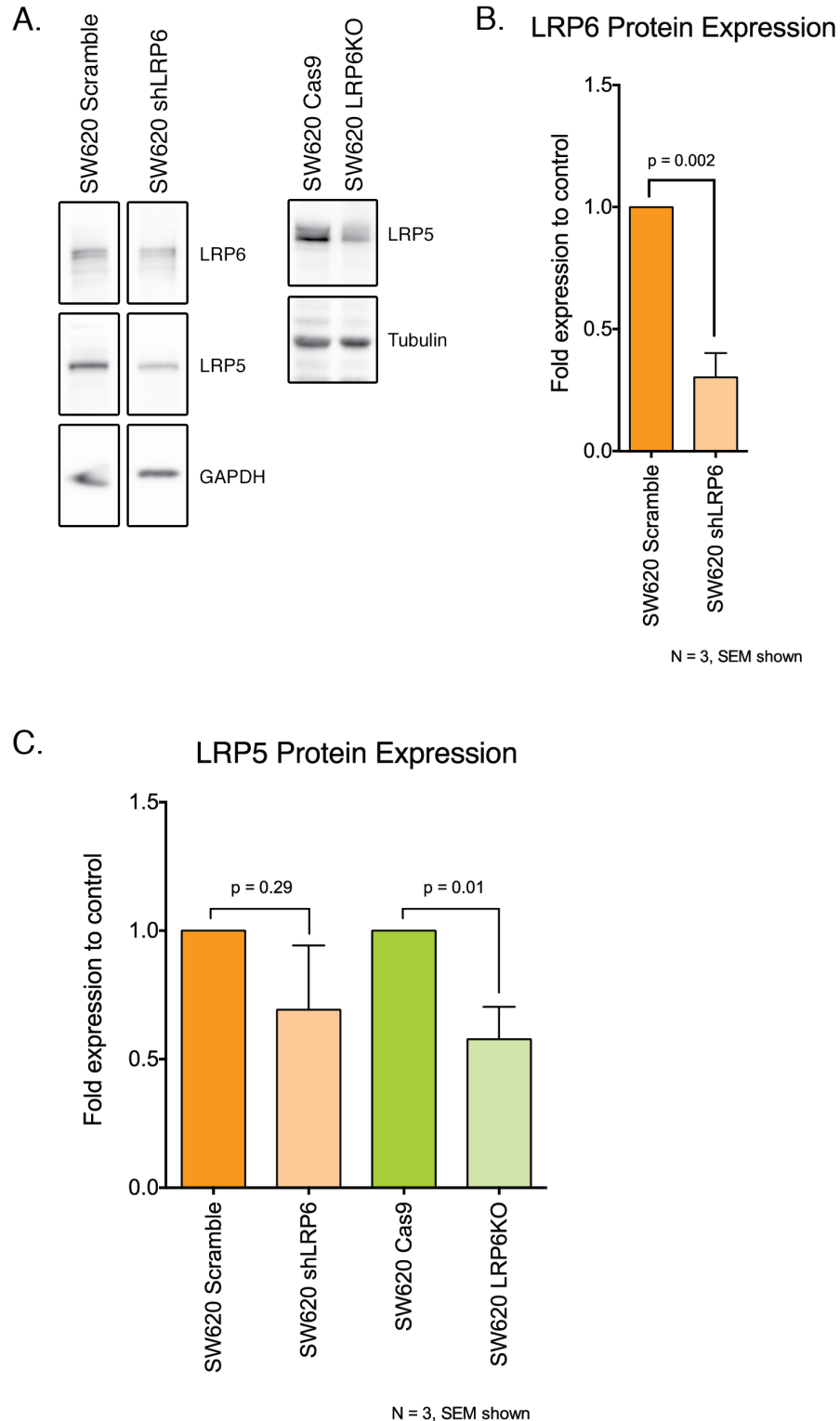


Figure A3.4. Loss of LRP6 induces a loss of LRP5 in SW620 cells

A. By Western blotting, we find a significant decrease in LRP5 protein in LRP6 knockout cells. To confirm that this was not an off-target effect of our construct, we used a lentiviral shRNA to knockout LRP6 (quantified in B). Total LRP5 was also decreased in the shRNA-transduced cells (quantification in C).

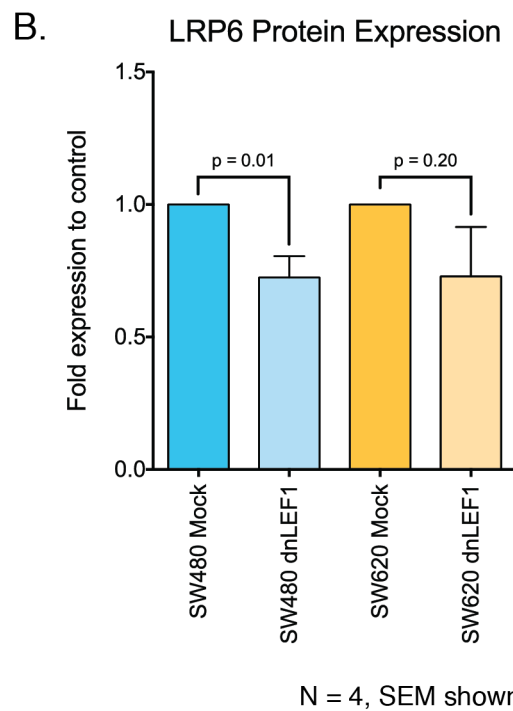
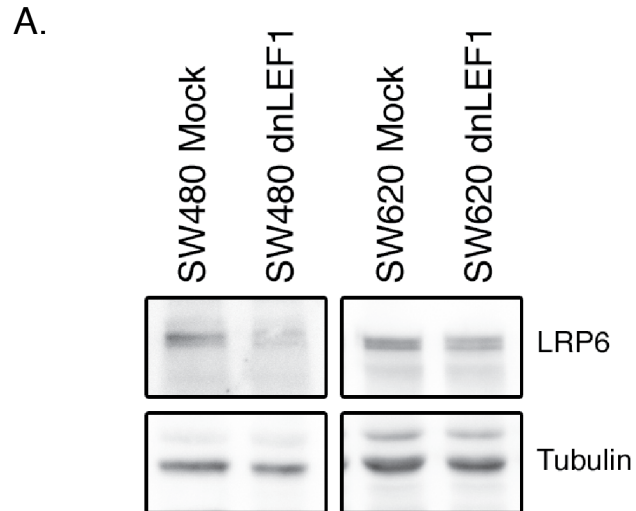
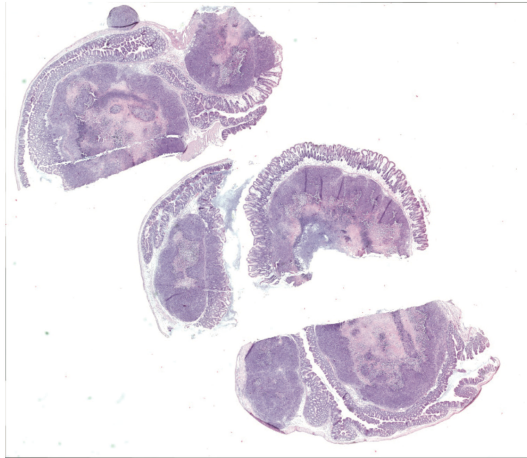


Figure A3.5. LRP6 protein expression is decreased in dnLEF1-expressing cell lines

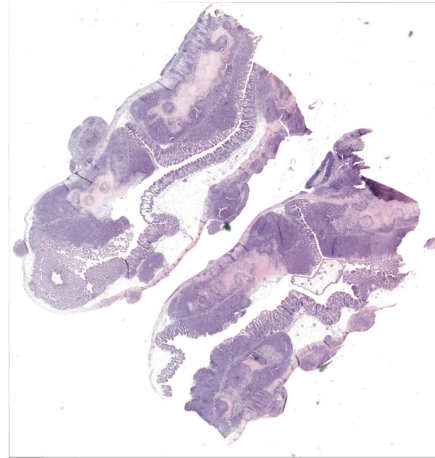
Total LRP6 protein is significantly decreased in SW480 dnLEF1 cells compared to Mock. A noticeable but not significant decrease in LRP6 is observed in SW620 dnLEF1 cells.

Mouse ID	Cell Line	Expression Vector
13-38	SW620	Mock
13-39	SW620	dnLEF1
14-01	SW480	Mock
14-02	SW480	dnLEF1
8760	SW620	dnLEF1
8761	SW620	Mock
8765	SW620	Mock
8767	SW620	dnLEF1
8768	SW620	dnLEF1
8721	COLO320	LRP6KO
8772	SW620	LRP6KO
8777	SW620	Cas9
8778	SW620	Cas9
8803	SW620	LRP6KO
8804	SW620	LRP6KO
8805	SW620	Cas9
8806	SW620	Cas9
8791	SW620	Cas9
8793	SW620	LRP6KO
8823	COLO320	LRP6KO
8840	COLO320	Cas9
8788	SW620	LRP6KO
8790	SW620	Cas9
8773	SW620	Mock
8774	SW620	dnLEF1
8775	SW620	dnLEF1
8782	SW620	Mock
8830	SW480	Mock
8831	SW480	Mock
8835	SW480	dnLEF1
8836	SW480	dnLEF1
8837	SW480	dnLEF1
8839	SW480	Mock
8825	COLO320	LRP6KO
8827	COLO320	Cas9
8849	COLO320	Cas9
8850	COLO320	LRP6KO

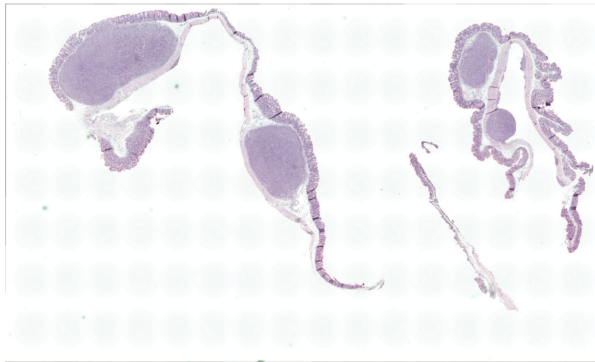
Figure A3.6. Orthotopic tumor sections for scoring



13-38



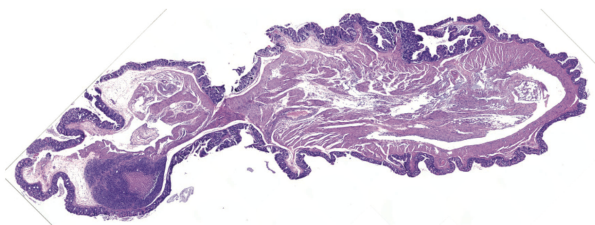
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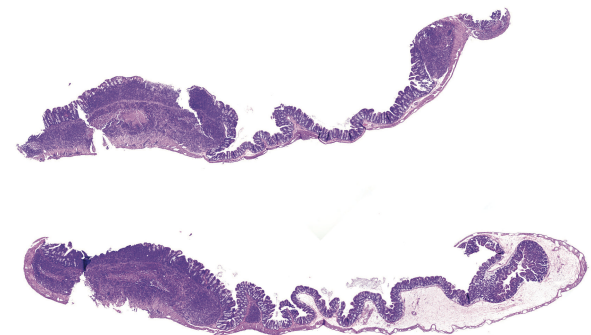
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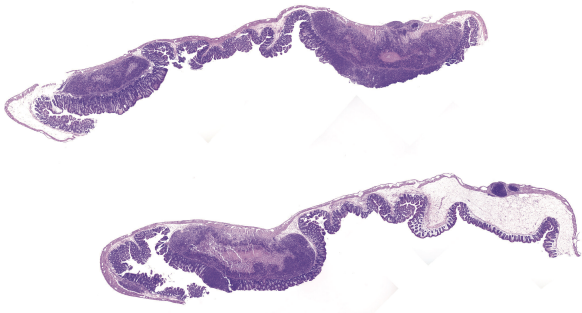


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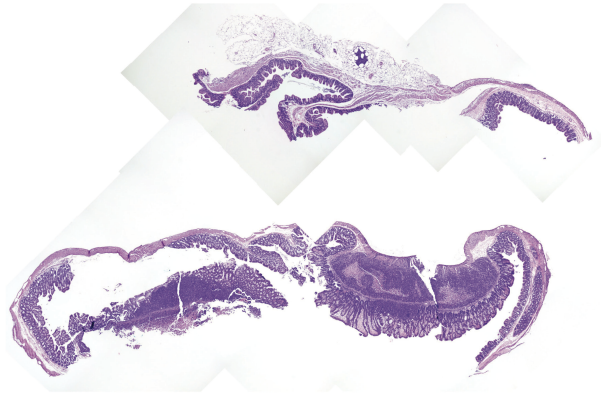


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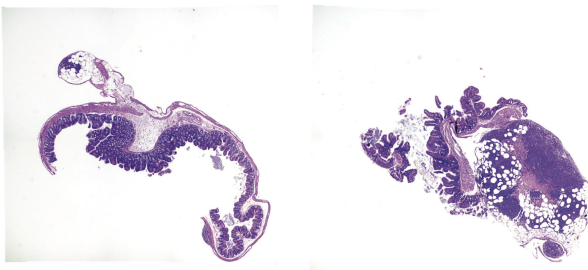
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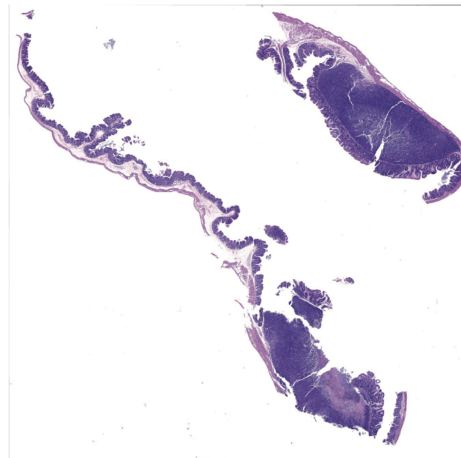
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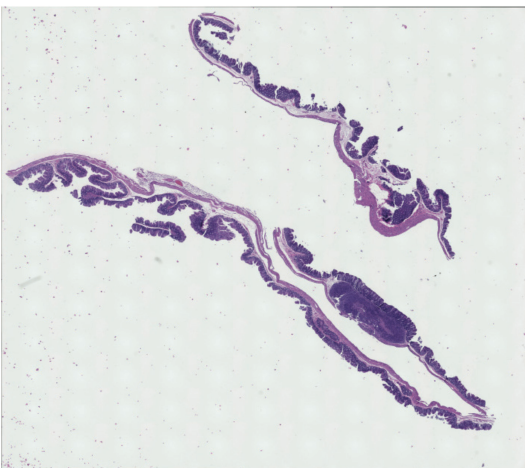
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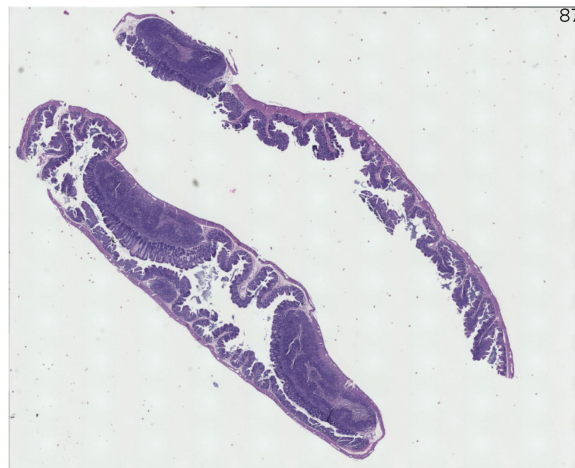
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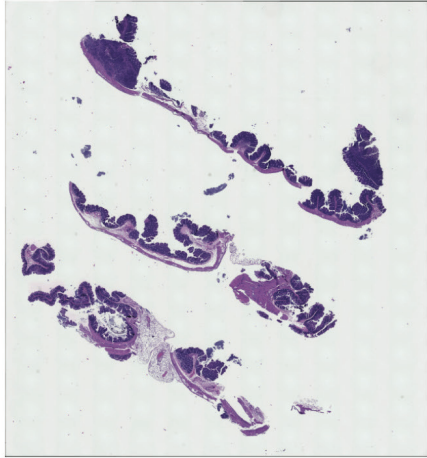


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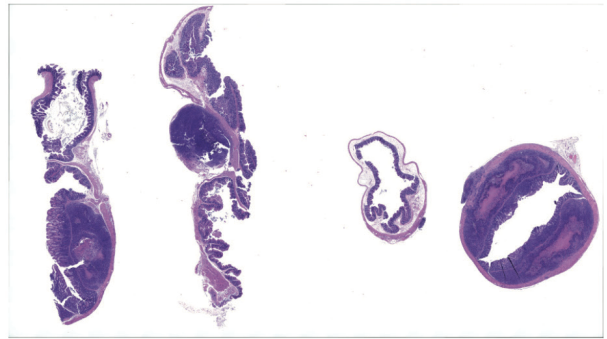


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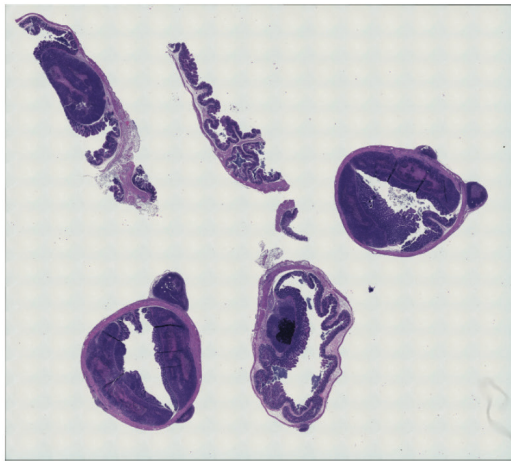




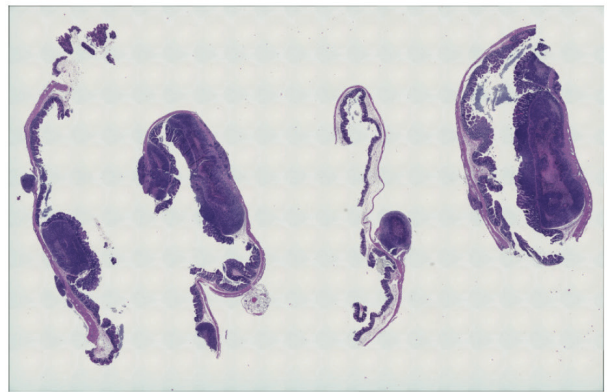
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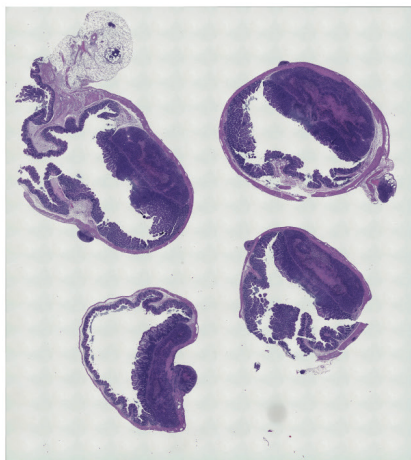
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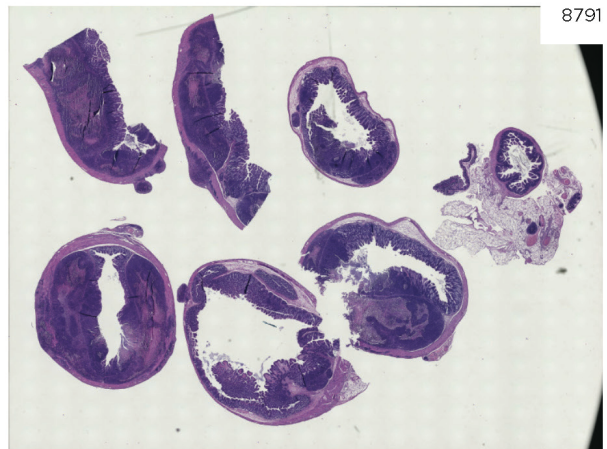
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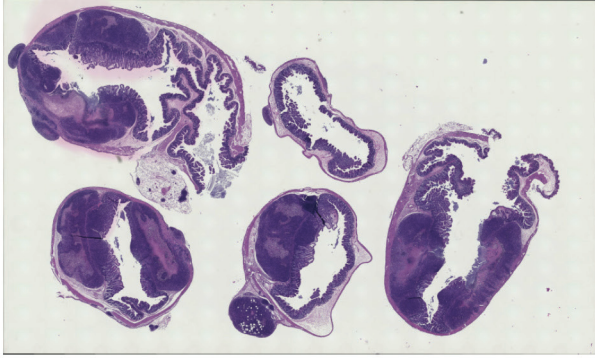


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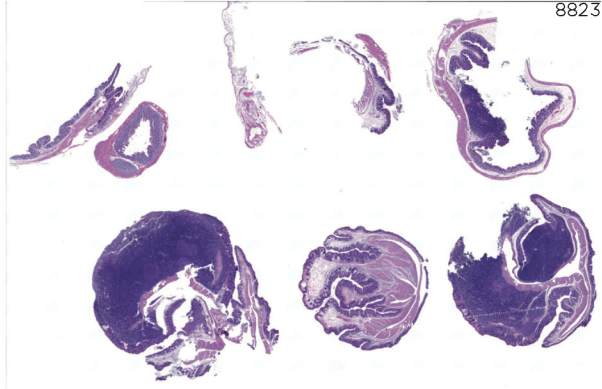


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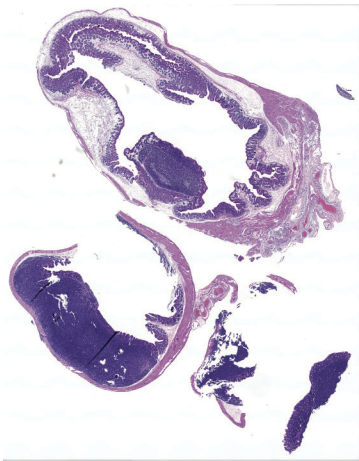
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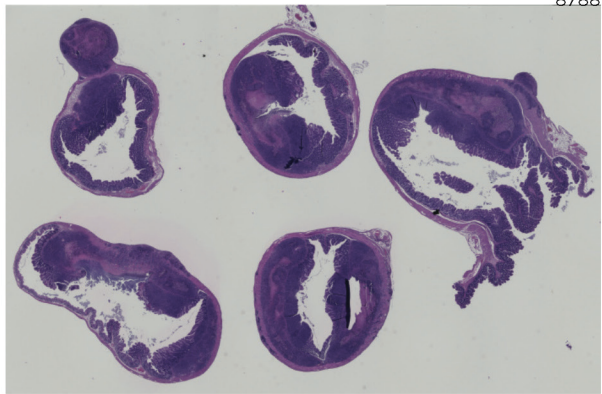
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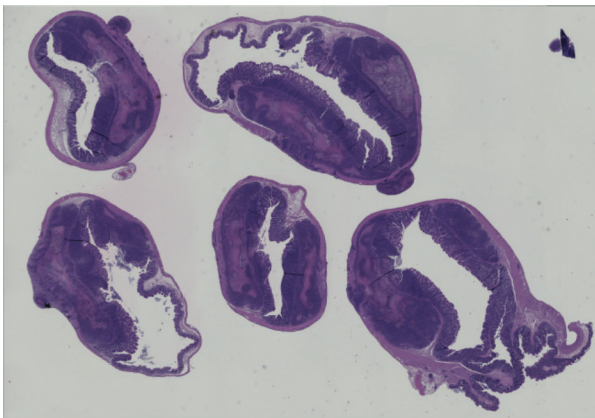
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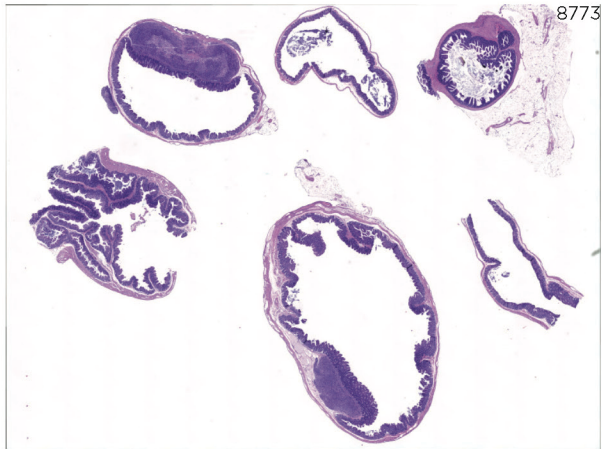
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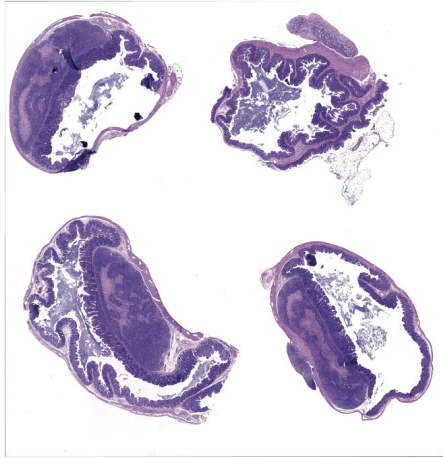


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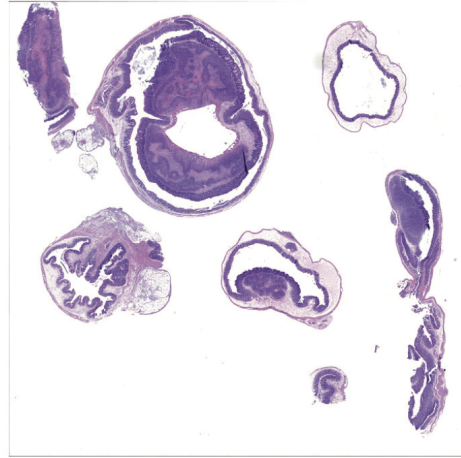


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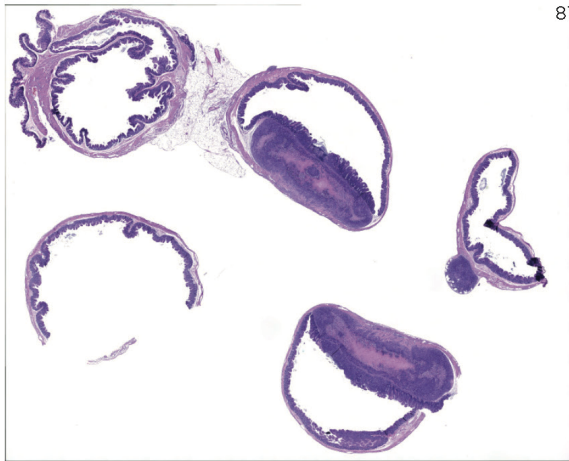




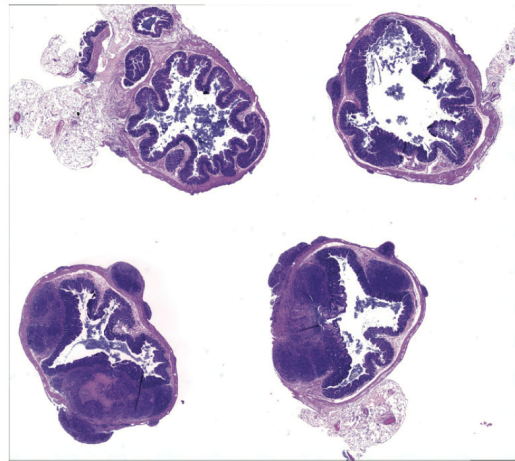
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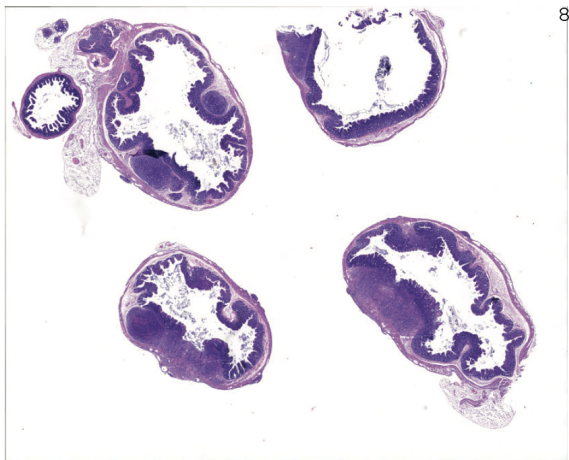
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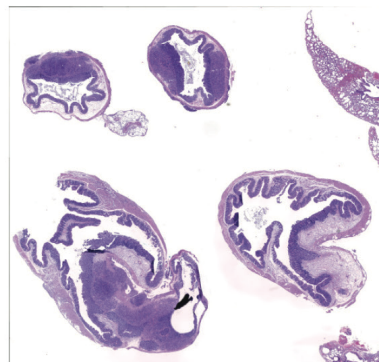
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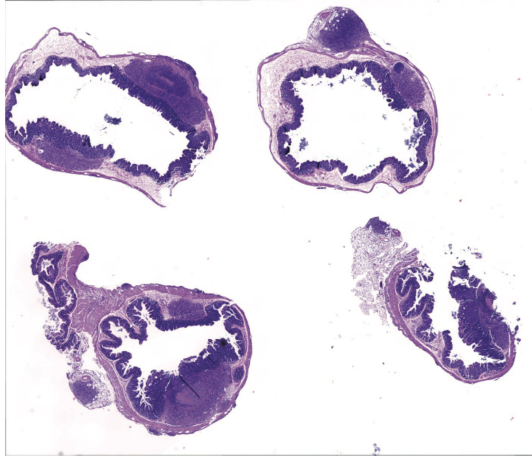
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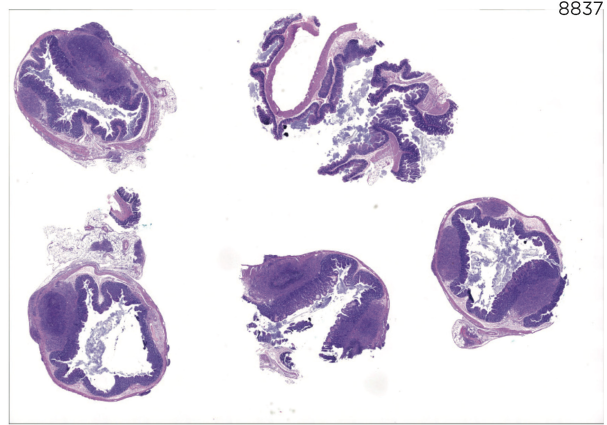
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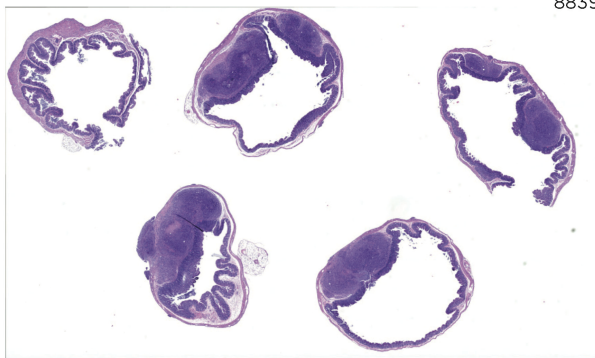
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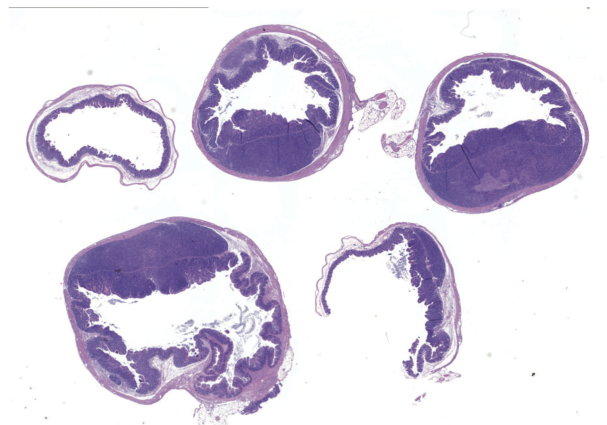
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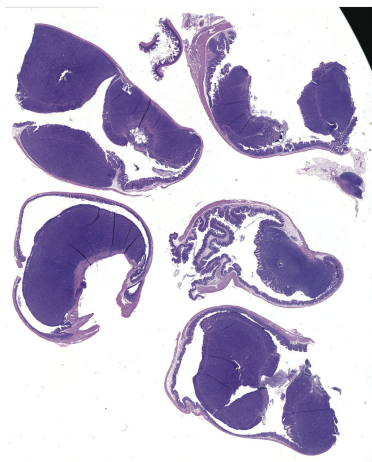
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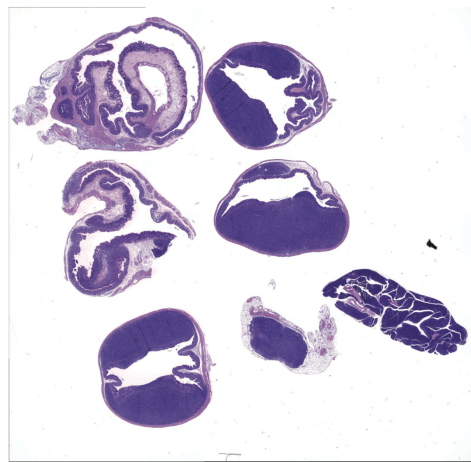
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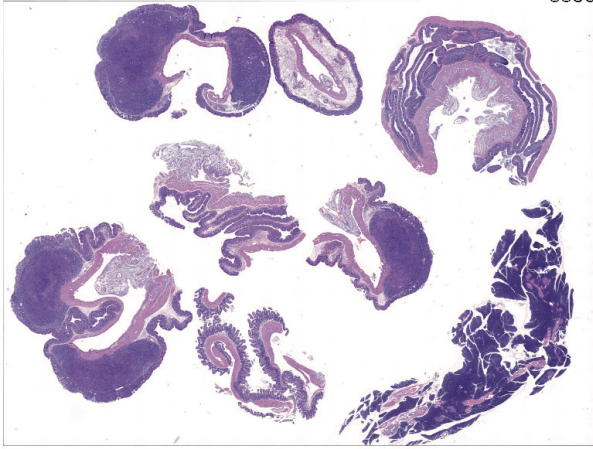


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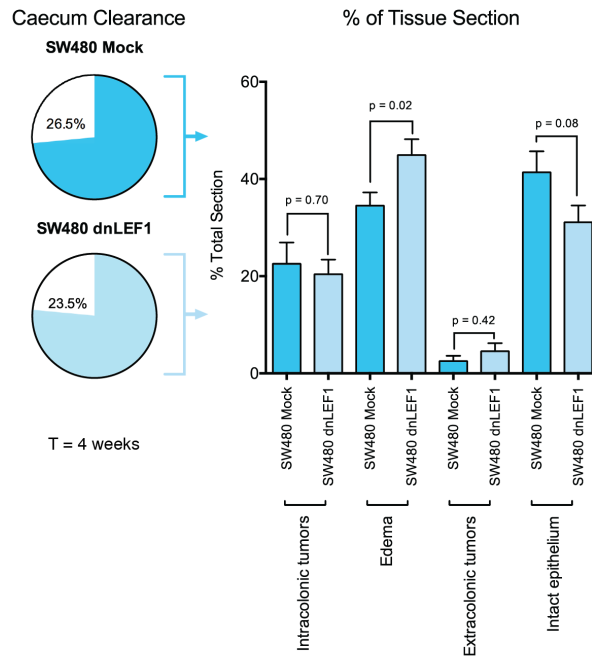


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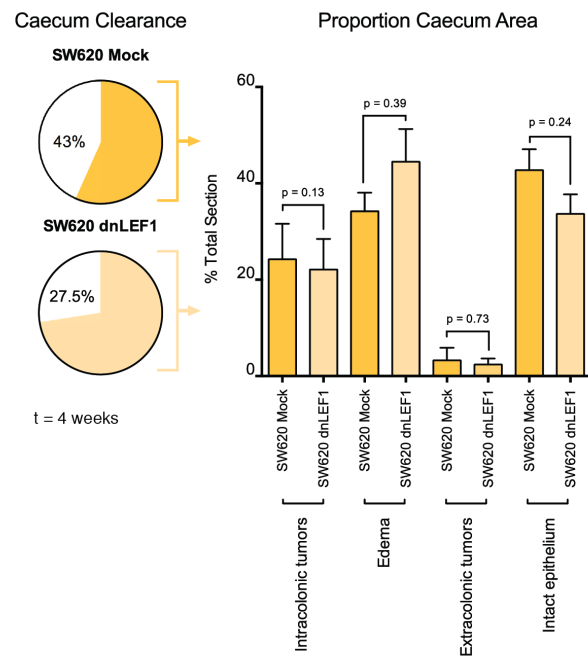
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A. SW480 Mock & SW480 dnLEF1



B. SW620 Mock & SW480 dnLEF1



C. SW620 Cas9 & SW620 LRP6KO

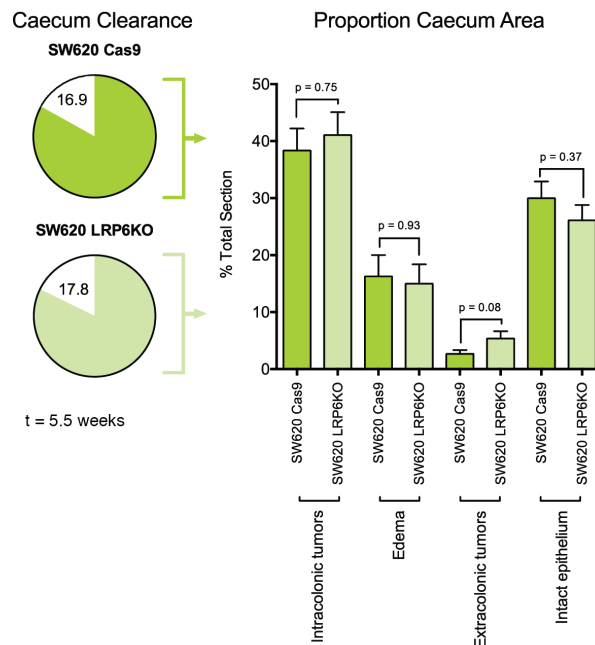


Figure A3.7. Quantification of orthotopic tumors

A. Percent of lumen clearance shows no significant difference between SW480 Mock and SW480 dnLEF1. A significant increase in edema and a decrease in intact epithelia are seen in SW480 dnLEF1. B. Percent of lumen clearance shows a significant decrease in cleared area in SW620 dnLEF1 compared to SW620 Mock. An increase in edema and a decrease of intact epithelia are seen in SW480 dnLEF1, but not to significance. C. No significant difference in lumen clearance observed in SW620 LRP6KO, but an increase in extracolonic tumors is observed.

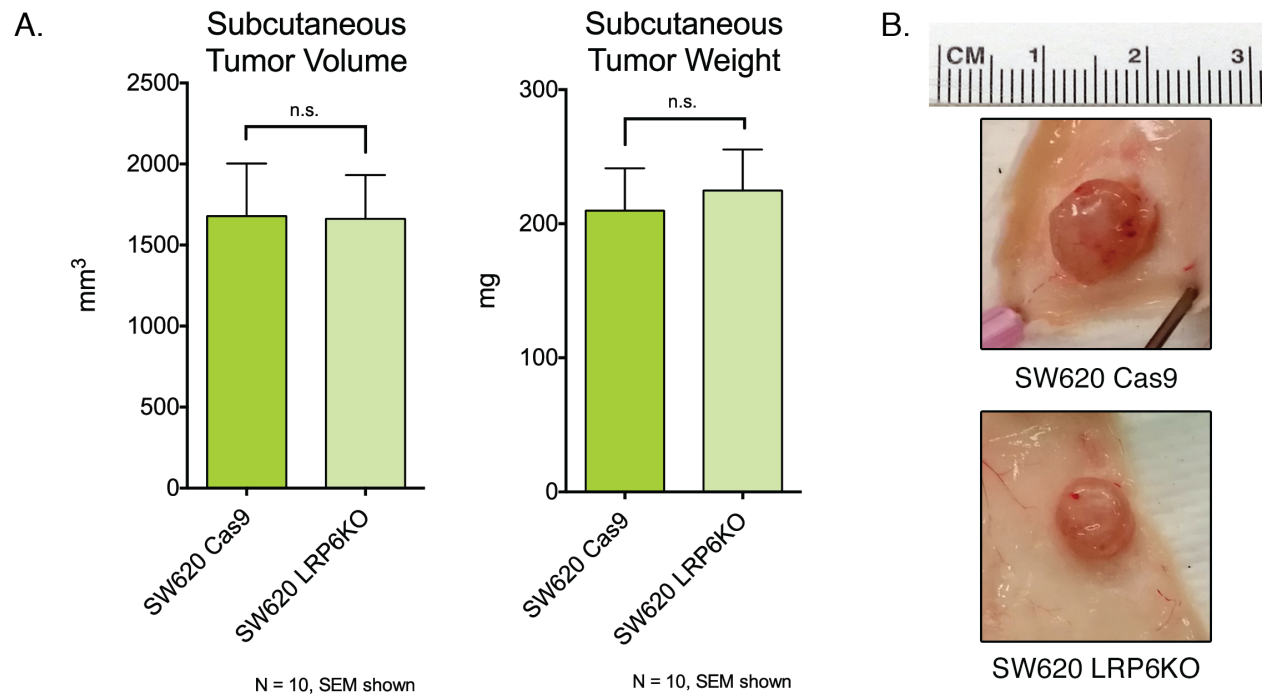


Figure A3.8. Subcutaneous tumors of SW620 Cas9 and LRP6KO

A. No significant difference in tumor volume and weight is seen between SW620 Cas9 and SW620 LRP6KO. B. Representative images of tumors at harvest.

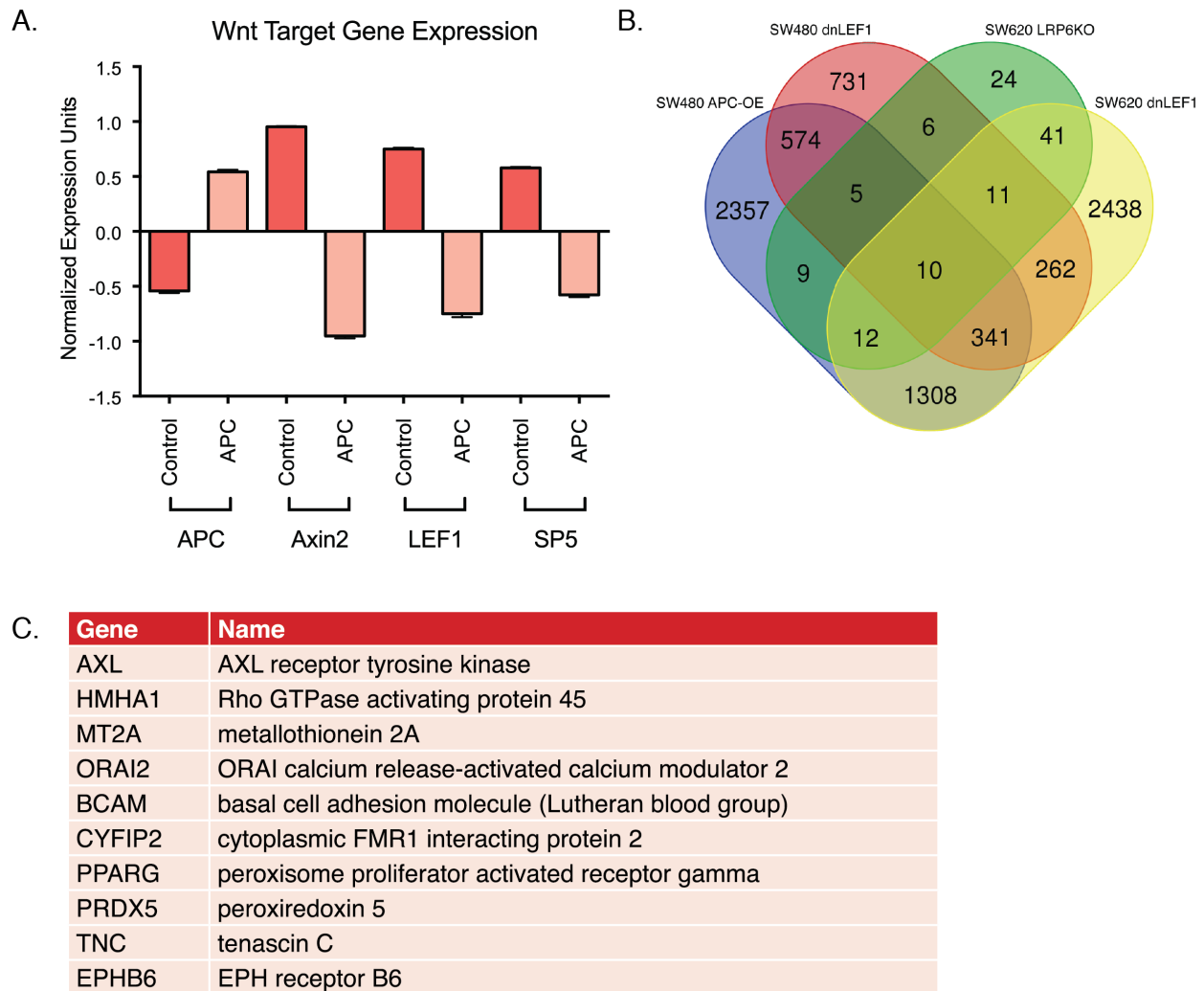
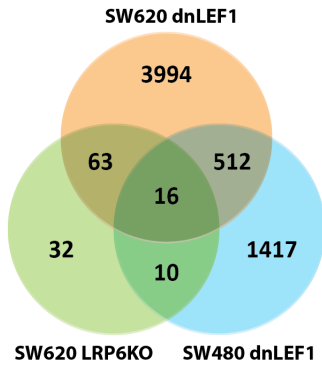


Figure A3.9. Overexpression of wildtype APC in SW480 cells leads to upregulation of genes associated with cell invasion.

A. Sequencing data from King, et al (NCBI GSE76307). APC overexpression in SW480 decreases known Wnt target genes *AXIN2*, *LEF1*, and *SP5*. B. Overlap of significantly upregulated genes from SW480 APC-overexpression, SW480 dnLEF1, SW620 LRP6KO, SW620 dnLEF1 shows 10 genes in common between all four cell types. C. Ten commonly upregulated genes.

A.



B.

Gene	Gene Name	Function
SEL1L3	SEL1L family member 3	Unclear function
MAN1A1	mannosidase alpha class 1A member 1	Transmembrane protein in golgi, metabolism of proteins
CA8	carbonic anhydrase 8	Lacks carbonic anhydrase activity (ironic!)
SCML1	Scm polycomb group protein like 1	DNA binding
KRT23	keratin 23	KRT23 KO decreased proliferation and affects DNA damage response in CRC cells. Marker of differentiated cells
NEBL	nebulette	Mutations associated with altered cardiac myofibril assembly and function
RCN1	reticulocalbin 1	Ca binding protein in ER, localizes to plasma membrane in endothelial cancer cell lines
CPLX2	complexin 2	Cytosolic proteins, part of synaptic vesicle exocytosis
DPEP1	dipeptidase 1	Membrane enzyme involved in metabolism of glutathione
HSPA12A	heat shock protein family A (Hsp70) member 12A	Response to heat stress
TC2N	tandem C2 domains, nuclear	Calcium ion binding
CD24	CD24 molecule	Expressed on granulocytes, B cells. Modulates growth and differentiation signals. In differentiated cells in normal colon
PLK2	polo like kinase 2	Role in cells undergoing rapid cell division
SSTR1	somatostatin receptor 1	Peptide hormone receptors
MAP1B	microtubule associated protein 1B	Microtubule assembly
RNF128	ring finger protein 128, E3 ubiquitin protein ligase	E3 ubiquitin ligase

Figure A3.10. Commonly downregulated genes between SW480 dnLEF1, SW620 dnLEF1, and SW620 LRP6KO cells

A. Overlap between significantly downregulated genes in SW480 dnLEF1, SW620 dnLEF1, and SW620 LRP6KO found 16 genes in common. B. The sixteen genes identified do not show overarching common functions.

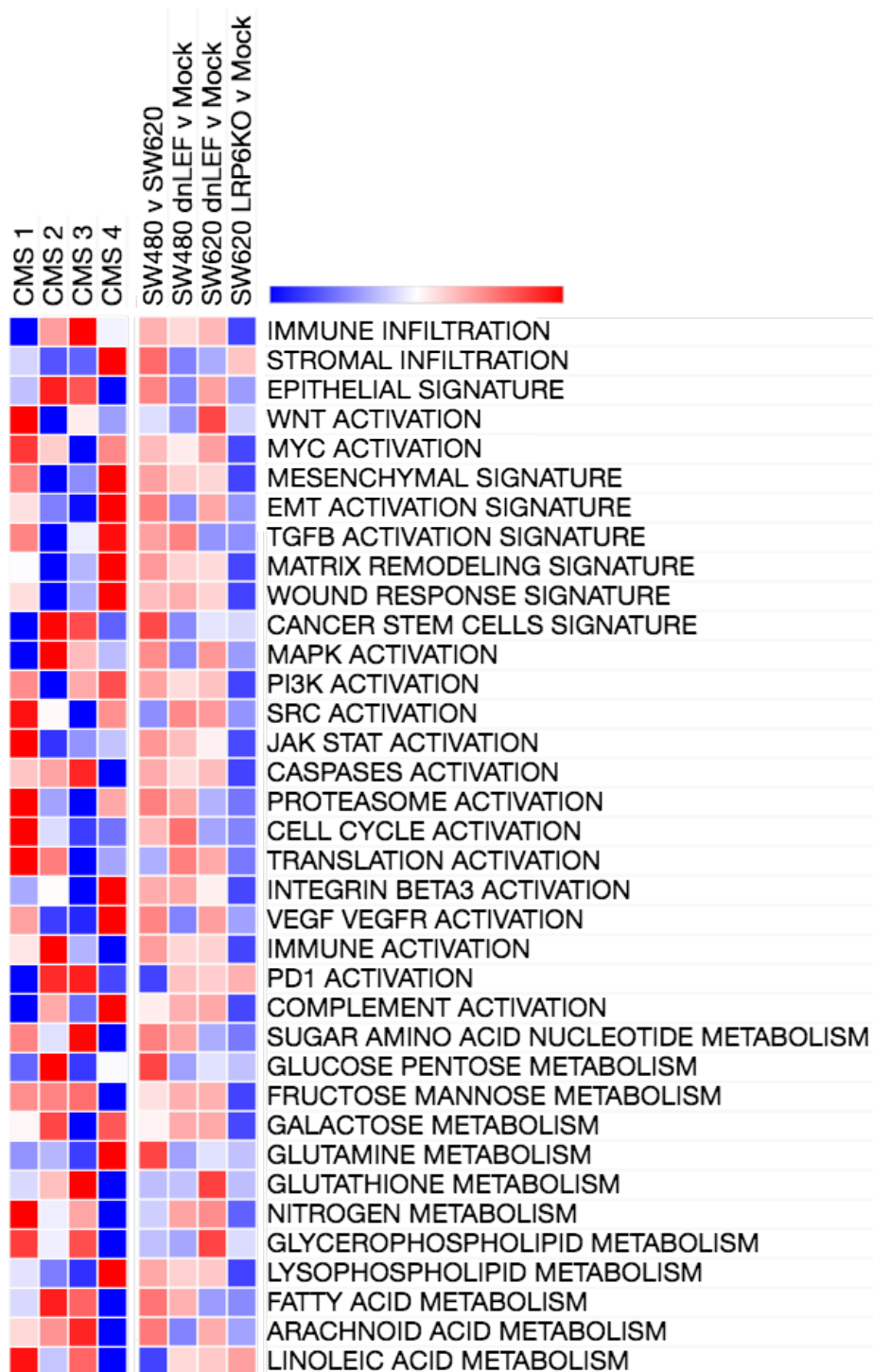


Figure A3.11. Geneset Enrichment Analysis subtypes of Wnt-reduced cell lines

Heatmap of enrichment scores for gene sets as defined in Guinney, et al to establish consensus molecular subtypes for colon cancer. Blue denotes under enrichment, red denotes over enrichment. Enrichment scores for CMS from Linnekamp et al. We find that compared to their parental lines, the dnLEF1 expressing lines trend towards CMS4. SW620 LRP6KO, due to the minor change in Wnt signaling, does not show significant enrichment compared to SW620 Cas9.