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Regulation and Therapeutic Targeting of
Wnt Signaling in Pancreatic Cancer

A dissertation submitted in partial satisfaction of the
requirements for the degree Doctor of Philosophy
in Cellular & Molecular Pathology

by

Michael Dawson Arensman

2015

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ABSTRACT OF THE DISSERTATION

Regulation and Therapeutic Targeting of Wnt Signaling in Pancreatic Cancer

by

Michael Dawson Arensman

Doctor of Philosophy in Cellular & Molecular Pathology

University of California, Los Angeles, 2015

Professor David Wayne Dawson, Chair

Pancreatic cancer, or pancreatic ductal adenocarcinoma (PDAC), is the fourth most common cause of cancer-related death and carries a dismal five-year survival rate of only 6%. Although our understanding of the molecular basis of the disease has improved over the past several decades, this increase in knowledge has not translated to improved therapeutics. PDAC is a highly heterogeneous disease, with a complex array of variable mutations, alterations of gene expression, and tumor subtypes. It is often unclear as to what is driving tumor progression and difficult to target candidate pathways. It has recently become evident that the Wnt/ β -catenin pathway is one such network that drives PDAC initiation, growth and metastasis, and that this critical developmental signaling cascade has the potential to be therapeutically targeted. This

dissertation examined the molecular mechanisms driving the Wnt/ β -catenin pathway and evaluated compounds to inhibit it in *in vitro* and *in vivo* models of PDAC. Endogenous Wnt/ β -catenin activity was found to be highly variable across PDAC cell lines and patient tumors, with WNT7B and SUMO2 identified as key activators and inhibitors of pathway activity, respectively. WNT7B activation of Wnt/ β -catenin signaling mediated an anchorage-independent growth phenotype, suggesting that this pathway represents a rational target for pharmacologic manipulation. Accordingly, the CBP/ β -catenin inhibitor ICG-001 effectively prolonged survival through induction of G1 arrest in PDAC models. However, ICG-001 inhibition of growth could not be attributed to effects on the Wnt signaling pathway, rather it acted through direct modulation of key regulators of G1/S transition. Alternatively, the vitamin D analog calcipotriol was investigated as a potential Wnt/ β -catenin inhibitor. Calcipotriol inhibited PDAC proliferation and Wnt/ β -catenin activity through upregulation of LDLRAP1 and destabilization of the Wnt co-receptor LRP6. Vitamin D receptor was also identified as a potential biomarker to predict and monitor response to vitamin D-based or Wnt/ β -catenin-targeted therapeutics. This dissertation provided valuable insights towards mechanisms regulating PDAC aggressiveness and characterized compounds currently under clinical evaluation for the treatment of PDAC.

The dissertation of Michael Dawson Arensman is approved.

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2015

DEDICATION

This work is dedicated to Dawson Teaforde, whose life was cut short by cancer,
and to all those who are dedicated to solving untreatable diseases.

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Calcipotriol targets LRP6 to inhibit Wnt signaling in pancreatic cancer. Mol Cancer Res. 2015. *Submitted Manuscript*

Arensman MD, Telesca D, Lay AR, Kershaw KM, Wu N, Donahue TR, et al. The CREB-binding protein inhibitor ICG-001 suppresses pancreatic cancer growth. Mol Cancer Ther. 2014;13:2303-14.

Arensman MD, Kovochich AN, Kulikauskas RM, Lay AR, Yang PT, Li X, et al. WNT7B mediates autocrine Wnt/beta-catenin signaling and anchorage-independent growth in pancreatic adenocarcinoma. Oncogene. 2014;33:899-908.

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REGULATION AND THERAPEUTIC TARGETING OF WNT SIGNALING IN PANCREATIC CANCER

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC), commonly referred to as pancreatic cancer, originates from the acinar cells in the pancreas which undergo acinar to ductal metaplasia (ADM) and pass through several stages of pre-malignant pancreatic intraepithelial neoplasias (PanINs)(1). PanINs ultimately progress to invasive adenocarcinoma and often metastasize to local lymph nodes, the liver, and other distant sites(2). During PanIN progression, mutations in key oncogenes and tumor suppressors including *KRAS*, *CDKN2A*, *TP53*, and *SMAD4* are commonly acquired(1, 3). However, PDAC becomes an incredibly heterogeneous disease, with an average of 63 genetic alterations per tumor(4) and innumerable changes in gene expression resulting in a myriad of tumor subtypes(5). While it remains unclear whether individual alterations are drivers or merely passengers of the disease, evidence is accumulating that the Wnt/ β -catenin pathway promotes tumorigenesis and may be a druggable target.

The Wnt/ β -catenin pathway, commonly referred to as the canonical Wnt pathway, is a critical developmental signaling cascade that is involved in nearly every aspect of embryonic development, especially axis patterning, as well as adult tissue homeostasis including maintenance of the colonic crypt and hair follicle(6, 7). In the absence of the Wnt ligand, constitutively expressed β -catenin is phosphorylated by components of the destruction complex, including APC, GSK3, Axin1/2 and CK1. Phosphorylation of β -

catenin leads to ubiquitination and subsequent degradation in proteasomes. Wnt ligands, of which there are 19, are palmitoylated and secreted by Porcupine and WLS, respectively. Wnt ligands, acting through either autocrine or paracrine signaling, bind to the Frizzled (Fz) receptors which cooperate with LRP5/6 coreceptors. Upon ligand binding a phosphorylation cascade is initiated involving LRP6, CK1, GSK3, Axin and Dsh, resulting in inactivation of the destruction complex and stabilization of β -catenin. β -catenin translocates to the nucleus where it interacts with TCF/LEF family transcription factors as well as other transcriptional co-activators including CBP, and activates the transcription of target genes. Multiple mechanisms exist to render cells more or less susceptible to Wnt signaling, including direct inhibitors (i.e., DKK1, sFRP1 and RNF43) and activators (i.e., RSPO2 and LGR5)(7-10), as well as cross talk with other signaling pathways such as Notch, Hedgehog and ATDC(11).

While Wnt signaling is one of the core signaling pathways mutated in PDAC(4), these mutations do not appear in key components of the pathway. For example, APC and CTNNB1 are commonly mutated in colon cancer resulting in hyper-activated canonical Wnt signaling. However, these mutations are rare in PDAC, and levels of Wnt/ β -catenin activation are variable across patient samples(11). While it is unclear what is driving Wnt/ β -catenin signaling in PDAC, mounting evidence suggests that this pathway is a driver of the disease. For example, knockdown of β -catenin in PDAC cell lines inhibits proliferation *in vitro*(12) and inhibition of Wnt signaling prevents PanIN formation *in vivo*(13). Therefore it is prudent to further explore the regulation and possibility of targeting Wnt signaling for the treatment of PDAC.

New treatments are desperately needed for PDAC as it carries one of the worst prognoses of all cancers, with an overall mean survival of only about 6 months(14) and a 5-year survival rate of about 6% in the United States(15). Surgical resection can significantly improve lifespan, but greater than 80% of patients present with locally advanced or metastatic disease which renders them surgically inoperable(1). Not only is PDAC aggressive in nature, it is also characteristically resistant to current cytotoxic chemotherapy. For example, the standard-of-care Gemcitabine only improves median survival by about 6 months(16), while newer combination therapies extend median overall survival to at most to one year(17). Failure of chemotherapy and the inability to target the most common driver, KRAS(18), suggests that PDAC must not be treated as a single disease but that therapeutics must be designed to target tumors based on their reliance on specific signaling pathways, such as Wnt/ β -catenin.

This dissertation seeks to better understand the regulators of Wnt signaling in PDAC as well as our ability to specifically target this pathway. Wnt7B was identified as the individual ligand driving activation of Wnt/ β -catenin signaling while SUMO2-modification was found to be a general inhibitor of the Wnt pathway in PDAC. Identification of these mediators will allow for the better design of Wnt-targeted therapeutics. The existing Wnt/ β -catenin inhibitors ICG-001 and calcipotriol were also investigated. ICG-001 effectively arrested PDAC growth *in vitro* and *in vivo*, but its growth inhibitory effects were found to be unrelated to its suppression of Wnt signaling. Calcipotriol, the vitamin

D analog, inhibited growth and Wnt activity to a similar extent as other tumor types, but we were able to uncover a novel mechanism of Wnt/ β -catenin pathway regulation. Insights towards these compounds will better guide future trials on how to administer ICG-001 and calcipotriol alone and in combination with other therapeutics. While significant work is still needed to improve the management of pancreatic cancer, targeting of the Wnt pathway, if applied correctly, remains a viable option for a subset of individuals with this devastating disease.

CHAPTER 1: WNT7B MEDIATES AUTOCRINE WNT/ β -CATENIN SIGNALING AND ANCHORAGE-INDEPENDENT GROWTH IN PANCREATIC DUCTAL ADENOCARCINOMA

Abstract

Developmental and cancer models show Wnt/ β -catenin-dependent signaling mediates diverse phenotypic outcomes in the pancreas that are dictated by context, duration and strength of activation. While generally assumed to be pro-tumorigenic, it is unclear to what extent dysregulation of Wnt/ β -catenin signaling impacts tumor progression in pancreatic adenocarcinoma (PDAC). In the present study, Wnt/ β -catenin activity was characterized across a spectrum of PDAC cell lines and primary tumors. Reporter and gene expression-based assays revealed wide heterogeneity in Wnt/ β -catenin transcriptional activity across PDAC cell lines and patient tumors, as well as variable responsiveness to exogenous Wnt ligand stimulation. An experimentally generated, pancreas-specific gene expression signature of Wnt/ β -catenin transcriptional activation was used to stratify pathway activation across a cohort of resected, early-stage PDAC tumors (N=41). In this cohort, higher Wnt/ β -catenin activation was found to significantly correlate with lymphovascular invasion and worse disease-specific survival (median survival time 20.3 versus 43.9 months, log-rank P=0.03). Supporting the importance of Wnt ligand in mediating autocrine Wnt signaling, Wnt/ β -catenin activity was significantly inhibited in PDAC cell lines by WLS gene silencing and the small-molecule inhibitor IWP-2, both of which functionally block Wnt ligand processing and secretion.

Transcriptional profiling revealed elevated expression of WNT7B occurred in PDAC cell

lines with high levels of cell autonomous Wnt/ β -catenin activity. Gene-knockdown studies in AsPC-1 and HPAF-2 cell lines confirmed WNT7B-mediated cell autonomous Wnt/ β -catenin activation, as well as an anchorage-independent growth phenotype. Our findings indicate WNT7B can serve as a primary determinant of differential Wnt/ β -catenin activation in PDAC. Disrupting the interaction between Wnt ligands and their receptors may be a particularly suitable approach for therapeutic modulation of Wnt/ β -catenin signaling in PDAC and other cancer contexts where Wnt activation is mediated by ligand expression rather than mutations in canonical pathway members.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is a highly lethal cancer noteworthy for its rapid clinical progression and resistance to therapy (19). While there has been recent significant progress in our understanding of the cellular and molecular basis of PDAC (2, 20), this knowledge has not yet translated into significant improvements in clinical outcomes. PDAC incidence has been slowly rising, while 5-year overall survival is 6% (21). A primary complicating factor is the remarkable genetic and epigenetic heterogeneity of pancreatic cancer (4, 22, 23). High throughput sequencing of the human PDAC exome reveals individual tumors average 63 genetic mutations, including a small number of high frequency mutations and a much larger number of heterogeneous lower frequency mutations (4). Interestingly, these highly variable alterations also define a core set of twelve signaling pathways and cellular processes in most PDAC tumors, including Wnt signaling (4).

The Wnt signaling pathway is highly conserved throughout vertebrate species and plays critical roles in development, tissue self-renewal and human diseases, including cancer (24). Wnts are secreted glycoproteins able to act as short-range ligands to activate receptor-mediated signaling via Frizzled (Fzd) receptors and LRP5/6 co-receptors or other novel receptor complexes. Numerous additional extracellular and intracellular proteins further transduce or regulate Wnt signaling (24-26). Multiple Wnt and Fzd isoforms exist and are variably expressed spatially and temporally during development and in adult organs. This complex pattern of expression contributes to a broad range of signaling outcomes that are highly context-dependent (25-27). Wnt signaling occurs through either Wnt/ β -catenin-dependent (“canonical”) or β -catenin-independent (“non-canonical”) pathways. Wnt/ β -catenin signaling involves an intracellular signaling cascade that stabilizes β -catenin (encoded by *CTNNB1*), a highly versatile protein involved in both cell adhesion and nuclear transcription. The net result is accumulation of β -catenin in the cytosol and its translocation to the nucleus where it complexes with the TCF/LEF family of transcription factors to activate target gene expression (24, 25).

Our understanding of Wnt/ β -catenin signaling in PDAC is evolving. While mutations in *CTNNB1*, *AXIN1* or *APC* are only rarely detected in PDAC (28-30), both pancreatic intraepithelial neoplasia (PanIN) and up to two-thirds of PDAC tumors have demonstrable Wnt/ β -catenin activation based on the surrogate measure of increased nuclear and/or cytoplasmic β -catenin (11, 12, 31). Most *in vitro* and *in vivo* studies addressing the biologic effects of Wnt/ β -catenin signaling in human PDAC cell lines indicate it is pro-tumorigenic (12, 32-34). While offering important insights into the

biochemical, transcriptional and phenotypic consequences of Wnt/ β -catenin signaling in PDAC, these studies commonly rely on a small number of PDAC cell lines and do not address the potentially confounding effects of variations in Wnt/ β -catenin signal strength, duration or context. As an example, *in vivo* developmental and cancer models reveal Wnt/ β -catenin signaling can mediate diverse and sometimes opposing phenotypic outcomes in the pancreas depending on its spatiotemporal context and relative levels of activation (11, 35). Moreover, even if the function of Wnt/ β -catenin signaling is consistently pro-tumorigenic in PDAC, it is unclear to what extent the pathway is variably activated in human tumors or whether such variations impact clinical behavior.

In this study we examine variations in Wnt/ β -catenin activation across a spectrum of PDAC cell lines and patient samples, identifying a subset of tumors that are distinguished by higher levels of Wnt/ β -catenin transcriptional activation and more aggressive clinical behavior. We further describe an important relationship between *WNT7B* expression, Wnt/ β -catenin signaling and anchorage-independent growth in PDAC cell lines with elevated pathway activation. We also conclude autocrine Wnt/ β -catenin signaling in PDAC can be primarily initiated and regulated by a single Wnt ligand, WNT7B, acting alone or in conjunction with other Wnt ligands. These results are of significance for future attempts to successfully deploy Wnt-directed therapy in this or other cancers.

Results

Wnt/ β -catenin signaling varies widely across pancreatic cancer cell lines

In order to determine the variability of Wnt/ β -catenin-dependent signaling across pancreatic cancer, Wnt/ β -catenin-dependent luciferase-based reporter assays were performed on multiple PDAC cell lines. Cell lines were transiently transfected with plasmid containing BAR (β -catenin activated reporter) driving luciferase expression or FUBAR (found unresponsive β -catenin activated reporter) identical to BAR with the exception of a mutation in the TCF/LEF binding motif abrogating β -catenin responsiveness. This BAR system has been previously described and shown to have greater sensitivity and dynamic range for β -catenin transcriptional activity relative to TOPFlash or SuperTOPFlash(36). HPDE, a non-transformed pancreatic ductal epithelial cell line, and several PDAC cell lines were found to have minimal baseline Wnt/ β -catenin reporter activity as reflected by BAR/fuBAR ratios of 0.5-2.0, hereafter referred to as “Low BAR” cell lines. In contrast, a subset of PDAC cell lines including AsPC-1, Capan-1 and HPAF-2 had far more significant baseline activation with BAR/fuBAR ratios of 5-20 (Figure 1-1A), hereafter referred to as “High BAR” cell lines. Expression of *AXIN2*, a widely documented endogenous transcriptional target of Wnt/ β -catenin activation (37), was also increased in High BAR lines compared to Low BAR lines (Figure 1-1B), confirming the validity of the BAR reporter.

Treatment of cell lines with either WNT3A ligand or the small-molecule GSK3 β inhibitor CHIR99021 significantly increased Wnt/ β -catenin signaling as measured by BAR-luciferase reporter assays (Figure 1-1C) and endogenous *AXIN2* expression (Figure 1-

1D). However, the relative-fold change observed after drug or ligand stimulation was greatest in Low BAR lines, reflecting their initial lower baseline. Therefore, while most PDAC cell lines had the capacity to respond to exogenous Wnt stimulation, only a subset demonstrated appreciable levels of cell autonomous Wnt/ β -catenin activity.

High Wnt/ β -catenin transcriptional signature predicts worse disease-specific survival

Given wide variation in baseline Wnt/ β -catenin activation across PDAC cell lines, we next explored the consequences of such variations in patient tumor samples. To date, the determination of Wnt/ β -catenin signaling in patient tumors has been primarily addressed by immunohistochemistry (IHC) for nuclear and/or cytoplasmic β -catenin expression, a widely recognized surrogate for Wnt pathway activation (26). However, published data regarding β -catenin IHC in PDAC are hard to interpret given significant differences in the reported frequencies of nuclear and/or cytoplasmic β -catenin expression, as well as variable and contradictory results for β -catenin IHC and its correlation with survival and other clinicopathologic variables (11). We therefore evaluated a pancreatic cancer-specific Wnt/ β -catenin transcriptional signature as an alternative measurement of Wnt/ β -catenin activation in patient tumors. To define this signature, the High BAR PDAC line AsPC-1 was transfected with either control or *CTNNB1* siRNA to generate gene expression profiles of Wnt/ β -catenin activation and inhibition, respectively. These profiles were then used as a training set to statistically model pancreas-specific Wnt/ β -catenin transcriptional activity in CREATE SIGNATURE, a publically available web-based application that employs Bayesian methods to determine and analyze gene expression signatures (38). The generated statistical

model (see Arensman et al (39)) assigned a relative Wnt/ β -catenin activity score that ranged from 0 (inactive) to 1 (high activity). The model was then used to evaluate a published microarray dataset (22) of 41 PDAC patient tumors linked to clinicopathologic information and survival data (see Arensman et al (39)). A variable and continuous range of low to high Wnt/ β -catenin transcriptional activity was noted across the 41 PDAC tumors (Figure 1-2A).

The potential clinical relevance of our defined pancreas-specific Wnt/ β -catenin signature was next evaluated by stratifying tumors into quartiles based on their activity score. Tumors in the lowest quartile of activity showed a trend toward improved survival relative to the top three quartiles (Figure 1-2B). Thereafter, patients were divided into two groups encompassing either low (bottom quartile) or high (combined top three quartiles) Wnt/ β -catenin transcriptional activity. These dichotomized groups showed no significant relationships with a variety of baseline clinicopathologic variables with the exception of a statistically significant correlation between high Wnt/ β -catenin activity and the presence of lymphovascular invasion ($P=0.01$, data not shown, see Arensman et al (39)). In univariate analysis, high Wnt/ β -catenin activity was associated with worse disease-specific survival (Hazard Ratio = 2.75, 95% CI 1.03-7.32, $P=0.039$) with lymphovascular invasion and increased tumor size also trending toward worse survival (data not shown, see Arensman et al (39)). Multivariate Cox regression analysis indicated high Wnt/ β -catenin activity was an independent predictor for worse disease-specific survival in a model with all other significant clinicopathologic variables retained after backward selection (Hazard Ratio = 3.16, 95% CI 1.19-8.40, $P=0.021$, data not

shown, see Arensman et al (39)). Kaplan-Meier analysis also verified high Wnt/ β -catenin activity was correlated with reduced disease-specific survival in the patient cohort (Figure 1-2C, median disease-specific survival time 20.3 vs. 43.9 months, log rank $P = 0.03$).

Wnt/ β -catenin activity in pancreatic cancer is mediated by autocrine Wnt ligand secretion

Wnt/ β -catenin reporter activity was next measured in PDAC cell lines following treatment with either IWP-2 or XAV939, small molecule compounds that inhibit Wnt/ β -catenin signaling by discrete mechanisms. IWP-2 inactivates the N-palmitoyltransferase PORCN to selectively inhibit the palmitoylation and secretion of Wnt ligands (40). XAV939 is a tankyrase inhibitor that augments β -catenin degradation through its stabilization of AXIN (41). IWP-2 significantly inhibited Wnt/ β -catenin reporter activity of High BAR lines at nanomolar concentrations (Figure 1-3A). XAV939 only partially inhibited Wnt/ β -catenin reporter activity at low micromolar concentrations, while higher concentrations were associated with increasing cytotoxicity (Figure 1-3B and data not shown). The reporter activities of Low BAR lines L3.6pl, MiaPaCa-2 and PANC-1 were unaffected by treatment with either IWP-2 or XAV939 (data not shown). Treatment with recombinant sFRP1, which can sequester Wnt ligands and prevent their binding to Fzd receptors, also significantly inhibited reporter activity in dose-dependent fashion (Figure 1-3C). The strong inhibitory effects of IWP-2 and sFRP-1 suggested that Wnt/ β -catenin activation in High BAR lines required effective Wnt ligand processing, secretion and receptor engagement. To address this further, BAR reporter

activity was measured following siRNA-mediated gene knockdown of *CTNNB1* or *WLS*, a key gene involved in the trafficking and secretion of Wnt ligands (42). Quantitative real-time PCR (qPCR) verified *CTNNB1* and *WLS* gene knockdown of 75-90% (data not shown). Inhibition of reporter activity seen with *WLS* knockdown was nearly identical to that observed with *CTNNB1* knockdown (Figure 1-3D), indicating that autocrine Wnt/ β -catenin signaling in High BAR lines is heavily dependent upon Wnt ligand secretion.

WNT7B mediates high levels of autocrine Wnt/ β -catenin activity in pancreatic cancer

Given the link between Wnt/ β -catenin activation and Wnt ligand secretion in PDAC cell lines, we sought to identify the specific ligands mediating this effect. Published RT-PCR and expression microarray analyses indicate nearly every one of the 19 Wnt genes is expressed in at least subsets of pancreatic cell lines (12, 22, 43). Therefore, to determine which Wnt genes are most abundant in pancreatic cancer cells we performed *in silico*-based analysis of published SAGE (serial analysis of gene expression) dataset of 24 human pancreatic tumors that excluded non-malignant cells through serial passage as *in vitro* cell lines or *in vivo* xenografts(4). *WNT3*, *WNT4*, *WNT5A*, *WNT7B*, *WNT10A* and *WNT11* were determined to be the most abundant WNT genes in pancreatic cancer cells on a per sequence tag basis (Figure 1-4A). Gene expression microarray analysis was next performed on multiple high BAR and low BAR PDAC cell lines to measure relative expression of Wnt genes. This revealed *WNT7B* expression to be the most highly and consistently increased Wnt gene in High BAR lines (Figure 1-4B). Direct evaluation of *WNT7B* expression by qPCR confirmed it was elevated in High BAR lines compared to Low BAR lines (Figure 1-4C). To directly link *WNT7B*

expression and Wnt/ β -catenin reporter activity, the High BAR lines AsPC-1 and HPAF-2 were transiently transfected with siRNAs targeting *WNT7B*. Knockdown of *WNT7B* significantly reduced *WNT7B* gene expression (Figure 1-4D) and resulted in corresponding significant reductions in Wnt/ β -catenin reporter activation (Figure 1-4E) and endogenous *AXIN2* expression (Figure 1-4F).

Additional knockdown of *WLS* in combination with *WNT7B* knockdown had no further effect on reporter activity or endogenous *AXIN2* expression (Figures 1-5A-B), suggesting other Wnt ligands provided no significant additive or complementary effects on *WNT7B* and its regulation of autocrine Wnt/ β -catenin signaling in these cell lines. Furthermore, individual siRNA-mediated knockdowns of various other Wnt ligands, including *WNT3*, *WNT3A*, *WNT4*, *WNT5A*, *WNT10A* and *WNT16*, did not significantly inhibit reporter activity in AsPC-1 (Figure 1-5C). Altogether, these data suggest that *WNT7B* is the key ligand driving Wnt/ β -catenin activity in PDAC lines.

WNT7B promotes an anchorage-independent growth phenotype

To determine cellular phenotypes linked to *WNT7B*-dependent autocrine Wnt/ β -catenin signaling, we stably depleted *WNT7B* in both AsPC-1 and HPAF-2 cell lines using two separate lentiviral shRNAs (Figure 1-6A). Stable *WNT7B* depletion significantly inhibited Wnt/ β -catenin transcriptional reporter activity in both cell lines (Figure 1-6B), but had no significant effect on cell viability or proliferation in standard monolayer culture as measured by MTT assay (Figure 1-6C) or BrdU incorporation (data not

shown). In contrast, stable *WNT7B* depletion significantly inhibited colony formation in both monolayer culture (Figure 1-6D) and soft agar (Figure 1-6E). HPAF-2 tumorsphere formation in serum-free, non-adherent conditions was also significantly inhibited by *WNT7B* depletion (Figure 1-6F), while AsPC-1 failed to form tumorspheres and was not evaluated.

Next, *WNT7B* knockdown was addressed in an *in vivo* orthotopic xenograft model of PDAC. AsPC-1 stably transduced with either control ($N=7$) or *WNT7B* ($N=8$) lentiviral shRNA were surgically implanted into the distal pancreas of immunocompromised mice. Survival was followed for 75 days until all but one mouse (shWNT7B) was sacrificed. While there was a trend towards increased survival in mice with tumors depleted of *WNT7B*, Kaplan-Meier survival curves revealed that there was not a statistically significant difference compared to control ($P=0.3$ Figure 1-6G). However, subsequent analysis of *WNT7B* expression in the tumors demonstrated that *WNT7B* knockdown was not maintained throughout the course of the study (Figure 1-6H). Therefore it is unclear if Wnt7B maintains an aggressive phenotype *in vivo*.

Further supporting Wnt ligand secretion and its role in autocrine signaling anchorage-independent growth, IWP-2 also significantly inhibited the non-adherent growth of High BAR PDAC lines in both soft agar and tumorsphere assays (Figure 1-7A-B). Likewise, treatment of High BAR PDAC cell lines with anti-WNT7B antibody partially but significantly inhibited Wnt/ β -catenin reporter activation and tumorsphere formation (Figure 1-7C-D).

Discussion

The origin and biological significance of heterogeneous levels of Wnt/ β -catenin activation across patient PDAC tumors remains unclear. Here we have interrogated variable Wnt/ β -catenin transcriptional activation across patient tumors and cell lines as assessed by promoter-reporter assays, endogenous *AXIN2* gene expression and an experimentally defined Wnt/ β -catenin transcriptional signature. We find that Wnt7B is the key ligand driving canonical Wnt/ β -catenin signaling and anchorage independent growth (Figure 1-7E). Our results parallel those of previous reports surveying PDAC cell lines by β -catenin-dependent transcriptional reporter assays (12, 32, 33) or endogenous *AXIN2* expression (44). We identify subsets of PDAC cell lines with either (i) minimal/low or (ii) higher levels of autocrine Wnt/ β -catenin activation and hypothesize that these and other variations in the context and/or strength of pathway activation could significantly alter the biochemical and phenotypic effects of Wnt/ β -catenin signaling in subsets of PDAC tumors. This variability may be a confounding factor when experimentally manipulating the Wnt/ β -catenin pathway to study its function in PDAC. For instance, it is unclear whether loss-of-function approaches employed in cell lines without appreciable Wnt/ β -catenin transcriptional activation truly reflect biological activities linked to Wnt/ β -catenin signaling. For example, Pasca di Magliano et al(12) and Wang et al(33) assess *in vitro* and *in vivo* proliferation in the context of β -catenin depletion in BxPC-3, L3.6pl, MiaPaCa-2 and PANC-1 PDAC lines, all of which were identified herein as having low endogenous Wnt/ β -catenin activity. Likewise, gain-of-function approaches that act to stabilize β -catenin will result in constitutively

hyperactivated levels of Wnt/ β -catenin signaling that are not typical of PDAC. Genetic mutation in *APC*, *AXIN* or *CTNNB1* that hyperactivate Wnt/ β -catenin signaling are rare in PDAC and instead occur at high frequencies in solid-pseudopapillary tumors, pancreatoblastomas and acinar carcinomas of the pancreas (11). Not surprisingly, transgenic mice expression a pancreas-specific *CTNNB1* mutation that stabilizes β -catenin develop solid-pseudopapillary tumors instead of PDAC. This mutation also blocks the formation of acinar-to-ductal metaplasia, mPanIN and PDAC when introduced into transgenic mice carrying oncogenic *Kras* (45, 46), indicating that high constitutive levels of Wnt/ β -catenin actually may actually inhibit the initiation of PDAC. We propose that a more accurate view of Wnt/ β -catenin signaling in PDAC required *in vitro* and *in vivo* studies that better account for variations in Wnt dosage and spatiotemporal context during pancreatic tumor development and progression.

Wnt ligand secretion was required for autocrine signaling in High BAR lines as demonstrated by both *WLS* gene silencing and IWP-2 treatment. Both Low and High BAR cell lines were able to respond to further exogenous Wnt ligand treatment, indicating paracrine sources of Wnt ligand originating from the tumor microenvironment could further impact pathway activity *in vivo*. In addition to paracrine-mediated Wnt ligand signaling, a variety of additional extracellular factors and signals have been previously shown to modulate Wnt/ β -catenin signaling PanIN and/or PDAC. Downregulation of secreted Wnt inhibitors from the tumor (34) or its associated stromal compartment (47, 48), as well as upregulation of RSPO2 (8) and sulfatases that potentiate Wnt ligand signaling (32), have been shown to promote Wnt/ β -catenin

signaling and pancreatic tumorigenesis. Additional extracellular signaling pathways that promote pancreatic tumorigenesis have also been shown to cross-talk with Wnt/ β -catenin signaling in PDAC, including TGF β , Hedgehog, Notch and fibroblast growth factor signaling (11). Beyond such extracellular mechanisms, tumor cell-associated changes in the expression, localization and/or activation of intracellular mediators of the pathway also facilitate Wnt/ β -catenin activation in PDAC (23, 33). Recently, additional factors including RNF43 and RSPO2 have been identified as critical mediators of WNT-dependent growth response in PDAC. RNF43 is a negative feedback regulator of Wnt signaling that suppresses membrane expression of the Wnt receptor Fzd. Several PDAC cell lines have RNF43 inactivating mutations, rendering them sensitive to Wnt stimulation and susceptible to the Wnt inhibitor LGK974(10). Indeed, HPAF-2 and Capan-2, two cell lines used in this study, harbor RNF43 mutations, which could explain their high endogenous Wnt activity and sensitivity to depletion of Wnt ligand. Expression of the Wnt enhancer RSPO2 is upregulated in PDAC cells with high endogenous Wnt activity, as well as a subset of patient tumors, and RSPO2 mediates stemness in PDAC cells(49). Overexpression of RSPO2 could provide additional means for Wnt pathway activation and aggressive tumor phenotype. These various mechanisms for augmenting Wnt/ β -catenin activation in PDAC appear to share the common requirement of a Wnt ligand/receptor-initiated signaling event.

Gene expression analysis and RNAi depletion studies presented here identify a significant role for *WNT7B* in PDAC cell lines with higher levels of autocrine Wnt/ β -catenin signaling. Our array profiling studies of Wnt ligands combined with the

observation that Wnt/ β -catenin pathway activation in High BAR PDAC lines is readily inhibited by knockdown of a single Wnt ligand supports a model in which Wnt/ β -catenin signaling can be regulated by the tissue-specific expression of specific ligands without appreciable ligand redundancy. This type of model would suggest that the targeting of an individual Wnt ligand may be sufficient to manipulate the pathway for therapeutic purposes if the ligand context is well understood (50). These cell lines offer a potential opportunity to further explore the function of WNT7B or other Wnt ligands that work individually or in synergy to initiate pathway activation.

Phenotypically, *WNT7B* was required for anchorage-independent cell growth and tumor sphere formation. Although we saw no specific effects on anchorage-dependent cell proliferation, our results are otherwise consistent with various reports that indicate Wnt/ β -catenin facilitates anchorage-independent growth and *in vivo* tumorigenicity in PDAC (12, 32-34). Recently, Wnt signaling has been implicated in pancreatic metastasis through its promotion of anchorage-independent growth and anoikis resistance (51). Wnt2 and a non-canonical Wnt signature are enriched in circulating tumor cells from mouse and human PDAC, while growth of human PDAC cell lines as tumorspheres variably induces the expression of multiple Wnt ligands, including WNT7B (51). Unfortunately, knockdown of WNT7B did not prolong survival in an orthotopic xenograft model of PDAC. However, recent studies suggest that depletion of Wnt/ β -catenin signaling *in vivo* prevents PDAC initiation and diminishes tumor growth(8, 13). It is therefore reasonable to speculate that a well-powered study with sustained reduction in Wnt7B levels would result in a less aggressive disease burden with

prolonged survival. Overall, accumulating data presented here and elsewhere in the literature indicate that both canonical and non-canonical Wnt signaling can facilitate anchorage-independent growth and metastasis in PDAC.

IHC for nuclear and/or cytoplasmic β -catenin is the most widely used surrogate for detecting Wnt/ β -catenin activation in patient tumors. However, the literature is plagued by variable and confounding conclusions regarding β -catenin IHC staining in PDAC(11). As an alternative to measuring the status Wnt/ β -catenin activation in primary human tumors, we adopted the novel approach of generating a pancreas-specific Wnt/ β -catenin transcriptional signature using a publically available web-based application, CREATE SIGNATURE (38). We identified a continuous range of low to high Wnt/ β -catenin transcriptional activity across tumors, suggesting that an ON/OFF model for Wnt signaling in PDAC is probably too simplistic and inadequate for describing its apparent range of activation and variable phenotypic consequences. Patients with a higher Wnt/ β -catenin transcriptional signature had significantly worse disease-free survival and increased histologic evidence of lymphovascular invasion. This finding must be viewed cautiously as it involved the retrospective analysis of a small cohort of patients and does not account for possible heterogeneity of signaling within a tumor due to spatiotemporal differences or associated non-malignant cell types. However, the findings are compatible with the various aforementioned *in vitro* and *in vivo* studies linking Wnt/ β -catenin to increased pancreatic tumorigenicity. As an example, the observed increase in lymphovascular invasion could be the consequence of augmented anchorage-independent growth and survival of circulating tumor cells. Similarly, high WNT2B

expression has been correlated with increased perineural invasion and decreased survival(52). Further retrospective and prospective evaluation of larger patient cohorts using a refined Wnt/ β -catenin transcriptional signature derived from multiple PDAC cell lines will be necessary to evaluate its potential clinical utility as a prognostic or predictive biomarker for PDAC.

The dependency on Wnt ligand (autocrine or paracrine) for initiating Wnt/ β -catenin signaling in PDAC suggests these tumors may be especially responsive to therapeutic approaches that inhibit Wnt ligand or extracellular receptors. IWP-2, which blocks Wnt ligand secretion, was an effective inhibitor of Wnt/ β -catenin activation and anchorage independent growth in high BAR PDAC lines. Unfortunately, IWP-2 is not suitable for *in vivo* use and existing approaches for inhibiting Wnt/ β -catenin *in vivo* are limited (11). However, the monoclonal antibody OMP-18R5, which binds to multiple FZD receptors to inhibit Wnt/ β -catenin signaling, was recently shown to effectively block *in vivo* tumor growth and improve chemotherapeutic response of primary human xenografts derived from pancreas and breast adenocarcinomas (53). LGK974, the small molecule Porcupine inhibitor, also impedes *in vivo* growth of multiple Wnt-driven diseases, including PDAC(10, 54). These preclinical data suggest promise for pharmacologic or genetic approaches that disrupt Wnt ligand interactions with their extracellular receptors. The role of Wnt/ β -catenin signaling as a master regulator of cellular transcription and cell fate also raise the possibility that variations in pathway activation may regulate the response of PDAC cells to chemotherapeutic agents. Given the potential efficacy of approaches targeting the proximal arm of the Wnt pathway, future

work will also need to focus on the development and optimization of predictive clinical biomarkers that identify subsets of tumors most likely to respond to Wnt/ β -catenin inhibitors.

Materials and Methods

Cell lines and Reagents

All human pancreatic cancer cell lines were cultured as previously described(55). AsPC-1, BxPC-3, Capan-1, HPAF-2, MiaPaCa-2 and PANC-1 were obtained from the American Type Culture Collection (ATCC, Rockville, MD). Dr. Timothy Donahue (University of California, Los Angeles) provided L3.6pl. HPDE, an immortalized, non-transformed pancreatic ductal epithelial cell line was kindly provided by Dr. Ming-Sound Tsao (Princess Margaret Hospital, Toronto, Canada) and cultured as previously described(55). Control and Wnt3A-expressing L cells (ATCC) were cultured in Dulbecco's Modified Eagle's Medium + 10% fetal calf serum. Conditioned media were collected in full growth media at 96 hours. Recombinant human sFRP-1 (R&D Systems, Minneapolis, MN) and anti-WNT7B antibody (Q-13, sc-26363) and corresponding isotype control normal goat IgG antibody (sc-2028) (Santa Cruz Biotechnology, Santa Cruz, CA) were purchased and used as indicated.

Quantitative Real-time PCR

RNA extraction, cDNA synthesis and SYBR Green-based quantitative PCR were performed as previously described(55) using an ABI-Prism 7700 sequence detector (Applied Biosystems, Foster City, CA). For primer sequences see Arensman et al(39).

Gene Knockdown

For transient gene knockdown, cell lines were transfected with 10-20 nM of duplexed siRNAs using Lipofectamine 2000 (Invitrogen/Life Technologies, Grand Island, NY).

For stable *WNT7B* knockdown, cells were infected with lentiviral shRNA particles and selected in puromycin (1µg/ml). *WNT7B* shRNAs TRCN0000061873 (shWnt7B #73) and TRCN0000061877 (shWnt7B #77) in pLKO.1 backbone were purchased from Open Biosystems (Lafayette, CO). For RNAi sequences see Arensman et al(39).

MTT Assay

AsPC-1 and HPAF-2 cells were plated at 5,000 and 2,000 cells per well, respectively in 96-well plates under normal growth conditions. MTT assays (ATCC) were carried out per manufacturer's instructions.

Soft Agar Assay

Stably transduced AsPC-1 or HPAF-2 cells (5,000 or 15,000 cells per well of 24 well plate, respectively) were plated as single cell suspensions in 0.4% Noble Agar overlaid on a base layer of 0.8% Noble Agar. Media (RPMI-1640 + 10% FBS) was replaced every 3 days. After 2-3 weeks, colonies were visualized by incubation with 10% MTT Reagent for 5 hours. Colonies greater than 200 microns in diameter were counted.

Clonogenic Assay

Stably transduced AsPC-1 or HPAF-2 cells were plated at 300 cells per well in 6-well plates in RPMI-1640 and 10% FBS. After 14 days, cell colonies were fixed in methanol, stained with crystal violet and counted.

Sphere Assay

HPAF-2 cells were suspended in Neurobasal Media (Invitrogen/Life Technologies) containing Glutamax, bFGF (20 ng/ml), EGF (20ng/ml) N-2 and B27 at 10,000 cells/well in 6-well ultra-low attachment plates (Corning, Lowell, MA). After 8 days, tumor spheres were visualized and counted by phase contrast microscopy.

β -catenin activated reporter (BAR) assays

Cell lines were transiently transfected with plasmid (pGL3 backbone) or stably transduced with lentiviral construct containing a β -catenin activated reporter (BAR) driving luciferase expression and separate construct with constitutive EF1 α promoter driving Renilla expression (normalization control). The BAR reporter construct has been previously described and is comprised of a 12 TCF/LEF binding motifs (5'-AGATCAAAGG-3'), each separated by distinct 5-base pair linkers upstream of a minimal promoter and firefly luciferase open reading frame (36). FUBAR (found unresponsive β -catenin activated reporter) is identical to BAR with the exception of a mutation in the TCF/LEF binding motifs that abrogates β -catenin responsiveness. Dual luciferase assays (Promega, Madison, WI) were performed to simultaneously measure firefly luciferase and Renilla expression.

Gene expression microarray and data analysis

Gene expression microarray analysis with Affymetrix U133 plus 2.0 oligonucleotide arrays was performed in the UCLA Clinical Microarray Core Facility. To determine relative expression of Wnt ligands across PDAC lines, total RNA was extracted from cells grown under normal conditions to 70-80% confluence. Microarray analysis was performed in the dChip Analysis software package using invariant set normalization. The signal intensity was summarized using the model-based expression index (MBEI) algorithm with mismatch probe option for background subtraction. For the pancreatic cancer-specific Wnt/ β -catenin-dependent gene signature, gene expression microarrays were performed on total RNA extracted from AsPC-1 cells 48 hours post transfection with either control or *CTNNB1* siRNAs to represent “Wnt activated” or “Wnt inhibited” training sets, respectively. These training sets were used to generate a statistical model in CREATE SIGNATURE, a publically available application utilizing Bayesian methods to process gene expression via a web-based interface in GenePattern (38). Default parameters were selected in CREATE SIGNATURE utilizing BinReg algorithm version 2 with both quantile and shiftscale normalization. A final statistical model of 50 genes and 1 metagene was generated and used to predict levels of pancreatic-specific Wnt/ β -catenin transcriptional activation in a previously published (22) microarray dataset of 41 PDAC patient tumor samples (deposited in NCBI GEO, GSE32688).

Orthotopic xenograft tumor assay

Animal work was performed with oversight by the UCLA Division of Laboratory Animal Medicine and approval from the UCLA Animal Research Committee. Orthotopic tumors were established and survival was measured as previously described(56).

Statistical Methods

SPSS 20.0 for Windows (IBM, Armonk, NY) was used for all statistical analyses.

Continuous variables were evaluated using Student t-tests. Chi square tests were used to evaluate the significance of dichotomized patient groups in relation to baseline clinicopathologic factors. Kaplan-Meier survival curves were evaluated by log-rank test. Univariate Cox regression analyses were performed to determine the prognostic significance of individual clinicopathologic variables. Multivariate Cox regression analysis was performed in stepwise fashion with backward selection using the Akaike Information Criterion. The level of significance for all statistical tests was defined as $\alpha = 0.05$.

Figures

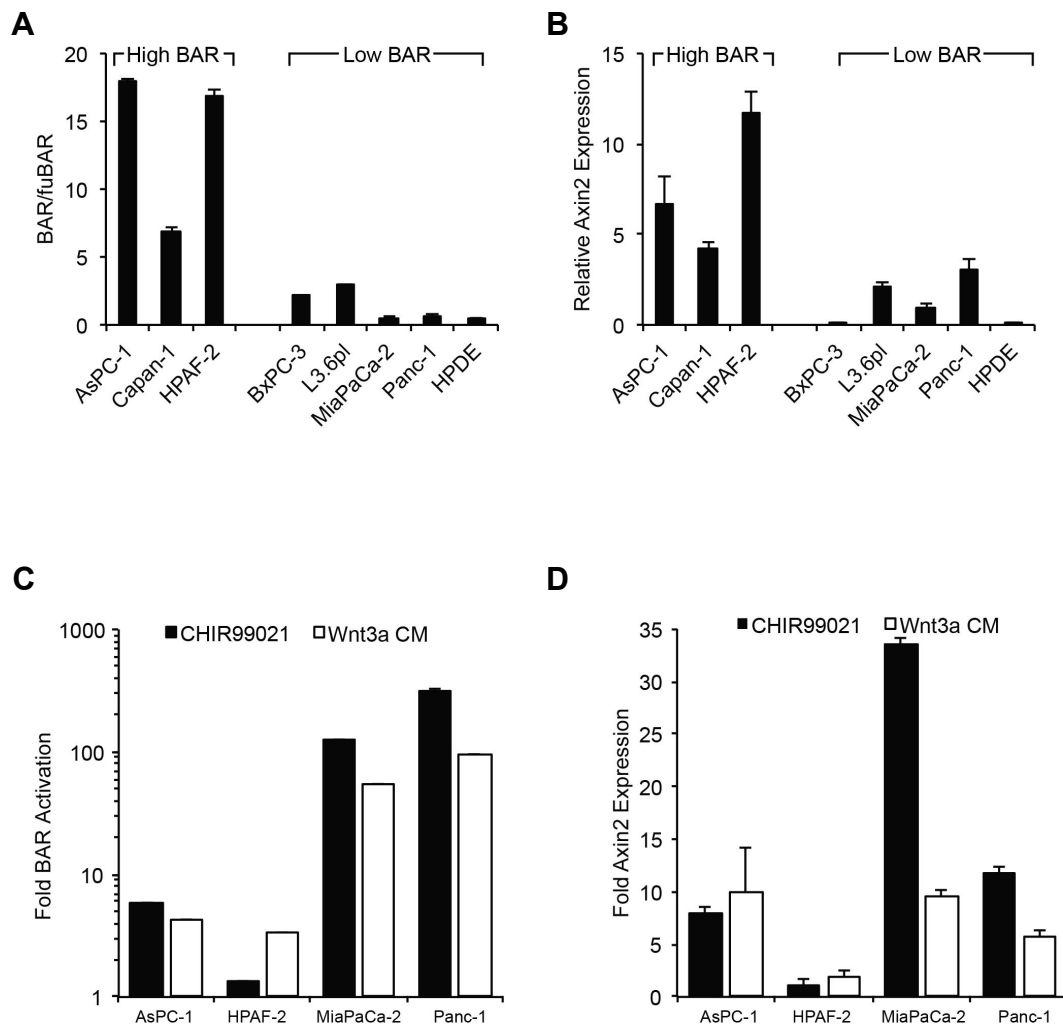


Figure 1-1. Endogenous Wnt/ β -catenin activity varies across PDAC cell lines. (A) BAR/fuBAR ratios for indicated cell lines as measured by dual luciferase assays 48 h after transfection with control Renilla and either BAR-luciferase or fuBAR-luciferase constructs. (B) *AXIN2* expression for indicated cell lines by qPCR. (C) BAR-luciferase activity and (D) *AXIN2* expression after treatment with CHIR990221 or Wnt3a-conditioned media shown relative to vehicle alone (dimethyl sulfoxide) or control L-cell-

conditioned media, respectively, as measured in cell lines with stable BAR-luciferase reporter. *ACTB* served as normalization control for qPCR.

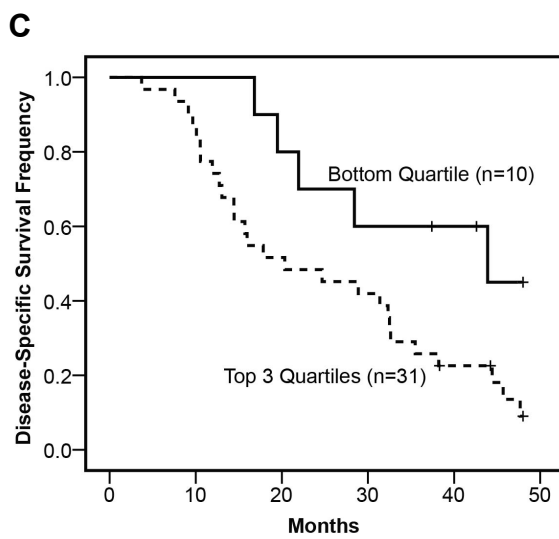
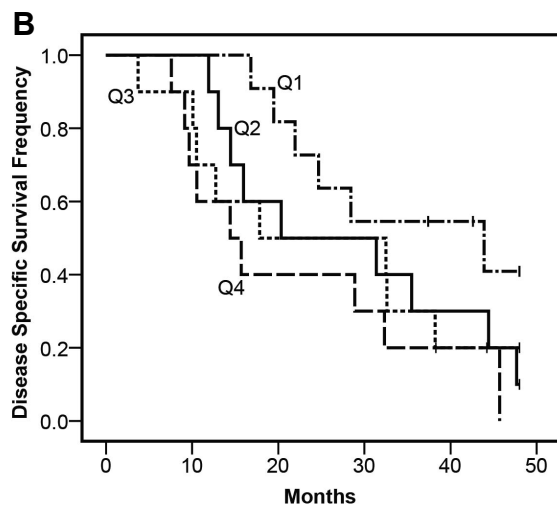
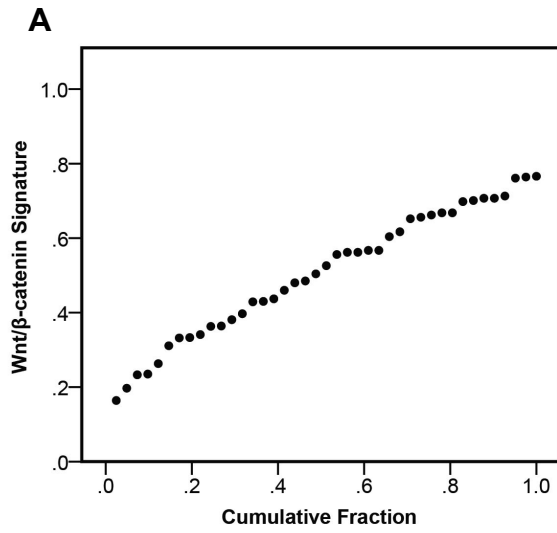


Figure 1-2. A high Wnt/ β -catenin signature predicts worse survival. (A) Wnt/ β -catenin transcriptional activity scores for primary human PDAC tumors ($N=41$) calculated using a model of pancreas-specific Wnt/ β -catenin activation generated in CREATE SIGNATURE. (B–C) Kaplan–Meier analysis of disease-specific survival for PDAC patients grouped by Wnt/ β -catenin transcriptional activity scores and separated into either (B) individual quartiles with Q1 to Q4 progressively indicating bottom to top or (C) bottom quartile (solid line) versus combined top three quartiles (dashed line). $P=0.03$, log-rank test.

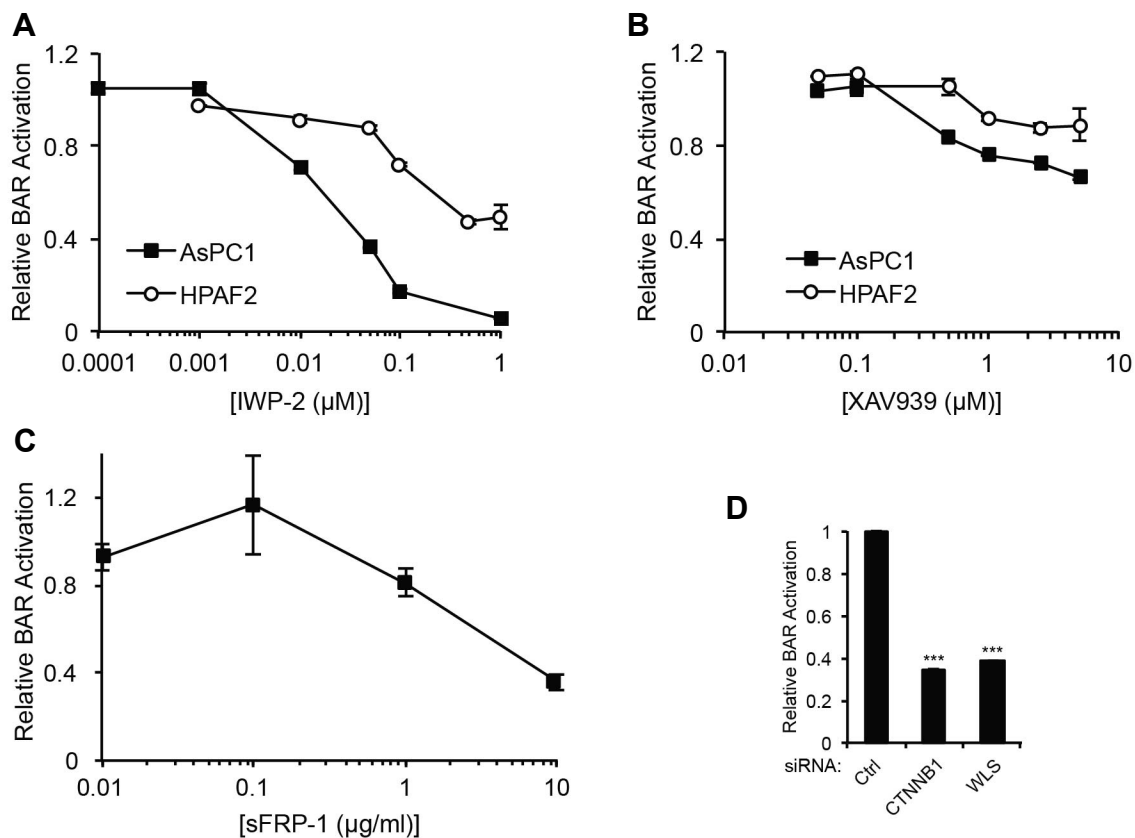


Figure 1-3. Wnt/ β -catenin signaling is dependent upon ligand secretion. Dose–response curves for (A) IWP-2, (B) XAV939 or (C) recombinant sFRP-1 in cell lines with

stable BAR-luciferase reporter. BAR-luciferase activity was measured 24–48h after treatment and normalized to treatments with an equivalent volume of vehicle alone (dimethyl sulfoxide). (D) BAR-luciferase activity for AsPC-1 transfected with control, *CTNNB1* or *WLS* siRNAs. *** $P < 0.001$.

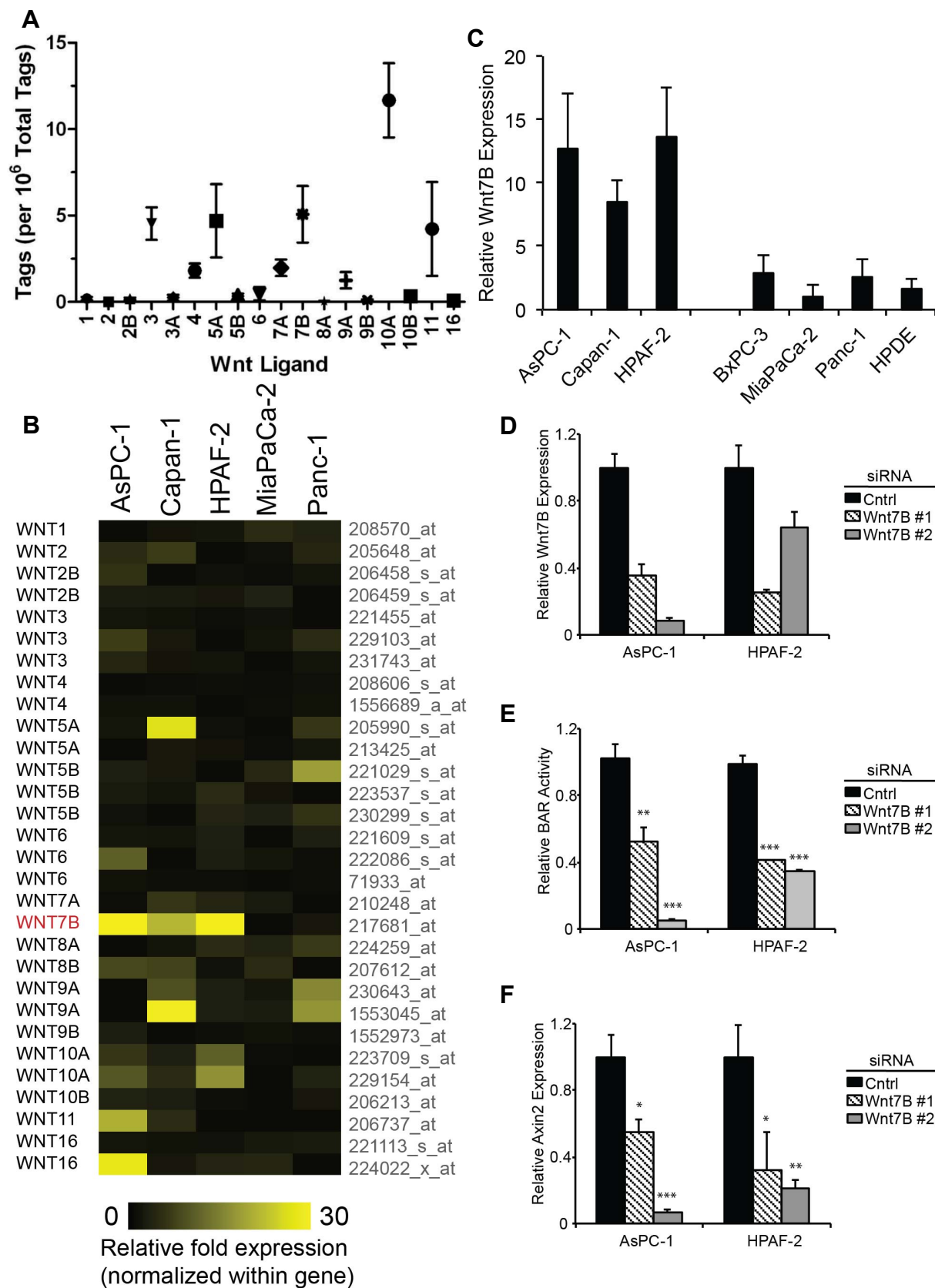


Figure 1-4. WNT7B drives Wnt/ β -catenin activity. (A) Overall combined average \pm standard deviation of *In silico* analysis of Wnt ligand expression of pancreatic cancer based on a published SAGE (serial analysis of gene expression) dataset of 24 PDAC tumors passaged either as in vitro cell lines or xenografts in nude mice. (B) Heatmap showing relative fold change in gene expression for each Wnt ligand across PDAC cell lines as determined by Affymetrix U133 plus 2.0 oligonucleotide arrays. (C) Relative *WNT7B* expression for indicated cell lines by qPCR. (D–F) Cell lines with stable BAR-luciferase reporter were transiently transfected with control or WNT7B siRNAs. At 48h post transfection, cells were analyzed by qPCR for (D) *WNT7B* or (F) *AXIN2* expression, as well as (E) BAR-luciferase activity. *ACTB* served as normalization control for qPCR. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

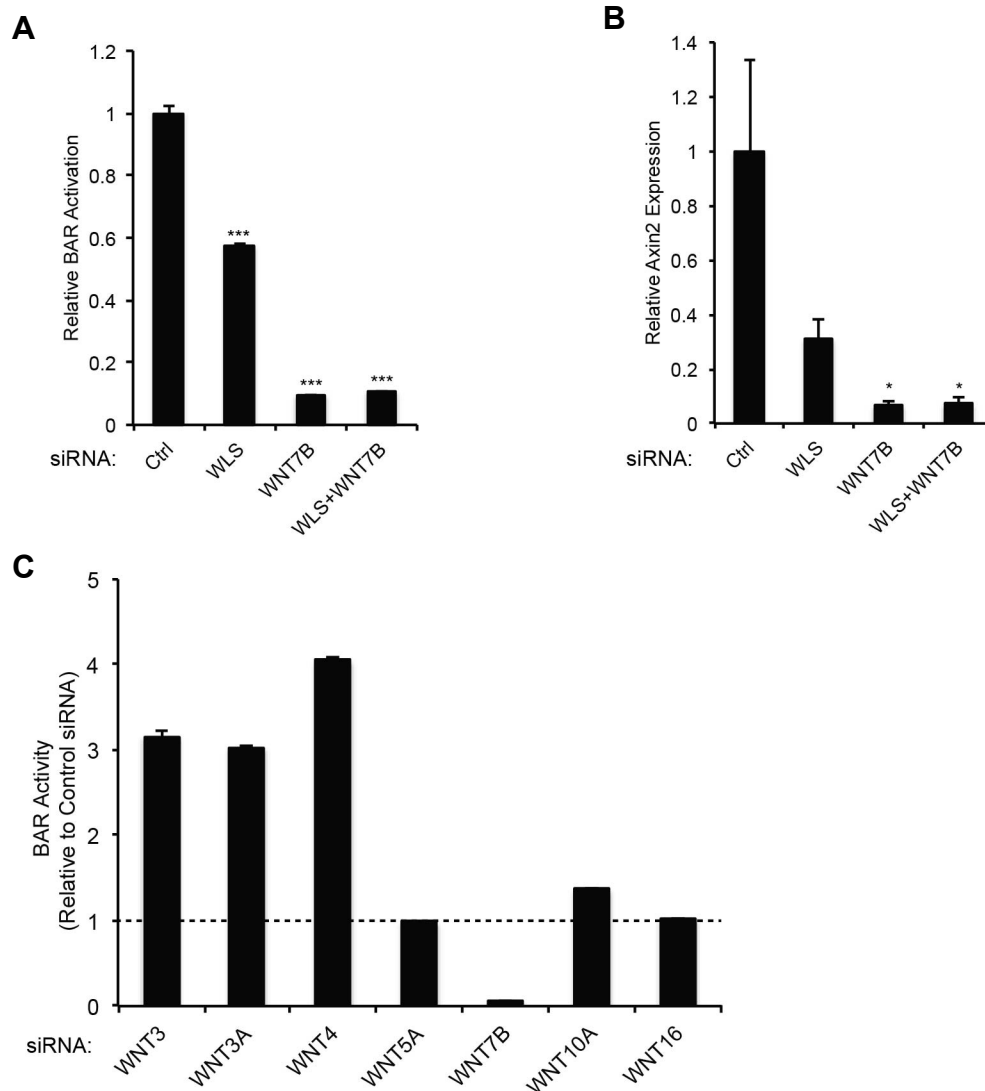


Figure 1-5. WNT7B is the primary ligand driving pathway activation. AsPC-1 with stable BAR-luciferase reporter were transiently transfected with 10nM each of the indicated individual or combinations of siRNAs. At 48 hours post-transfection, cells were analyzed for (A, C) BAR-luciferase reporter activity or (B) *AXIN2* expression by qPCR with *ACTB* as normalization control for qPCR. All data were normalized to measurements observed with corresponding control siRNAs. The dashed line in panel C indicates position of normalized control siRNA and shows that only WNT7B siRNA

transfection led to appreciable inhibition of BAR-luciferase reporter. At least 75% knockdown of each gene by its corresponding siRNA was confirmed by qPCR (data not shown). * $P < 0.05$, *** $P < 0.001$.

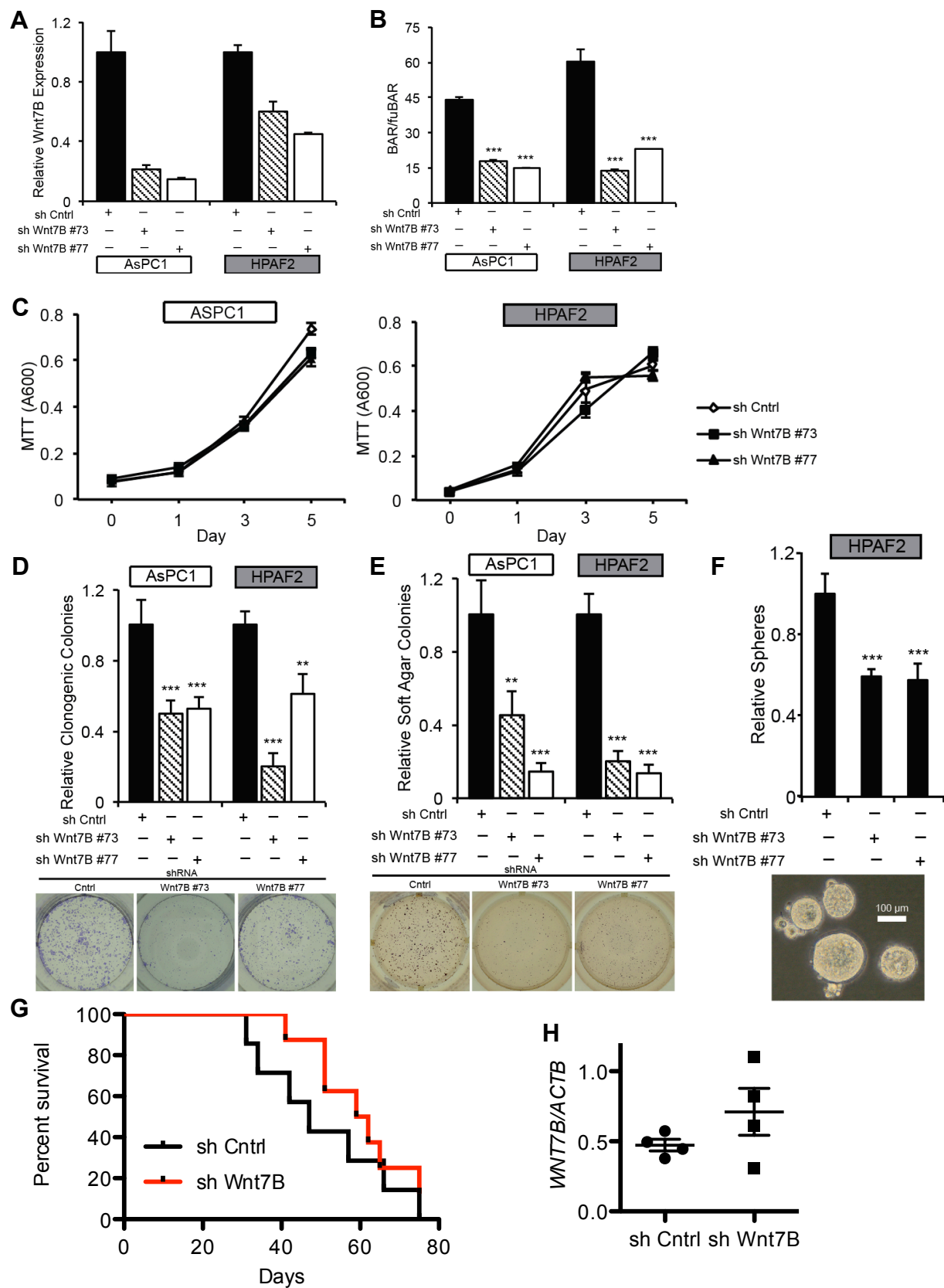


Figure 1-6. Wnt7B promotes anchorage-independent growth *in vitro*. (A-F) AsPC-1 and HPAF-2 cells stably transduced with either control or WNT7B short-hairpin RNA lentiviral constructs were assayed for (A) *WNT7B* expression by qPCR, (B) BAR/fuBAR ratios by dual luciferase, (C) adherent cell growth by MTT, (D) clonogenicity in monolayer cultures, (E) colony formation in soft agar and (F) tumorsphere growth in non-adherent conditions. All data are shown relative to control short-hairpin RNA for the corresponding cell line. Representative images of clonogenic, soft agar and tumorsphere assays show HPAF-2 cells (D-F). (G) Kaplan-Meier survival curve for mice after orthotopic implantation of AsPC-1 stably transduced with either control or WNT7B short-hairpin RNA lentiviral constructs. (H) *WNT7B* expression by qPCR in representative tumors from deceased mice as in (G). *ACTB* served as normalization control for qPCR. Scale bar, 100 μ m. ** $P < 0.01$, *** $P < 0.001$.

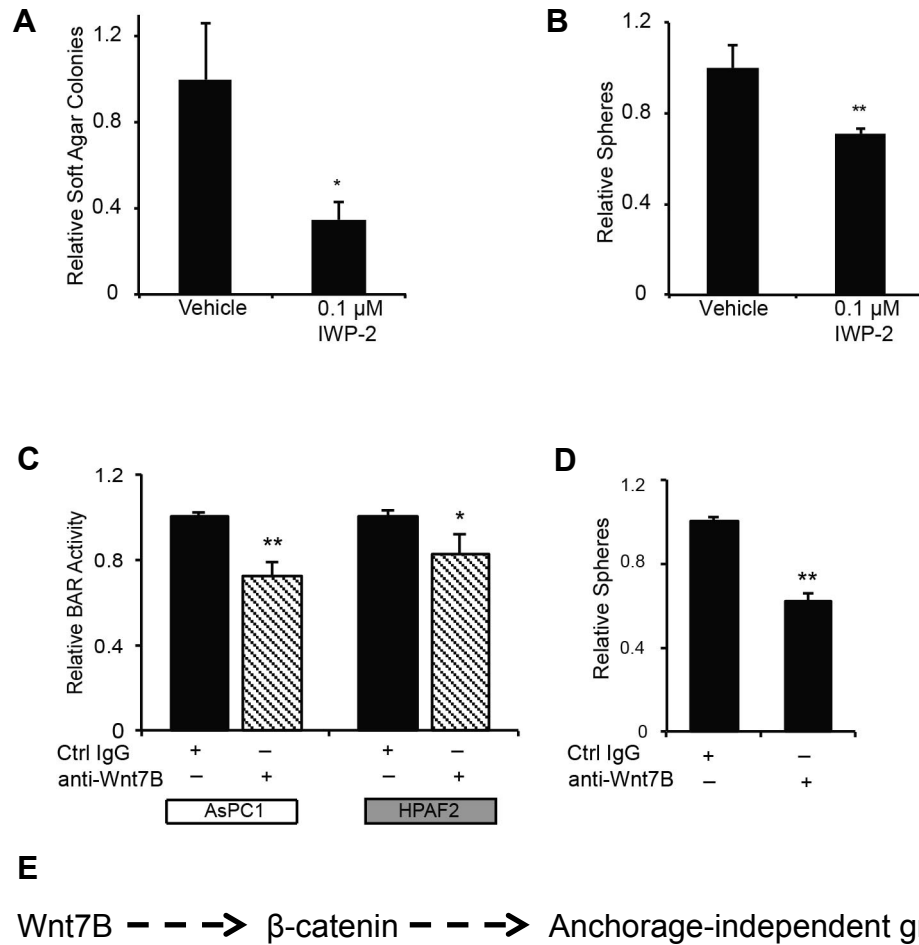


Figure 1-7. Pharmacologic Wnt inhibitor IWP-2 reduces anchorage-independent growth. (A) AsPC-1 soft agar growth or (B) HPAF-2 tumorsphere growth was measured in the context of vehicle (dimethyl sulfoxide) or 0.1 μ M IWP-2 treatment every 72 h. (C) BAR-luciferase reporter assays at 48 h in indicated cell lines with stable BAR-luciferase reporter and (D) HPAF-2 tumorsphere growth were measured following treatment every 48h with 20 μ g/ml anti-WNT7B antibody or matched isotype control antibody. (E) Schematic demonstrating the role of Wnt7B in PDAC. * $P=0.05$, ** $P=0.01$.

CHAPTER 2: SUMO2 MODIFICATION INHIBITS WNT/ β -CATENIN SIGNALING IN PANCREATIC CANCER

Abstract

Pancreatic ductal adenocarcinoma (PDAC) is currently the fourth leading cause of cancer-related death and is characterized by its aggressive nature and resistance to cytotoxic chemotherapy. Recent studies reveal the importance of Wnt/ β -catenin signaling in pancreatic cancer progression and aggressive clinical behavior. Further insights into mechanisms responsible for dysregulation of Wnt/ β -catenin signaling in PDAC should provide a deeper understanding of its role in tumorigenesis and could lead to the identification of novel clinical biomarkers or therapeutic approaches. Here we investigated the action of sumoylation in altering Wnt/ β -catenin signaling in PDAC. Individual knockdown of key genes regulating various steps in the sumoylation cascade activated Wnt/ β -catenin-mediated transcriptional activity, suggesting that sumoylation acts as an inhibitor of Wnt/ β -catenin signaling. This effect was specifically mediated through SUMO2, as SUMO2 knockdown, but not SUMO1 knockdown, activated Wnt reporter activity. Global proteomic analysis identified several known and novel SUMO2 substrates that may suggest a role of sumoylation in the regulation of nuclear import or nuclear sequestration of key Wnt transcription factors.

Introduction

Wnt/ β -catenin signaling is under the control of a variety of post-translational modifications (57), most notably phosphorylation, acetylation and ubiquitination. Another important post-translational modification is sumoylation, which involves covalent attachment of small ubiquitin-like modifier (SUMO) proteins to target proteins. SUMO's are a family of 12 kDa ubiquitin-like proteins that act on cellular targets similar to, but distinct from that of ubiquitin. In mammals, SUMO has four isoforms, SUMO1, SUMO2, SUMO3 and SUMO4. SUMO2 and SUMO3 have 95% sequence homology and are commonly referred to as SUMO2/3(58, 59). Similar to ubiquitination, the process of covalently attaching SUMO to target proteins requires sequential enzymatic reactions involving SUMO activating enzymes (E1), SUMO conjugating enzymes (E2) and SUMO ligases (E3) (58, 59). Unlike ubiquitin modification, which primarily target proteins for degradation, SUMO modifications have much more diverse functions including modification of protein-protein interactions, subcellular localization, stability and activity(59).

Sumoylation is involved in many cellular processes and pathways including DNA repair, transcriptional activation, nuclear body formation, protein turnover and signal transduction(58, 60). Several components of the Wnt pathway are known to be sumoylated, which can result in both positive and negative regulation of the pathway. For example, sumoylation of LEF1, a β -catenin/TCF co-transcription factor, negatively regulates its transcriptional activity by promoting its sequestration into nuclear bodies(61). Conversely, TCF4 is also sumoylated and subsequently localized to

nuclear bodies, but this results in positive regulation of Wnt signaling(62). Sumoylation of TBL1 and TBLR1, two β -catenin transcriptional coactivators, enhances the formation of the TBL1-TBLR1- β -catenin complex and recruitment to β -catenin-target promoters, resulting in transcriptional activation of Wnt target genes (63). β -catenin itself is also sumoylated, resulting in active Wnt signaling (64). Additionally, sumoylation of Wnt pathway components can have effects on different pathways, such as SUMO-modified Axin, which increases JNK signaling (65). Finally, some sumoylation events remain unexplained, such as sumoylation of GSK3 which affects its subcellular localization and kinase activity, but has unknown consequences on downstream Wnt signaling (66).

Given these multiple potential connections between sumoylation and Wnt signaling, and the importance of Wnt signaling in PDAC, we sought to investigate the global effect of sumoylation on Wnt/ β -catenin activity in PDAC. Using gene knockdown and a Wnt transcriptional reporter, we found that sumoylation has a net-negative regulatory effect on the Wnt/ β -catenin pathway that was mediated specifically by SUMO2/3 (hereafter collectively referred to as SUMO2) and not SUMO1. We additionally identified several known and novel SUMO2 substrates in PDAC cells through global proteomic analysis that provide putative targets for further exploring the mechanism linking sumoylation to Wnt pathway inhibition.

Results

Knockdown of sumoylation activates Wnt signaling in PDAC cell lines

An siRNA screen previously performed in the Dawson lab measuring BAR-luciferase activity in the AsPC-1 cell line revealed that knockdown of *SUMO2*, *PIAS1*, *UBE2I* and *SAE2* (*UBA2*) resulted in an increase in reporter activity (Figure 2-1A). To further explore the effects of sumoylation on Wnt signaling in PDAC, Wnt/ β -catenin activity was measured using a Wnt/ β -catenin-dependent luciferase-based reporter BAR (36, 39) in the context of siRNA-mediated knockdown of the aforementioned key regulators of the sumoylation cascade, including *SUMO2*, *PIAS1* (an E3 ligase), *UBA2* (SAE2 protein, an E1 activating enzyme) and *UBE2I* (an E2 conjugating enzyme) (Figure 2-2A). Wnt reporter activity was increased after knockdown of all four components of the sumoylation cascade in the PDAC cell line AsPC-1 (Figure 2-2B), a cell line with high endogenous Wnt/ β -catenin activity (39). Similar experiments were also performed in MiaPaCa-2 and PANC-1 cells, two PDAC lines with low endogenous Wnt/ β -catenin activity (39), but knockdown of *SUMO2* and *PIAS1* had no effect on BAR (Figure 2-2C-D and data not shown). However, upon stimulation with Wnt3A conditioned media, knockdown of *SUMO2* and *PIAS1* lead to a further significant increase in BAR activity induced in response to Wnt3A treatment (Figure 2-2D). Therefore, sumoylation appears to function as a negative rheostat for Wnt/ β -catenin transcriptional activity in circumstances where the Wnt pathway is activated in PDAC.

We also further investigated the global effects of sumoylation on the 293T human embryonic kidney cell line. Previously, sumoylation has been shown to be responsible for the sequestration of LEF1 into nuclear bodies resulting in inhibition of Wnt/ β -catenin signaling (61). As seen with PDAC lines, siRNA knockdown of *SUMO2*, *PIAS1*, *UBA2* and *UBE2I* all significantly increased BAR activity in 293T cells (Figure 2-2E). This is in line with our PDAC findings and others in the literature(61), suggesting that sumoylation may act as a global inhibitor of Wnt/ β -catenin activity.

Since knockdown of sumoylation-related genes increased Wnt/ β -catenin signaling, we assessed whether overexpression of SUMO2 might have an opposing effect on Wnt activity. BAR activity was measured in AsPC-1 stably overexpressing histidine-tagged SUMO2 (8HisSUMO2, Figure 2-2F Input lanes). However, SUMO2 overexpression had no significant effect on BAR activity compared to control cells expressing GFP (Figure 2-2G). This suggests that additional enzymes or signals may be necessary for SUMO2 to impinge upon Wnt signaling.

Inhibition of Wnt signaling by sumoylation is mediated through SUMO2 and not SUMO1

Many studies of sumoylation and its relationship to Wnt/ β -catenin signaling focus on SUMO1(62, 63, 65, 67, 68). However, there is precedence that there may be context-dependent utilization of different SUMO isoforms. For instance, SUMO modification of LEF1 by PIAS was shown to preferentially favor SUMO2/3 over SUMO1 (61).

Therefore, we established whether inhibition of Wnt signaling was specific to either SUMO1 or SUMO2 in PDAC. While knockdown of SUMO2 significantly increased BAR

activity, knockdown of SUMO1 had no effect on the Wnt reporter (Figure 2-3A-B), indicating that SUMO2 specifically mediates inhibition of Wnt/ β -catenin signaling to the exclusion of SUMO1.

Identification of SUMO2-modified proteins

Given that SUMO2 had an overall inhibitory effect on Wnt/ β -catenin activity, we sought to identify which proteins were SUMO2-modified in PDAC in an attempt to identify key mediators of Wnt/ β -catenin signaling. Lysates from AsPC-1 cells stably expressing Histidine-tagged SUMO2 were affinity purified using Nickel beads (Figure 2-2F and Figure 2-4A) followed by mass spectrometry. Western blot for RanGAP1, a known SUMO2 substrate, confirms specificity of the affinity purification (Figure 2-4B). Mass spectrometry analysis revealed enrichment for over 200 SUMO2-modified proteins, although none of the aforementioned sumoylated Wnt regulators were identified. A list of SUMO2-modified proteins that may have potential roles in Wnt/ β -catenin signaling is presented in Figure 2-4C.

Discussion

It is becoming clear that Wnt/ β -catenin signaling is a driver of PDAC in at least a subset of tumors. For example, Wnt/ β -catenin is required for initiation of PDAC(13) and mediates anchorage-independent growth as well as stemness in a subset of PDAC lines(39, 49). While Wnt/ β -catenin signaling is aberrantly active in a subset of patients (11, 12, 31, 39), mutations in key components of the pathway, such as *CTNNB1*, *AXIN1* and *APC*, are only rarely detected(28-30), so it remains unclear as to what is driving

Wnt/ β -catenin activity. One possibility is through post-translational modifications such as sumoylation. Therefore, sumoylation was evaluated in an attempt to identify additional key regulators of Wnt/ β -catenin signaling in PDAC.

Sumoylation has been previously shown to affect Wnt/ β -catenin signaling through multiple mechanisms. Specific SUMO1-modification of TCF4 increases its interaction with β -catenin(62), and SUMO1 modification of TBL1, and TBLR1 releases them from their repressor complex to recruit β -catenin to target promoters(63, 69). However, these mechanisms do not appear to be relevant in our study, as we observed SUMO1 to have no significant impact on Wnt/ β -catenin activity in PDAC. Additionally, our data conversely suggests sumoylation has a net inhibitory effect on Wnt signaling. In this regard our observations are in line with several other proposed mechanisms for sumoylation, such as stabilizing and promoting the activity of GSK3 β or Axin in the destruction complex or segregation of LEF1 into nuclear bodies(61, 65, 66, 70). However, these previously proposed mechanisms also focused on the role of SUMO1 modifications, without addressing the specific role of SUMO2. These mechanisms also do not appear to be at work in PDAC because our mass spectrometry analysis of SUMO2-modified proteins did not reveal GSK3, Axin, LEF1 or any other key component of canonical Wnt signaling.

We found that stable overexpression of SUMO2 has no effect on Wnt/ β -catenin activity when it was expected that increased SUMO2-modification would inhibit Wnt activity. It has been documented that cells already contain a large free pool of SUMO2 and that

SUMO2 is not the rate-limiting step for sumoylation, so overexpression of SUMO2 may have no additional effect(71). Another possible explanation is the reversible nature of SUMO(72), where any additional SUMO2 conjugation would be rapidly reversed by SENPs (SUMO proteases)(71), limiting the ability of additional SUMO2 to modify its targets. This desumoylation, as well as the observation that only a small fraction of a particular SUMO-modified substrate exists under steady-state(71) could also contribute to the difficulty in detecting relevant SUMO2-modified proteins by mass spectrometry.

While our mass spectrometry did not reveal previously described SUMO-modified proteins involved in Wnt signaling, we did identify several proteins that may have connections with the Wnt/ β -catenin pathway. RanGAP1 and RanBP2 are both part of the nuclear pore complex and are involved in the nuclear import of β -catenin and TCF-4(68). SUMO2-modification of either of these proteins could disrupt the nuclear localization of the critical Wnt transcription factors. PML is critical for nuclear body formation, and its sumoylation could be responsible for sequestration of LEF1 or other proteins involved in β -catenin mediated transcription(71, 73). TRIM28 is a binding partner of WTX, which modulates Wnt signaling(74) and TRIM28 is also part of a family of SUMO E3 enzymes and could have a role in PML nuclear bodies(75). SP100 also interacts with PML nuclear bodies(76). Therefore, it is tempting to speculate that SUMO2 could inhibit Wnt signaling by preventing nuclear import of critical Wnt transcription factors or by sequestering key Wnt transcription factors such as β -catenin, TCF or LEF into nuclear bodies.

In conclusion we have shown that the sumoylation cascade along with SUMO2, but not SUMO1, inhibit Wnt/ β -catenin signaling in PDAC and possibly in other mammalian systems (Figure 2-4D). While it remains unclear what is the direct target of SUMO2-modification, our mass spectrometry analysis revealed several candidates involved in nuclear transport and nuclear body formation. More work is required to determine the effects of sumoylation on these targets and how it relates to Wnt signal transduction. Additionally it will be pertinent to determine growth phenotypes when sumoylation is affected as Wnt signaling has been shown to be critical for PDAC progression.

Materials and Methods

Cell lines and reagents

All human cell lines were cultured as previously described(39). AsPC-1, MiaPaCa-2, PANC-1 and 293T were obtained from the American Type Culture Collection (ATCC). Control and Wnt3A conditioned media were collected from L cells (ATCC) as previously described(39).

Real-time PCR

RNA extraction, cDNA synthesis and SYBR Green-based quantitative PCR were performed as previously described(39). All values were normalized to *ACTB* expression. Primers used are *ACTB* Forward 5'- CCAACCGCGAGAAGATGA and Reverse 5'- CCAGAGGCGTACAGGGATAG, *SUMO2* Forward 5'- CAGGATGGTTCTGTGGTGCAGTTT and Reverse 5'- TTGATTGGTTGCCCGTCAAATCGG, *PIAS1* Forward 5'-

GCGGACAGTGCGGAACTAAA and Reverse 5'- ATGCAGGGCTTTTGTGAGAAG, *UBE2I* Forward 5'- TCCTCCACCTGTCCGCTAC and Reverse 5'- GCCACGAAACCAAATGGGTG, and *UBA2* Forward 5'- AGGAAACCTCCAGTTCCGTT and Reverse 5'- GTTTGCAGCAGAGGTGACAA.

Gene knockdown

Cells were transfected for 48 hours with 5nM Invitrogen stealth siRNA SUMO2 (18420622), PIAS1 (18175978), UBA2 (18178676), Invitrogen silencer select siRNA UBE2I (4390824), Santa Cruz siRNA SUMO1 (sc-29498) or Invitrogen negative universal control siRNA (12935-200) in combination with Lipofectamine 2000 (Life Technologies) per manufacturer's instructions.

Wnt reporter assays

Baseline Wnt reporter activity was measured in dual luciferase assays (Promega, Madison, WI) as previously described(39) following transient co-transfection with control plasmid with constitutive EF1 α promoter driving Renilla expression (serving as a normalization control) and either BAR (beta-catenin activated reporter, 12 TCF response elements driving luciferase expression) or fuBAR (found unresponsive BAR, contains mutated TCF response elements). In other experiments, Wnt reporter activity was measured in AsPC-1, 293T or MiaPaCa-2 stably transduced with the BAR reporter and Renilla control as previously described(56).

Western blots

Lysates were prepared using RIPA buffer with 1% SDS. SDS-PAGE and immunoblotting were performed as previously described(56) with antibodies specific for SUMO2 (Abcam ab81371), SUMO1 (Cell Signaling 4930), PIAS1, (Cell Signaling 3550) or tubulin (Santa Cruz sc-5546). Silver stain (Life Technologies 24612) was performed per manufacturer's instructions.

SUMO2 overexpression

SUMO2 with N-terminal 8xHistidine tag was cloned into the MSCV-GFP-IRES-PURO retroviral vector and transduced into AsPC-1 cells as previously described(55). Primers for PCR amplification of human SUMO2 cDNA are Forward 5'-GTACACCGGTGCCGCCATGCATCATCACCATCACCATCACCACGCCGACGAAAAGCCCAAG and Reverse 5'-TGCATGCATGCGGCCGCTCAGTAGACACCTCCCGTCTGC.

Affinity purification and mass spectrometry

5 sub-confluent 15cm plates of AsPC-1 cells were collected with trypsin and trypsin inhibitor, and washed in phosphate buffered saline. Cells were then lysed in 1mL lysis buffer (pH 7.4, 50mM imidazole, 300mM sodium chloride, 20mM sodium phosphate, 8M urea) for 1 hour at 4 degrees Celsius. Cells were sonicated 3x for 10 seconds, while resting on ice between steps. Ni-NTA agarose beads were prewashed 2x in lysis buffer. From the lysis supernatant, protein concentration was measured by Bradford assay, and 10mg of protein was added to 20µL of Ni-NTA agarose beads; total volume

was brought to 1mL with lysis buffer, and solution was rocked at 4 degrees Celsius overnight. Beads were then washed 10x in 500 μ L lysis buffer, and either used for mass spectrometry or eluted in 10 μ L 6x SDS buffer and 50 μ L lysis buffer by boiling. 20 μ L of the resultant solution was then loaded to 10% polyacrylamide gels for analysis. Mass spectrometry and analysis was performed by Ben Major (University of North Carolina at Chapel Hill).

Figures

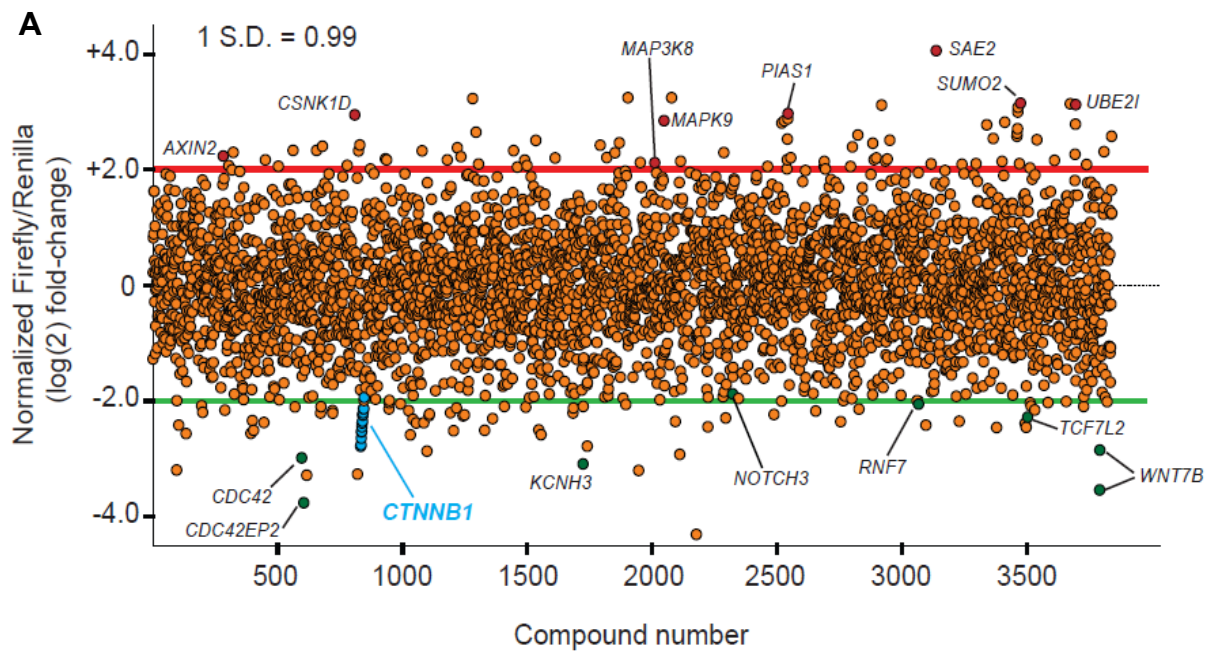


Figure 2-1. Sumoylation genes in an siRNA screen for Wnt/ β -catenin regulators.

(A) Previously performed siRNA screen measuring BAR-luciferase activity reveals components of sumoylation cascade.

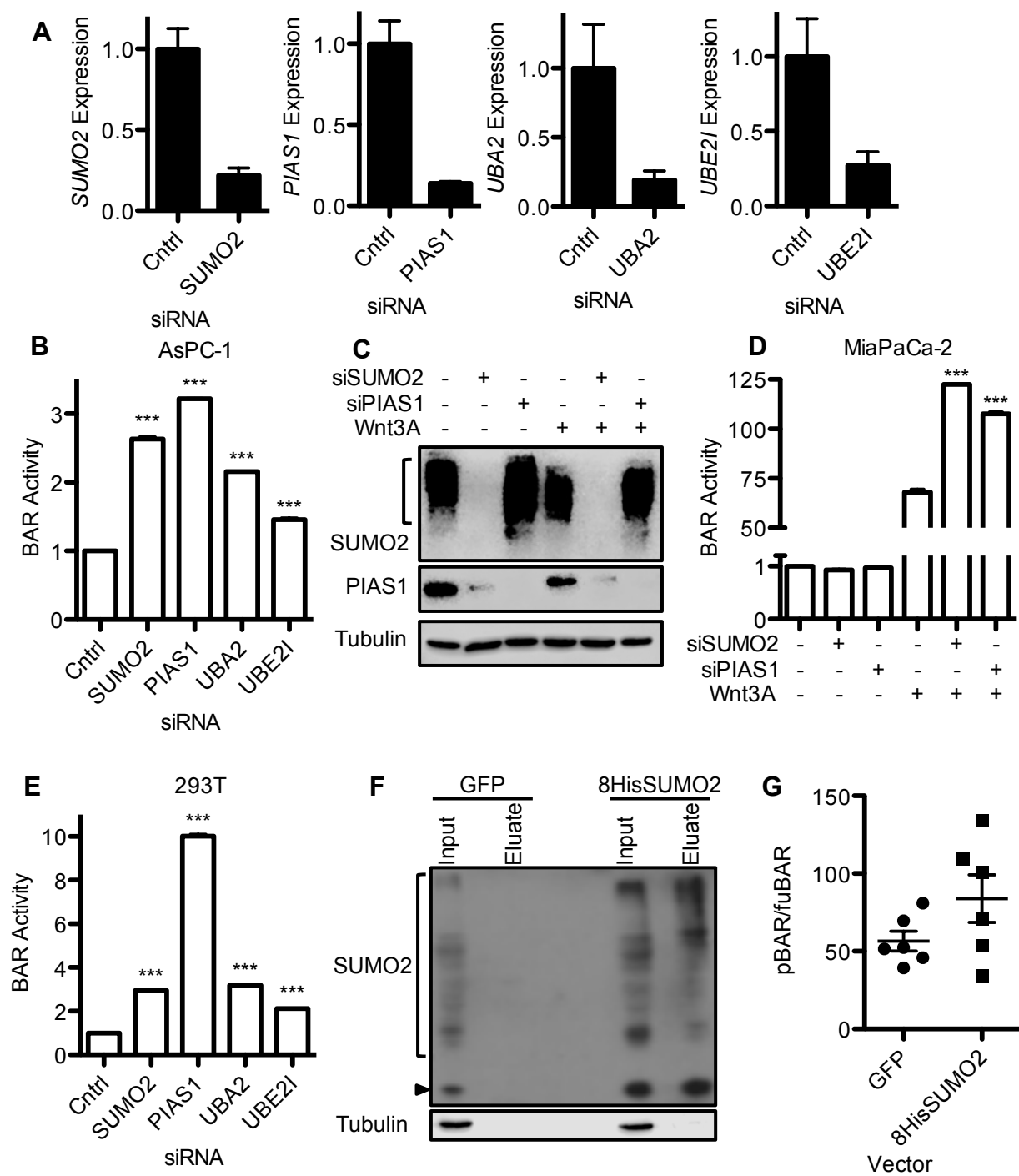


Figure 2-2. Sumoylation negatively regulates Wnt signaling. (A) qPCR for indicated genes and (B) BAR-luciferase activity after 48 hour transfection with 5nM siRNA in AsPC-1. (C) Western blot for SUMO2, PIAS1 and tubulin (loading control)

after 48 hour treatment of MiaPaCa-2 with indicated siRNA and 50% L-cell (control) or Wnt3A conditioned media. (D) BAR-luciferase activity after treatment as in (C). (E) BAR-luciferase activity in 293T after treatment as in (A). (F) Western blot for SUMO2 and tubulin (loading control) after AsPC-1 were stable transduced with GFP control or Histidine-tagged SUMO2 expression vector (8HisSUMO2). Input lanes demonstrate overexpression of SUMO2 with 8HisSUMO2 vector. Eluate lanes demonstrate enrichment of SUMO2-modified proteins after affinity purification with Nickel beads. (G) BAR/fuBAR ratios for AsPC-1 after treatment as in (H) as measured by dual luciferase assays 48 h after transfection with control Renilla and either BAR-luciferase or fuBAR-luciferase constructs. For SUMO2 western blots, brackets indicate sumoylated proteins and arrowheads indicate free-SUMO2. All values for indicated siRNA are shown relative to control siRNA. *** $P < 0.001$.

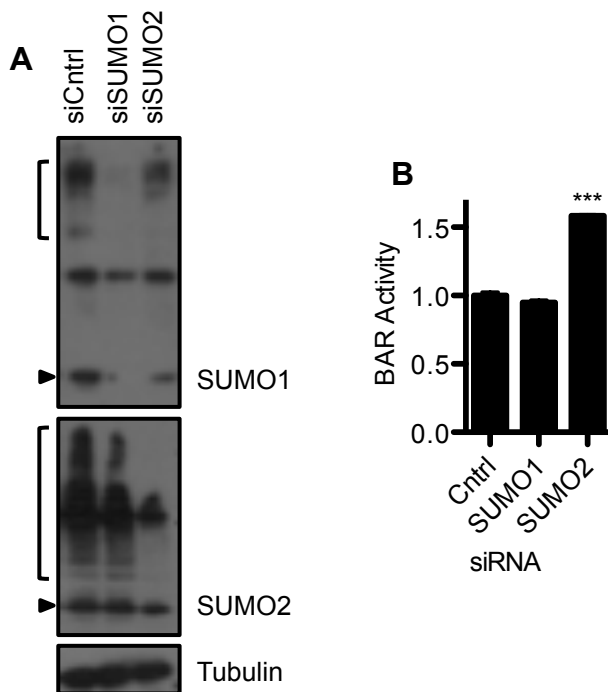


Figure 2-3. SUMO1 does not inhibit Wnt signaling in PDAC. (A) Western blots for SUMO1, SUMO2, and tubulin (loading control) and (B) BAR-luciferase activity were determined for AsPC-1 cells at 48 hours post-transfection with 20nM of indicated siRNA. All values are shown relative to control siRNA. For SUMO western blots, brackets indicate sumoylated proteins and arrowheads indicate free-SUMO. ** $P < 0.01$.

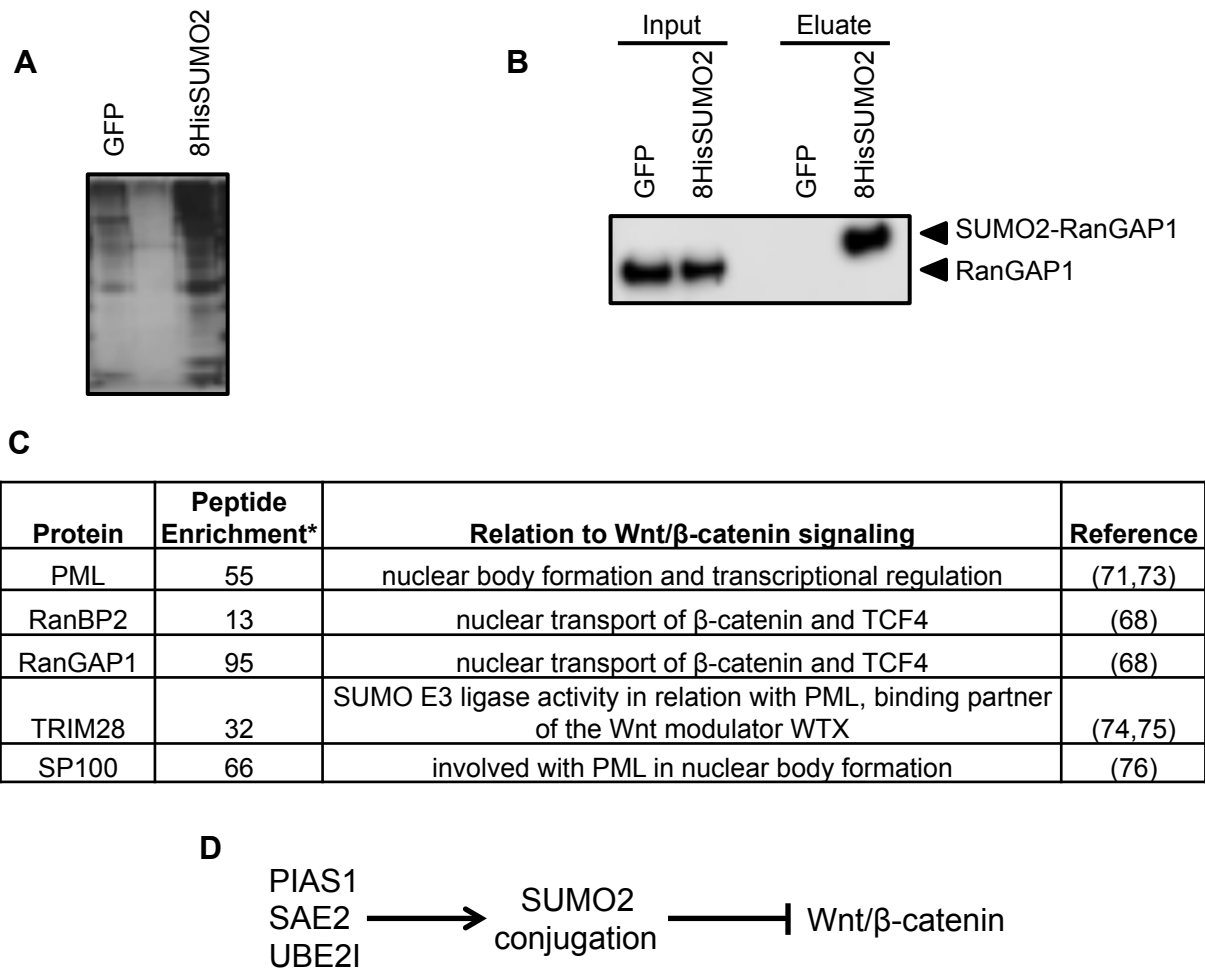


Figure 2-4. Identification of SUMO2-modified proteins in PDAC. Lysates from AsPC-1 cells stably transduced with either GFP control or histidine-tagged-SUMO2 (8HisSUMO2) were affinity purified with Ni-NTA agarose beads. (A) Silver stain of final Ni-NTA purified fractions demonstrates enrichment of proteins covalently bound to 8HisSUMO2. (B) Western blot for RanGAP1 before (input) and after (eluate) affinity purification of AsPC-1 lysates treated as in (A). (C) List of SUMO2-modified proteins identified in mass spectrometry of AsPC-1 that may be involved in the regulation of the Wnt/ β -catenin pathway. (D) Schematic representing effects of sumoylation on Wnt signaling.

CHAPTER 3: THE CREB BINDING PROTEIN INHIBITOR ICG-001 SUPPRESSES PANCREATIC CANCER GROWTH

Abstract

Pancreatic ductal adenocarcinoma (PDAC) is a highly lethal cancer due in part to a lack of highly robust cytotoxic or molecular-based therapies. Recent studies investigating ligand-mediated Wnt/ β -catenin signaling have highlighted its importance in pancreatic cancer initiation and progression, as well as its potential as a therapeutic target in PDAC. The small-molecule ICG-001 binds cAMP-responsive element binding (CREB)-binding protein (CBP) to disrupt its interaction with β -catenin and inhibit CBP function as a coactivator of Wnt/ β -catenin-mediated transcription. Given its ability to inhibit Wnt/ β -catenin-mediated transcription in vitro and in vivo, as well as its efficacy in preclinical models of colorectal cancer and other Wnt-driven diseases, we examined ICG-001 and its potential role as a therapeutic in PDAC. ICG-001 alone significantly inhibited anchorage-dependent and -independent growth of multiple PDAC lines, and augmented in vitro growth inhibition when used in combination with gemcitabine. ICG-001 had only variable modest effects on PDAC apoptosis and instead mediated PDAC growth inhibition primarily through robust induction of G₁ cell-cycle arrest. These effects, however, seemed decoupled from its inhibition of Wnt/ β -catenin-mediated transcription. DNA microarrays performed on PDAC cells in the context of ICG-001 treatment revealed ICG-001 altered the expression of several genes with well-established roles in DNA replication and cell-cycle progression, including direct actions on SKP2 and CDKN1A. ICG-001 also significantly prolonged survival in an in vivo orthotopic

xenograft model of PDAC, indicating ICG-001 or derived compounds that disrupt CBP activity are potentially useful small-molecule therapeutics for pancreatic cancer.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is among the deadliest of cancers, with an overall five-year survival rate of approximately 6%(77). PDAC is difficult to treat because it frequently presents at an advanced, non-operative stage and is highly resistant to cytotoxic or targeted molecular therapy(19). While our understanding of the molecular and cellular basis of PDAC continues to expand, present therapeutic options remain limited and offer only modest survival benefits for most patients.

Wnt/ β -catenin signaling is a critical developmental signaling pathway whose dysregulation is strongly implicated in the pathogenesis of many types of cancers(7). Canonical mutations in *CTNNB1*, *APC* and *AXIN* that lead to constitutive hyperactivation of the pathway occur only infrequently in PDAC(11, 35). Nevertheless, perturbations in the timing, context and strength of Wnt/ β -catenin signaling promote the development and progression of PDAC(11, 35). Ligand-mediated Wnt/ β -catenin signaling is essential for pancreatic cancer initiation and progression(13) and has been linked to aggressive tumor behavior(39). Wnt pathway activation as detected by nuclear and/or cytoplasmic accumulation of β -catenin is observed in 10-65% of pancreatic intraepithelial neoplasia (PanIN)(12, 31), increasing with higher PanIN grade and in invasive PDAC(33). Genetic or pharmacologic inhibition of various steps in the

Wnt pathway has also been shown to prevent *in vitro* (12, 33, 39) and *in vivo* tumor growth (10, 13, 33, 53, 78), implicating Wnt signaling as therapeutic target in PDAC.

Although previously plagued by poor *in vivo* pharmacokinetics, several novel Wnt/ β -catenin inhibitors have demonstrable *in vivo* activity and are now in various stages of preclinical or early clinical development. These include naturally occurring compounds, small molecule inhibitors, blocking antibodies and peptide antagonists (11, 79, 80). Among them is ICG-001, identified in a screen of small molecules that inhibited Wnt/ β -catenin transcriptional activity in a colorectal cancer cell line(81). ICG-001 selectively blocks the interaction of β -catenin with its transcriptional co-activator cyclic-AMP-response-element-binding protein (CBP) without disrupting β -catenin interaction with highly homologous p300. One significant effect ascribed to ICG-001 and its disruption of Wnt/ β -catenin transcription is decreased expression of the apoptosis inhibitor *BIRC5* (aka survivin protein), which leads to activation of caspase-3/7-mediated apoptosis(81-84). ICG-001 was first shown to slow colorectal cancer xenograft growth and intestinal polyp formation in the *Apc*^{min} mouse model(81) and has been subsequently shown to have *in vivo* efficacy in other Wnt-driven disease models, including rodent models of pulmonary fibrosis(85), renal interstitial fibrosis(86), acute lymphoblastic leukemia(87), chronic myocardial infarction(88), dermal fibrosis(89) and salivary tumorigenesis(90). The ICG-001-derived compound PRI-724 is now under investigation as a Wnt inhibitor in early phase clinical trials for advanced solid tumors (NCT01302405) and myeloid malignancies (NCT01606579)(91).

Given the importance of Wnt signaling in pancreatic carcinogenesis, we have now explored therapeutic potential and mechanism of action of ICG-001 in PDAC. ICG-001 significantly inhibited *in vitro* and *in vivo* PDAC growth by inducing G1 cell cycle arrest through effects that were largely decoupled from its activity as a Wnt inhibitor. Instead, ICG-001 appeared to more broadly impact CBP function as a co-transcriptional activator, directly or indirectly perturbing the expression of numerous genes with key roles in cell cycle progression.

Results

ICG-001 inhibits in vitro PDAC cell line growth

ICG-001 (Figure 3-1A) effects on *in vitro* anchorage-dependent PDAC cell growth were addressed by MTT cell viability assays. ICG-001 showed dose dependent cell growth inhibition of AsPC-1, L3.6pl, PANC-1 and MiaPaCa-2 cell lines at IC₅₀ values of 5.48 μ M, 14.07 μ M, 3.43 μ M and 3.31 μ M, respectively (Figure 3-1B). ICG-001 also significantly inhibited anchorage-independent growth in soft agar assays (Figure 3-1C). Addressing the potential for combined use of ICG-001 with cytotoxic chemotherapy, concurrent treatment with 5 μ M ICG-001 and 5 nM gemcitabine further increased growth inhibition relative to individual treatments (Figure 3-1D).

ICG-001 inhibits in vivo PDAC tumor growth

ICG-001 was next addressed in an *in vivo* orthotopic xenograft model of PDAC. AsPC-1 tumor cells were injected into the distal pancreas of nude mice 10 days prior to the start of treatment. Tumor-bearing mice were then randomized into four different

treatment arms, including vehicle (N=12), ICG-001 (N=12), gemcitabine (N=11) or ICG-001 + gemcitabine (N=12). Treatment was continued for a total of 8 weeks, after which all surviving animals were followed until the end of the study (120 days). ICG-001 significantly improved survival compared to vehicle alone as determined by Cox proportional hazard analysis (HR=0.207, 95%CI: 0.079-0.543, $P=0.001$). Gemcitabine also significantly improved survival compared to vehicle (HR=0.123, 95%CI: 0.045-0.334, $P<0.001$). There was no survival difference between gemcitabine alone versus ICG-001 alone (HR=0.592, 95% CI: 0.250-1.399, $P=0.23$). In line with *in vitro* results, the greatest survival benefit relative to vehicle was seen with combined gemcitabine + ICG-001 (HR= 0.074, 95%CI: 0.026, 0.212, $P<0.001$). Although this combination improved survival relative to ICG-001 alone (HR=0.360, 95% CI: 0.152-0.848, $P=0.02$), there was no significant improvement relative to gemcitabine alone (HR=0.608, 95% CI: 0.254-1.455, $P=0.66$). Kaplan-Meier survival curves (Figure 3-2A) also demonstrated improved survival relative to vehicle control (mean survival 46 ± 3.1 days) for ICG-001 (mean survival 64 ± 7.1 days, log-rank $P=0.01$), gemcitabine (mean survival 83 ± 5.1 days, log-rank $P<0.00001$) and combined ICG-001 + gemcitabine (mean survival 95 ± 6.0 days, log-rank $P<0.00001$) treatments.

The *in vivo* biological effects of ICG-001 were further addressed by gross and histologic analysis of mice at necropsy. Using a previously described scoring system that awards points based on tumor infiltration and metastasis (92), tumor dissemination was quantified at the time of death. No differences were observed between the four treatment arms (Figure 3-2B), indicating that animals were reproducibly sacrificed at

time points of equal tumor burden. All tumors significantly involved the pancreas with direct extension into adjacent spleen. Metastatic tumor deposits were observed in all animals, most frequently involving the lung, small bowel and diaphragm (Figure 3-2C-H).

Pharmacologic inhibition of Wnt signaling could have potential deleterious effects on normal somatic stem cell maintenance and tissue homeostasis(91). However, no clinical evidence of ICG-001 cytotoxicity was observed on the basis of body weight measurements, as there was no significant difference in weight across treatment arms throughout the study (data not shown). Furthermore, H&E evaluation of uninvolved liver and intestine revealed no histologic changes to suggest cytotoxicity in animals sacrificed during treatment with ICG-001 or vehicle control. Small bowel from both treatment groups revealed normal villous-crypt ratios and no changes in apoptotic or mitotic activity within crypts, while liver showed no evidence of hepatitis, cholestasis or hepatocellular injury (i.e., ballooning change or apoptosis) (Figure 3-2I-N).

A mitotic index calculated on tumors harvested during drug treatment revealed a significant decrease in mitotic activity in ICG-001 versus vehicle control treated tumors (Figure 3-2O). Quantitative real-time PCR (qPCR) for three well-established Wnt target genes in tumors harvested during drug treatment also revealed a significant decrease in *MMP7* expression with ICG-001 versus vehicle control treatment, but no differences in *BIRC5* or *AXIN2* expression (Figure 3-2P).

ICG-001 induces G1 arrest and has variable effects on Wnt/ β -catenin signaling

We next explored mechanisms underpinning ICG-001 effects on pancreatic growth. As previously shown in colorectal cancer(81), ICG-001 disrupted the interaction between CBP and β -catenin in AsPC-1 cells as measured by co-immunoprecipitation (Figure 3-3A). To examine ICG-001 effects on Wnt/ β -catenin signaling, PDAC cell lines transduced with a β -catenin-activated luciferase reporter (BAR) (36, 39) were treated with increasing concentrations of ICG-001. ICG-001 exhibited a strong, dose-dependent inhibition of reporter activity in AsPC-1 (Figure 3-3B), a cell line characterized by higher levels of autocrine Wnt/ β -catenin activity(39). By comparison, high concentrations of ICG-001 only weakly inhibited reporter activity in PANC-1 and MiaPaCa-2 cells, and not at all in L3.6pl cells (Figure 3-3B). Parallel effects of ICG-001 on the expression of the endogenous Wnt/ β -catenin target gene *AXIN2* were also observed, with the exception of L3.6pl cells demonstrating disparate inhibition of *AXIN2* expression at high ICG-001 concentration (Figure 3-3C). Of note, ICG-001 significantly inhibited the growth of multiple PDAC cell lines at lower concentrations of ICG-001 that had corresponding little or no effect on Wnt/ β -catenin transcriptional activity (compare Figure 3-1B and Figure 3-3B-C).

ICG-001 has been shown to induce apoptosis via downregulation of the apoptosis inhibitor *BIRC5* (survivin), a known Wnt/ β -catenin target gene (81-84). ICG-001 treatment decreased the expression of *BIRC5* transcript and survivin protein levels in all four PDAC cell lines (Figure 3-3D-E). Despite this significant inhibition of survivin expression, ICG-001 had no significant effects on AsPC-1 apoptosis as determined by

cleaved PARP, caspase-3, caspase-7 or annexin-V-propidium iodide (PI) flow cytometry (Figure 3-3F-G). PANC-1 and MiaPaCa-2 cells showed mild increases in cleaved PARP, caspase-3 and caspase-7 with no appreciable differences in apoptosis by annexin-V-PI staining, while only L3.6pl showed significantly increased apoptosis as measured by annexin-V-PI flow staining (Figure 3-3F-G). These modest changes indicated apoptosis was not the primary mechanism for the observed inhibition of PDAC growth by ICG-001.

In order to account for the dramatic growth inhibitory effects of ICG-001, it was hypothesized that instead of inducing apoptosis ICG-001 perturbed cell cycle. Therefore, ICG-001 effects on cell cycle were assessed by propidium iodide staining and flow cytometry. ICG-001 treatment robustly induced G1 arrest in all PDAC cell lines (Figure 3-4A), a finding consistent with the decreased mitotic activity observed in ICG-001 treated orthotopic tumors (Figure 3-2O). Notably, significant G1 arrest was observed in all four cell lines at 10 μ M ICG-001, a concentration for which only AsPC-1 showed appreciable inhibition of Wnt transcriptional activity.

If ICG-001 exerts anti-proliferative effects by inhibiting β -catenin-mediated transcription, this effect should be phenocopied by knockdown of β -catenin expression. However, siRNA-mediated *CTNNB1* gene knockdown failed to induce G1 arrest in AsPC-1 cells, whereas parallel ICG-001 treatment did induce significant G1 arrest (Figure 3-4B). Furthermore, while exogenous Wnt3A ligand stimulation fully rescued ICG-001 inhibition of Wnt reporter activity across the cell lines (Figure 3-4C), it had no effect on ICG-001

mediated G1 cell cycle arrest (Figure 3-4D). Altogether, these data indicate that ICG-001 mediates growth arrest in PDAC cell lines through mechanisms discrete from its action as an inhibitor of Wnt signaling.

ICG-001 inhibits the expression of genes involved in cell cycle

To explore mechanisms underlying ICG-001 inhibition of cell cycle in PDAC, gene expression microarrays were performed on AsPC-1 cells after 6 and 24 hours of treatment with ICG-001. In total, 569 transcripts (419 upregulated and 150 downregulated) showed altered expression in response to ICG-001 treatment ($P < 0.01$; 1.67-fold change at 6 hours or 2-fold change at 24 hours; 0.2% median false discovery rate) (Figure 3-5A, for complete list see Arensman et al (56)). Several of these changes were further validated by qPCR in separate ICG-001 treatment experiments (Figure 3-5B). To explore biological processes linked to ICG-001 treatment, we performed enrichment analysis of gene ontology (GO) functional annotations. In line with phenotypic observations, the top five GO terms for genes downregulated by ICG-001 were DNA replication, DNA metabolic processes, DNA replication initiation, DNA-dependent DNA replication and cell cycle ($p < 0.00013$). Top GO terms for genes upregulated by ICG-001 included multicellular organismal homeostasis, negative regulation of cell proliferation, cell cycle arrest, regulation of angiogenesis and regulation of cell proliferation ($p < 0.001$).

Although its inhibition of PDAC cell proliferation did not appear mechanistically linked to its effects on Wnt/ β -catenin transcriptional activity, ICG-001 did inhibit several known

Wnt transcriptional targets, including *MMP7* and *LGR5* that were further validated by qPCR (Figure 3-5B). To address ICG-001-regulated genes representing actual Wnt transcriptional targets in PDAC, we compared ICG-001 results with a second microarray study comparing gene expression changes in AsPC-1 transfected with either control or *CTNNB1* siRNA. In the context of greater than 80% *CTNNB1* knockdown, there were 441 altered transcripts (absolute fold change ≥ 1.33 ; median false discovery rate 10.4%), including 228 upregulated and 213 downregulated genes (for complete list see Arensman et al (56)). Top GO terms for genes downregulated by *CTNNB1* knockdown included cell fate commitment, tube morphogenesis, tube development, morphogenesis of a branching structure and angiogenesis ($p < 0.0005$). Top GO terms for genes upregulated by *CTNNB1* siRNA included response to wounding, cell adhesion, biological adhesion, response to inorganic substance and regulation of cell migration ($p < 0.0002$). Cross-referencing both ICG-001 and *CTNNB1* siRNA microarray results revealed 117 overlapping transcripts (Figure 3-5C, for complete list see Arensman et al (56)). GO functional annotations were only marginally enriched in this overlapping dataset ($P < 0.01$), but included regulation of cell proliferation, positive regulation of developmental processes, homeostatic process, response to wounding and regulation of cell cycle. Therefore, ICG-001 regulates a set of genes distinct from β -catenin.

Among the most highly regulated genes identified in the ICG-001 arrays were *SKP2* and *CDKN1A* (p21), both key regulators of G1 progression. We therefore explored whether these genes were direct transcriptional targets of CBP that are altered in response to ICG-001 treatment. After 1 hour ICG-001 treatment, chromatin immunoprecipitation

(ChIP) revealed reduced CBP occupancy at *SKP2* and increased CBP occupancy at *CDKN1A* (Figure 3-6A), corresponding respectfully to aforementioned decreased *SKP2* and increased *CDKN1A* gene expression results (Figure 3-5A). Further detailed time-course analysis of gene and protein expression revealed *SKP2* transcript levels were reduced within 4 hours while *CDKN1A* transcript levels were increased within 2 hours following ICG-001 treatment (Figure 3-6B-C). Corresponding decreased SKP2 protein expression and increased p21 protein expression were also seen within 4-6 hours of ICG-001 treatment (Figure 3-6D). MYC protein levels were also decreased after 6 hours, although *MYC* transcript levels were unchanged (Figure 3-6D-E), indicating a post-transcriptional mechanism of MYC protein levels occurs with ICG-001 treatment.

Discussion

At least subsets of PDAC are dependent upon Wnt/ β -catenin signaling for initiation and progression (12, 13, 31, 33, 39, 43), warranting exploration of ICG-001 and other Wnt inhibitors as potential therapeutics. PRI-724, an ICG-001 derivative, is presently under investigation in combination with gemcitabine in a phase 1 clinical trial for patients with metastatic pancreatic cancer (NCT01764477). Additional compounds with greater specificity for Wnt pathway antagonism are also undergoing clinical evaluation in PDAC, including the function-blocking Fzd7 antibody Vantictumab (NCT02005315), the Fzd8 fusion protein OMP-54F28 (NCT02050178), and the small-molecule Porcupine inhibitor LGK974 (NCT01351103).

We have demonstrated that ICG-001 significantly inhibits *in vitro* PDAC growth through induction of G1 cell cycle arrest and improves survival in an *in vivo* orthotopic xenograft model of PDAC. Despite augmented PDAC growth inhibition *in vitro*, combined ICG-001 and gemcitabine did not further improve survival over gemcitabine alone in the xenograft model. It is possible that robust induction of G1 arrest with ICG-001 compromises the action of cytotoxic chemotherapy such as gemcitabine, which primarily kills cancer cells undergoing DNA synthesis in S phase. An analogous situation has been observed with CDK4 inhibitors that also induce G1 arrest and can reduce the efficacy of cytotoxic chemotherapy in certain circumstances(93). Further optimization of the dosing and delivery of ICG-001 (or PRI-724) is obviously needed to resolve whether ICG-001 can be used effectively in combination with gemcitabine. Intriguingly, ICG-001 reduced expression of *RRM1* in AsPC-1 microarrays. *RRM1* expression directly correlates with gemcitabine resistance(94) and worse overall survival in PDAC(95, 96). Furthermore, ICG-001 was shown to act synergistically with conventional chemotherapy to prolong survival in NOD/SCID mice engrafted with primary acute lymphoblastic leukemia(87). Thus, when used appropriately in combination with cytotoxic regimens ICG-001 may still ultimately provide additional survival benefit in PDAC or other tumors.

ICG-001 treatment and β -catenin knockdown regulated an overlapping set of genes in AsPC-1, including known targets of canonical Wnt signaling (i.e., *MMP7*, *EDN1*, *LGR5*, *RNF43* and *PORCN*). Originally identified in a small molecule library screen of compounds that inhibited β -catenin/TCF-dependent reporter activity, ICG-001 inhibits

Wnt transcriptional activity by binding to the N-terminal domain of CBP and preventing its interaction with β -catenin(81). This not only has the potential to disrupt CBP-dependent β -catenin-TCF transcription, but also may increase the pool of β -catenin available to interact with p300. Like CBP, p300 functions as a transcriptional co-activator or co-repressor in a context-dependent manner (91, 97, 98). Kahn and colleagues have proposed a model of CBP/p300 differential co-activator usage whereby TCF- β -catenin-CBP-mediated transcription drives a non-differentiated proliferative state and ICG-001 induces a switch to TCF- β -catenin-p300 mediated transcription that favors cellular differentiation(97, 99). In keeping with this model, ICG-001 strongly induced G1 cell cycle arrest in PDAC cell lines and inhibited the expression of certain genes linked to pluripotency, including *LGR5* that marks progenitor cells in intestinal crypts and *DCLK1* which was recently shown to mark a unique tumor initiating cell population in PDAC(100). ICG-001 also regulated many additional genes unaltered by β -catenin knockdown, including several linked to DNA replication and cell cycle regulation. Beyond β -catenin, ICG-001 may more generally alter gene expression by disrupting CBP interaction with any of a number of its known protein binding partners which includes many transcription factors(101). As an example, ICG-001 was recently shown to block CBP binding to γ -catenin, resulting in increased p300/ γ -catenin interaction and decreased *BIRC5* expression(87, 102).

ICG-001 can induce caspase-3/7-mediated apoptosis in colon cancer cells via downregulation of *BIRC5*/survivin (81). Although ICG-001 treatment reduces *BIRC5* transcript and survivin protein levels, there were only variable and modest effects on

apoptosis in PDAC cell lines. Rather, ICG-001 induced robust G1 cell cycle arrest, which is perhaps not unexpected given CBP/p300 histone acetyltransferase activity is essential for cell proliferation and orderly G1/S transition(103). Several groups find Wnt/ β -catenin signaling promotes cellular proliferation in PDAC(12, 35) and while Wnt/ β -catenin transcriptional targets *MYC* or *CCND1* are commonly linked to its regulation of cell cycle, transcript levels of these genes were not altered in PDAC cell lines in response to ICG-001 treatment.

ICG-001 dramatically inhibited both anchorage-dependent (MTT assay) and anchorage-independent (soft agar assay) growth in all four PDAC lines that were examined. Inhibition of anchorage-dependent growth was not expected because we have previously shown that knockdown of the canonical ligand WNT7B and subsequent inhibition of Wnt/ β -catenin signaling did not decrease anchorage-dependent growth(39). Another unexpected finding was that ICG-001 inhibited the growth of PDAC cell lines that have relatively low to absent levels of endogenous Wnt/ β -catenin activity(39) and presumably do not rely on Wnt/ β -catenin for growth. Moreover, growth inhibition and G1 cell cycle arrest was observed in multiple PDAC cell lines at ICG-001 concentrations with little or no measurable effect on Wnt reporter activity or endogenous *AXIN2* expression. Altogether these data indicate that ICG-001 inhibition of PDAC cell proliferation is decoupled from its function as a Wnt inhibitor.

ICG-001 treatment altered the expression of numerous genes linked to DNA replication and cell cycle in AsPC-1 microarray analysis, both downregulating genes that promote

(i.e., *SKP2*, *CDC25A*, *CDC7*, *CCNE* and *MCM2-MCM6*) and upregulating genes that block (i.e., *CDKN1A*, *CDKN1C*, *CDKN2B*, *MXD1*, *MXI1* and *BTG1*) cell cycle progression. ICG-001 induced rapid changes in CBP occupancy on *SKP2* and *CDKN1A* regulatory regions with corresponding changes in mRNA and protein levels, implicating *SKP1* and *CDKN1A* as direct targets of ICG-001-mediated cell cycle arrest (Figure 17F). *SKP2* is a substrate component of the SCF E3 ubiquitin-protein ligase complex that promotes cell cycle progression through ubiquitination and subsequent degradation of several proteins linked to cell cycle inhibition, including p21, p27, p57, p130 and RASSF1A among others(104). In contrast, ICG-001 increased CBP occupancy at *CDKN1A* promoter site in AsPC-1 cells, perhaps by increasing the availability of CBP to different subsets of transcription factors mediating *CDKN1A* expression. *CDKN1A* encodes the cyclin-dependent kinase inhibitor p21 that oppose the function of multiple cyclin-CDK complexes that mediate G1 and S cell cycle progression. Increased p21 levels with ICG-001 could be due to increased *CDKN1A* expression via CBP co-transcriptional activation as well as decreased *SKP2* mediated degradation.

Although *MYC* transcript levels were unchanged, ICG-001 reduced *MYC* protein levels within 6 hours with corresponding increases in several known targets of *MYC*-mediated transcriptional repression involved in cell cycle (i.e., *CDKN1A*, *CDKN1C* and *CDKN2B*). It is intriguing to further speculate how ICG-001 might directly antagonize *MYC* through additional post-translational mechanisms linked to its protein stability or function. *MXD1* and *MXI1*, which were both upregulated in response to ICG-001 treatment, can inhibit

the transcriptional activity of MYC by competing for its important heterodimerization partner MAX(105). CBP/p300-mediated acetylation has been shown to stabilize MYC protein and function(106), which could also be perturbed by ICG-001. Finally, ICG-001 could indirectly disrupt MYC activity through its aforementioned downregulation of SKP2, which has been shown to ubiquitylate MYC to license its function as a transcriptional activator (107).

In conclusion, ICG-001 appears to robustly inhibit PDAC growth through both direct and indirect mechanisms that mutually reinforce the induction of G1 cell cycle arrest. While further studies are needed to expand upon precise mechanisms underpinning its transcriptional and phenotypic activities, ICG-001 appears to more broadly disrupt CBP function beyond its role as co-transcriptional activator of Wnt/ β -catenin signaling (Figure 3-6F). These studies should not only further examine the therapeutic potential of ICG-001 and its derivatives in PDAC and other cancers, but also hopefully better inform future drug combination approaches and patient selection.

Materials and Methods

Cell Lines and Reagents

All cell lines were cultured as previously described(39). AsPC-1, MiaPaCa-2 and PANC-1 were obtained in 2005 from the American Type Culture Collection. L3.6pl was obtained in 2010 from Hong Wu (UCLA). ICG-001 was purchased from Selleck Chem and gemcitabine was kindly provided by Timothy Donahue (UCLA). Wnt3a and L-cell conditioned media were prepared as previously described(39).

Cell growth, proliferation and apoptosis assays

MTT cell viability assays (ATCC) were carried out per manufacturer's instructions and initial plating at 5,000 (AsPC-1 and L3.6pl) or 3,000 (MiaPaCa-2 and PANC-1) cells per well in 96-well plates. Soft agar assays were performed as previously described(39) in 48-well format. Media and drug were replenished once every 3-4 days. For cell cycle analysis, treated cells were stained using hypotonic DNA staining buffer: 0.1mg/ml propidium iodide (Calbiochem), 20µg/ml RNaseA, 1mg/ml sodium citrate and 0.3% Triton X-100. Flow cytometry analysis was performed in the UCLA Flow Cytometry Core using a Becton Dickinson FACSCalibur cytometer and using ModFit LT v3.1 software. Annexin V-PI flow cytometry analysis was performed with Cellquest v3.3 software using an Annexin V FITC Apoptosis Detection Kit (BD Biosciences).

Orthotopic Xenograft Tumor Assay

Animal work was performed with oversight by the UCLA Division of Laboratory Animal Medicine and approval from the UCLA Animal Research Committee. Orthotopic tumors were established by injecting 5×10^5 AsPC-1 cells suspended in 50% Matrigel (BD Biosciences)/RPMI into surgically-exposed pancreatic tails of 6-week old female athymic nude mice (Charles River) as previously described(108). Intraperitoneal drug injections were begun 10 days after tumor cell injections in animals randomized to four treatment arms: vehicle alone (20% PEG300, 5% solutol, 3.75% dextrose, 1% DMSO in PBS) (n=12), ICG-001 (5 mg/kg 6 days/week) (n=12), gemcitabine (25 mg/kg 2 days/week) (n=11) or ICG-001 + gemcitabine (n=12). Treatment was for 8 weeks and

then discontinued for the remaining duration of the study (up to 120 days post-surgery). Animals were monitored daily and sacrificed when reaching a Body Conditioning Score of 2 (109) or palpable tumor of 1.5 cm in diameter per institutional guidelines. Full necropsy was performed at the time of death with snap frozen and formalin-fixed tissue sections taken of primary and metastatic tumors, as well as normal spleen, lung, liver, small and large intestine. Histologic analysis was performed with H&E stained sections by a practicing surgical pathologist (DWD) blinded to treatment arms. Ten random high power fields per tumor were analyzed for mitotic counts. A previously described dissemination score awarding points based on tumor infiltration and metastasis was used to quantify tumor spread(92).

Wnt reporter assays

Dual luciferase assays (Promega) were performed as previously described(39) on cell lines stably transduced with lentiviral constructs containing β -catenin-activated reporter (BAR) driving firefly luciferase expression and separate normalization control construct with constitutive EF1 α promoter driving Renilla expression.

Gene Knockdown

Lipofectamine 2000 was used per manufacturer's instructions to transfect AsPC-1 cells with 20 nM control siRNA (#12935-200) or *CTNNB1* s437 (#4390824) siRNA (all purchased from Life Technologies). Western blots to verify knockdown were performed using β -catenin (Sigma, C2206) and tubulin (Santa Cruz, sc-5546) antibodies.

Real-time PCR

RNA extraction, cDNA synthesis and SYBR Green-based quantitative PCR were performed as previously described(39). For a list of primers used see Arensman et al(56).

Western Blots and Immunoprecipitation

Western blots were performed as previously described (39) using antibodies specific for β -catenin (Sigma C2206), PARP (Cell Signaling Technology (CST) 9542), Cleaved Caspase-3 (CST 9664), Cleaved Caspase-7 (CST 9491), SKP2 (CST 2652), MYC (Santa Cruz sc-40), p21 (CST 2947) or tubulin (Santa Cruz sc-5546). CBP co-immunoprecipitations were performed on nuclear extracts as previously described(110) with anti-CBP (Santa Cruz, sc-369) or control isotype-matched IgG (Abcam, ab46540) antibodies.

Chromatin Immunoprecipitation

AsPC-1 cells (70% confluency) were crosslinked with formalin. Chromatin immunoprecipitation (ChIP) was performed using the Pierce Agarose ChIP Kit (Thermo Scientific) on chromatin digested with 2U micrococcal nuclease. Digested chromatin extracts were precleared with 20 ug agarose beads and incubated overnight with beads pre-bound with either 5 μ g IgG control or CBP antibody (Abcam, ab46540 and ab2832, respectively) blocked with 1 μ g fish sperm DNA. PCR was performed using primers (see Arensman et al(56)) covering gene regulatory regions for *CDKN1A* (-231 to -117, relative to transcriptional start site, TSS) and *SKP2* (+1772 to +1870 relative to TSS).

Gene expression microarray and data analysis

RNA was extracted from three biological replicates of AsPC-1 cells 6 and 24 hours post-treatment with 10 μ M ICG-001 or vehicle control (DMSO) or in separate experiments from two biological replicates of AsPC1 cells transfected for 48 hours with either 20 nM control or *CTNNB1* siRNA. Affymetrix U133 plus 2.0 oligonucleotide arrays were processed in the UCLA Clinical Microarray Core. Arrays are available in Gene Expression Omnibus database repository (GSE57728). Data analysis was performed with dChip Analysis software package(111) using invariant set normalization and signal intensities summarized using the MBEI (model-based expression index) algorithm with mismatch probe option for background subtraction. Initial gene filtering criteria was set at >10% present call and variance across samples of $0.4 < \text{standard deviation/mean} < 1000$. ICG-001-regulated genes were determined by comparative analysis of ICG-001 versus vehicle control (selection criteria >1.67-fold change and $P < 0.01$ at 6 hours or >2-fold change and $P < 0.01$ at 24 hours). β -catenin-regulated genes were determined by comparative analysis of *CTNNB1* versus control siRNA transfection (selection criteria ≥ 1.33 -fold change and >50 absolute change). Functional enrichment was examined using default parameters in the web-based Database for Annotation, Visualization and Integrated Discovery (DAVID, v6.7) (<http://david.abcc.ncifcrf.gov>) (112). Analysis was limited to the GO biological process FAT (GOTERM_BP_FAT) category terms and ranked on P -values after removal of categories with less than 6 annotated genes.

Statistics

Student *t* tests were used to compare continuous variables. The Cox proportional hazard model was used to estimate hazard ratios between treatment groups. The assumption of proportionality was assessed both by visual inspection of Kaplan-Meier survival curves and formal analysis of Schoenfeld residuals (p.val=0.13). Kaplan-Meier survival curves and log rank tests were performed using GraphPad Prism.

Figures

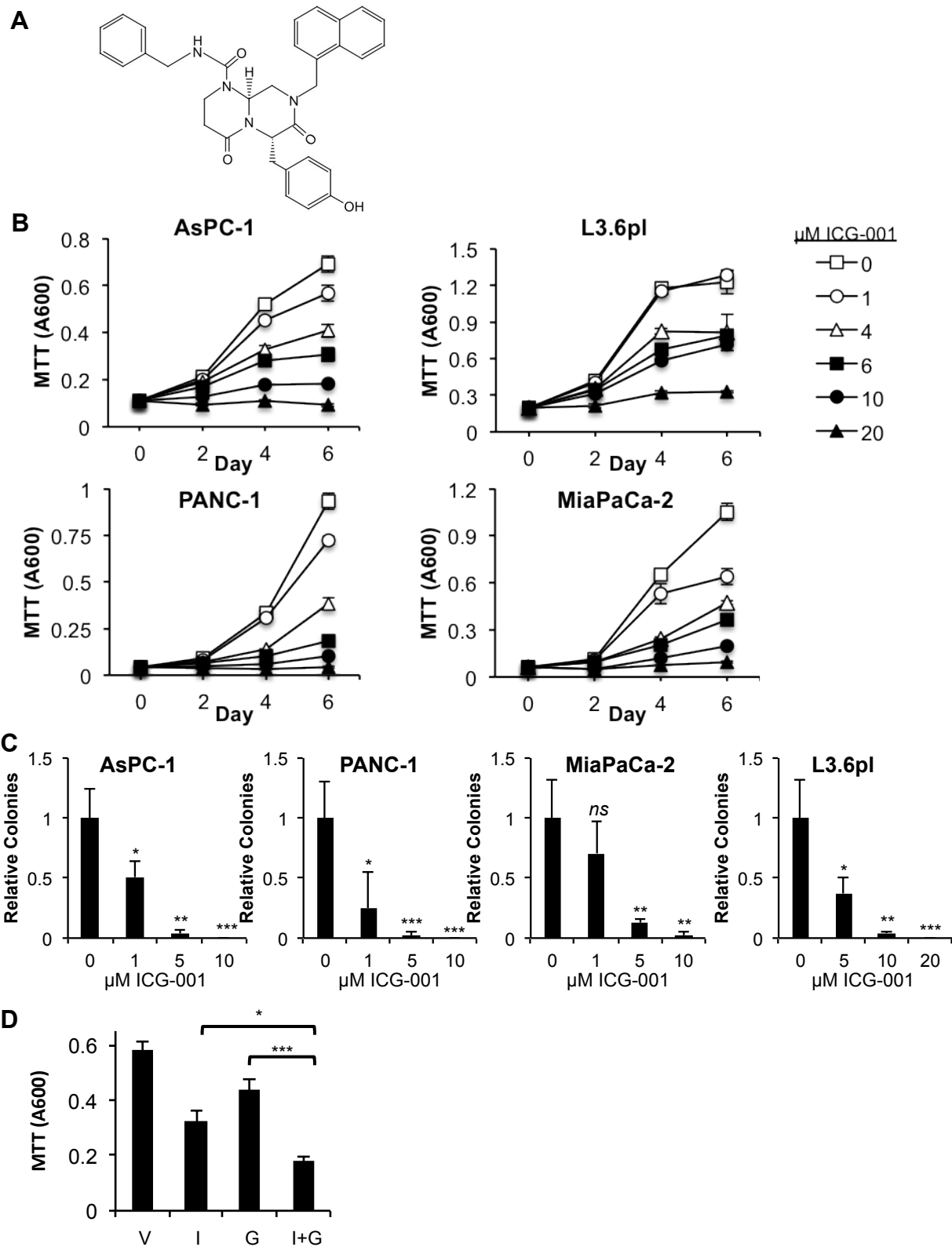


Figure 3-1. ICG-001 inhibits *in vitro* anchorage dependent and independent growth of pancreatic cancer lines. (A) Chemical structure of ICG-001. (B) MTT cell viability and (C) soft agar assays were performed at the indicated concentrations of ICG-001. (D) MTT assay on AsPC-1 cells treated for 6 days with vehicle (V), 5 μ M ICG-001 (I), 5 nM gemcitabine (G) or 5 μ M ICG-001 plus 5 nM gemcitabine (I+G). Representative experiment from at least three replicates is presented for each cell line. * P <0.05, ** P <0.01, *** P <0.001, *ns*=not significant.

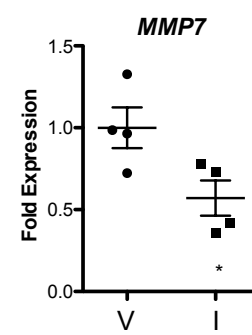
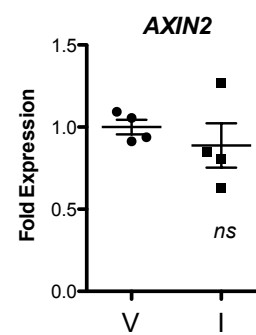
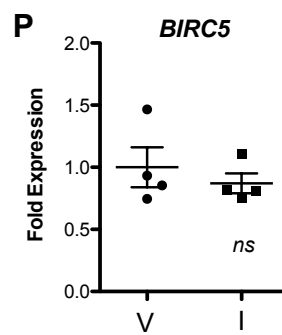
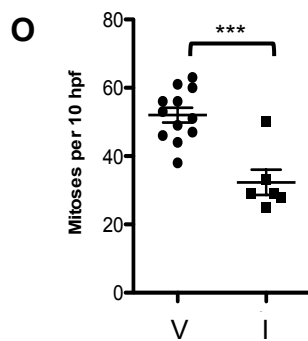
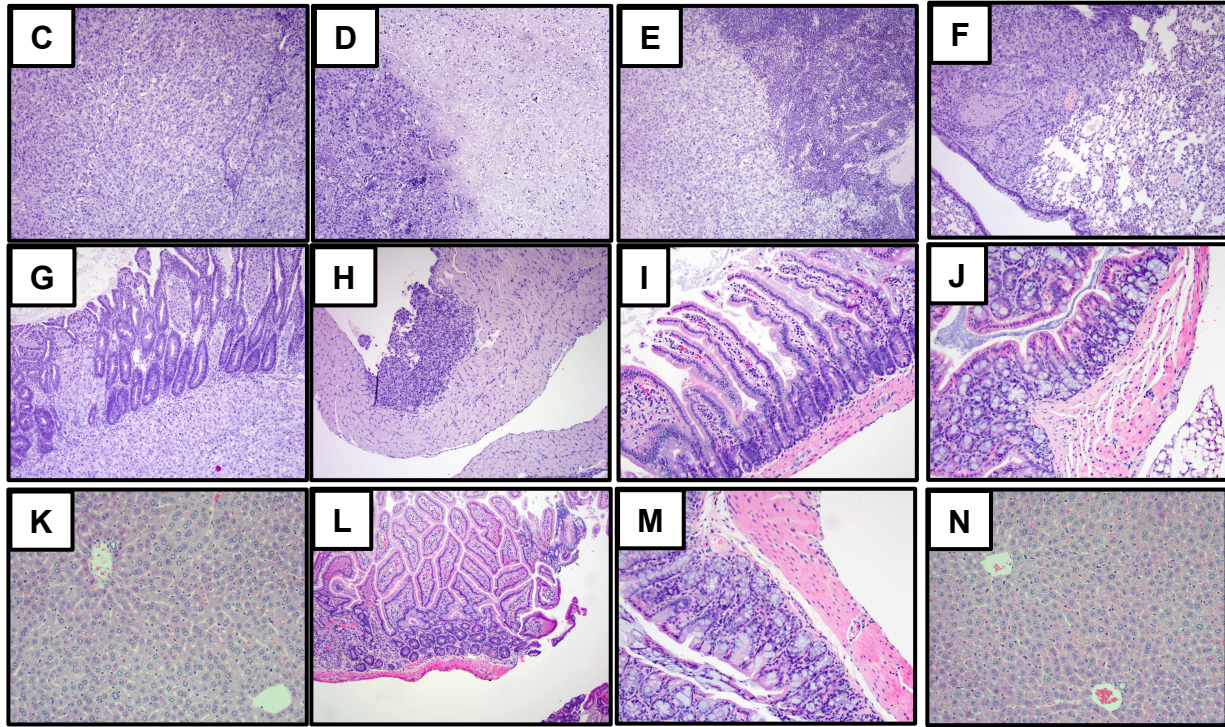
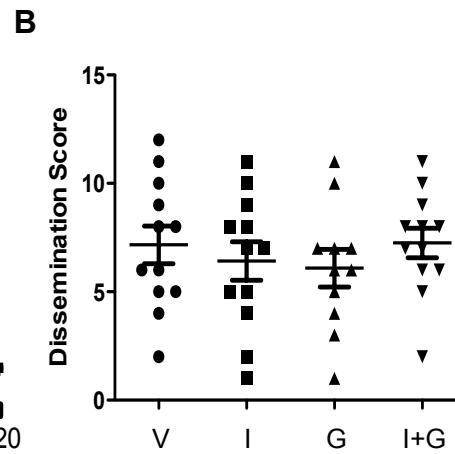
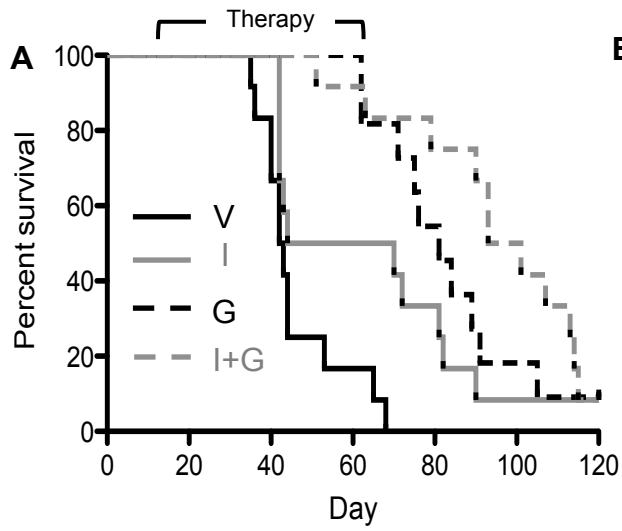


Figure 3-2. ICG-001 shows *in vivo* efficacy against pancreatic cancer. (A) Kaplan-Meier survival curves for mice treated with vehicle (V), ICG-001 (I), gemcitabine (G) or ICG-001 plus gemcitabine (I+G). (B) Tumor dissemination scores were determined at the time of sacrifice for all mice in the four treatment arms. (C-N) Representative H&E images (20x objective) of the orthotopic xenograft model include (C) viable primary tumor, (D) primary tumor with large area of geographic necrosis, (E) direct extension of tumor into spleen and distant metastatic tumor nodules involving (F) lung, (G) small bowel and (H) diaphragm. Representative sections of normal tissues with no histopathologic abnormalities are shown for mice sacrificed 28-32 days into their treatment course with (I-K) vehicle or (L-N) ICG-001. Sections include (I,L) small bowel, (J,M) colon and (K,N) liver showing no architectural or cytologic abnormalities to suggest cytotoxicity. (O) Mitotic counts in 10 random high power fields and (P) qPCR analysis of indicated Wnt gene targets normalized to *ACTB* housekeeping gene were determined in primary tumors taken from mice still receiving treatment at time of death. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, *ns*=not significant.

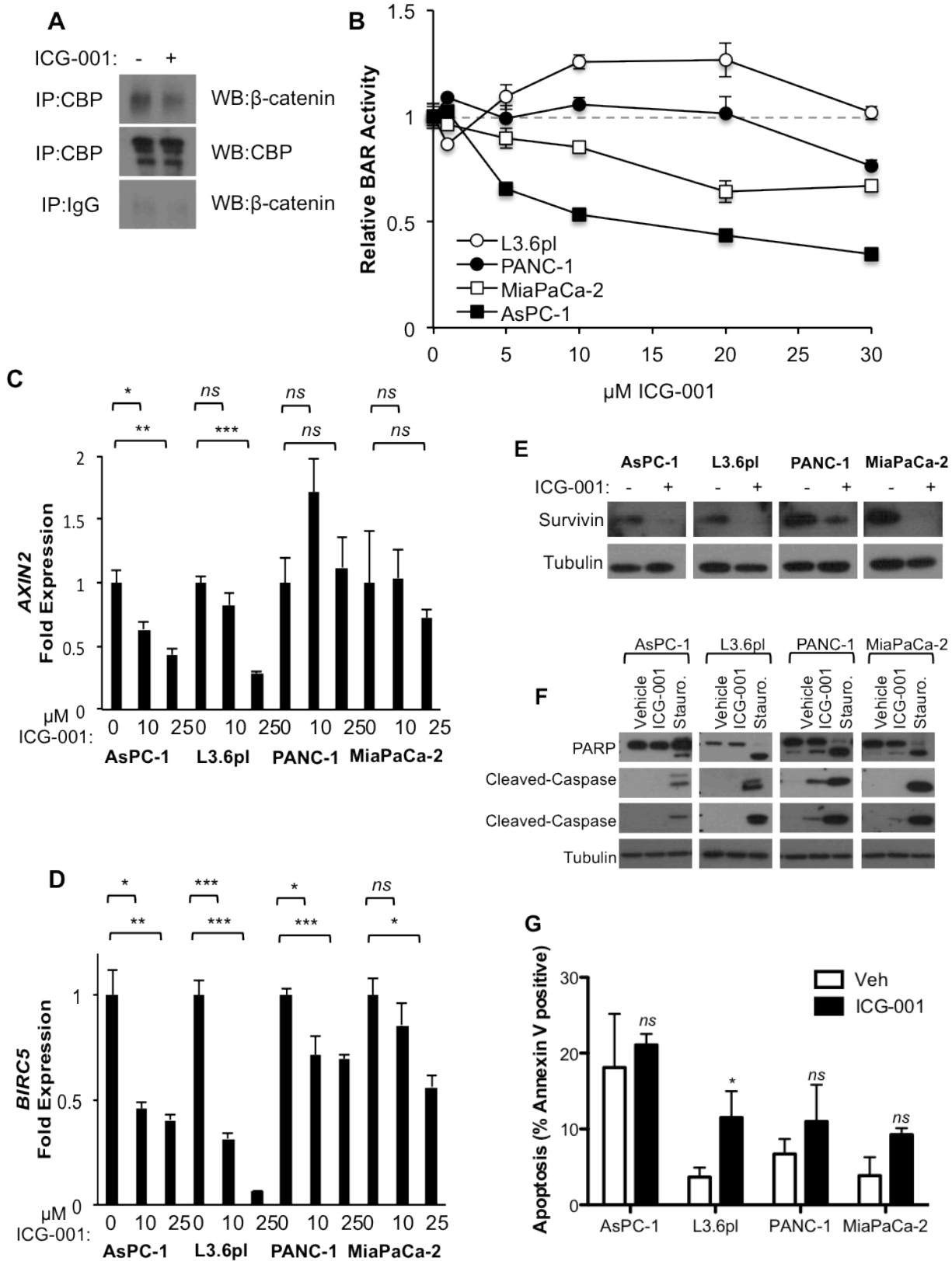


Figure 3-3. ICG-001 variably influences Wnt transcriptional activity and apoptosis. (A) Nuclear extracts from AsPC-1 cells treated with vehicle or 30 μ M ICG-001 for 6 hours were immunoprecipitated with anti-CBP or control IgG antibodies followed by western blots for β -catenin and CBP. (B) PDAC cell lines carrying stably integrated Wnt/ β -catenin luciferase reporter (BAR) were treated with indicated concentrations of ICG-001 and measured in dual luciferase reporter assays at 24 hours. Luciferase is normalized to Renilla and shown relative to vehicle. (C) *AXIN2* and (D) *BIRC5* transcript levels were determined by qPCR in PDAC cell lines treated with indicated concentrations of ICG-001. All values were normalized to *ACTB* and shown relative to vehicle. One representative experiment from at least two biological replicates is shown. (E) Western blot for survivin and tubulin (loading control) after 48 hours vehicle or 10 μ M ICG-001. (F) Cells were incubated with vehicle, 10 μ M ICG-001 or 500 nM staurosporine (Stauro, positive control for apoptosis) for 24 hours and analyzed by western blot for PARP, cleaved-caspase 3, cleaved-caspase 7 and tubulin (loading control). (G) Vehicle or 10 μ M ICG-001 treated cells were analyzed by flow cytometry for apoptosis by annexin V-PI staining at 48 hours. One representative experiment from three replicates is shown. * P <0.05, ** P <0.01, *** P <0.001, *ns*=not significant.

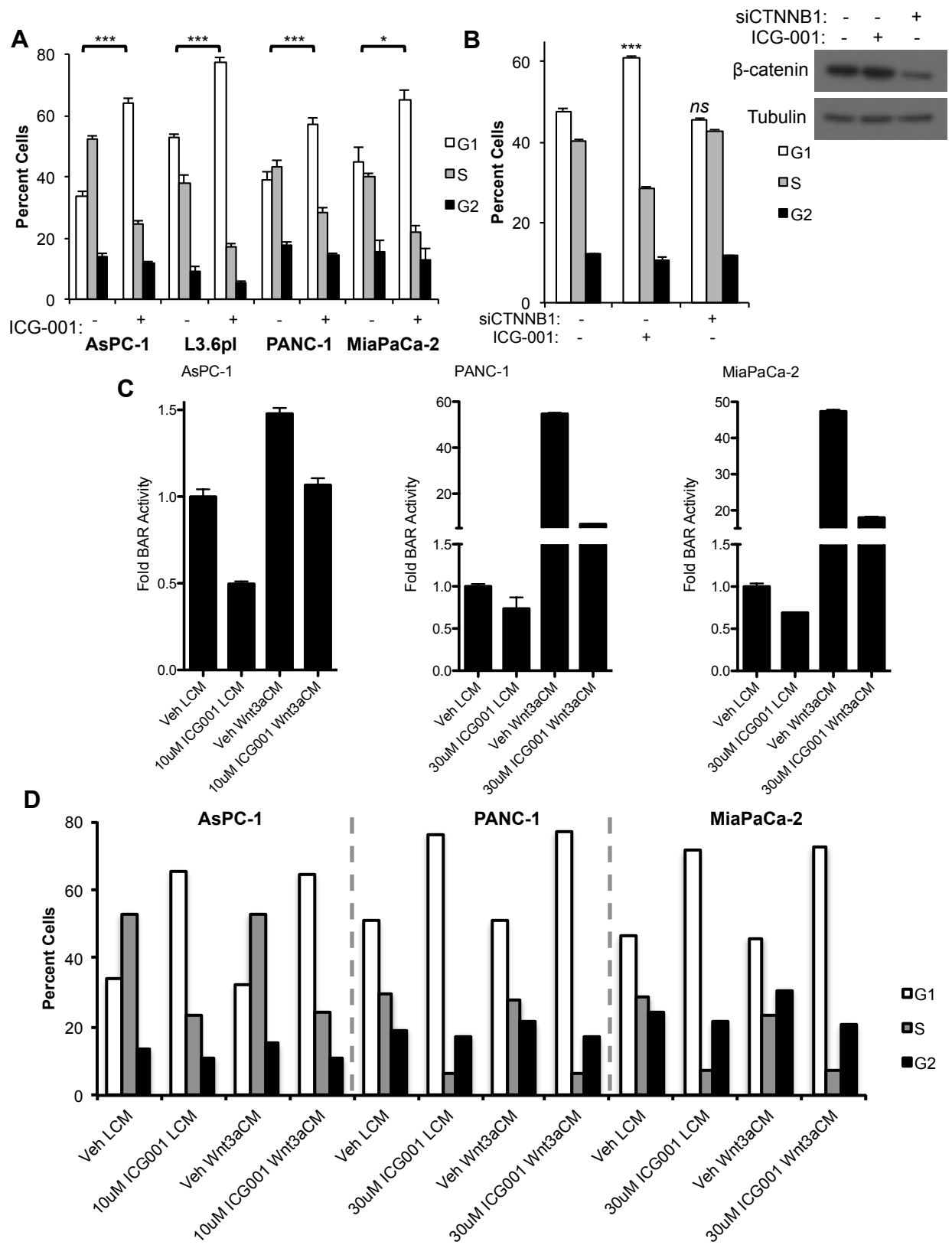


Figure 3-4. ICG-001 robustly induces G1 cell cycle arrest. (A) Vehicle or 10 μ M ICG-001 treated cells were analyzed by flow cytometry for cell cycle by propidium iodide staining at 24 hours. (B) Cell cycle distribution was determined for AsPC-1 cells treated simultaneously with indicated combinations of vehicle, 10 μ M ICG-001, control siRNA (20 nM) or CTNNB1 siRNA (20nM) for 48 hours. Western blot confirmed >75% knockdown of β -catenin with CTNNB1 siRNA. One representative experiment of three replicates is shown. Wnt3a conditioned media (C) rescued ICG-001 inhibition of the BAR reporter but (D) did not rescue ICG-001 mediated G1 arrest. Indicated cell lines were treated with either 50% Wnt3a media or L-cell conditioned media (control) in combination with specified concentrations of ICG-001 or vehicle control. Dual luciferase reporter assays and propidium iodide flow analysis were performed at 24 hours.

*P<0.05, **P<0.01, ***P<0.001, *ns* = not significant.

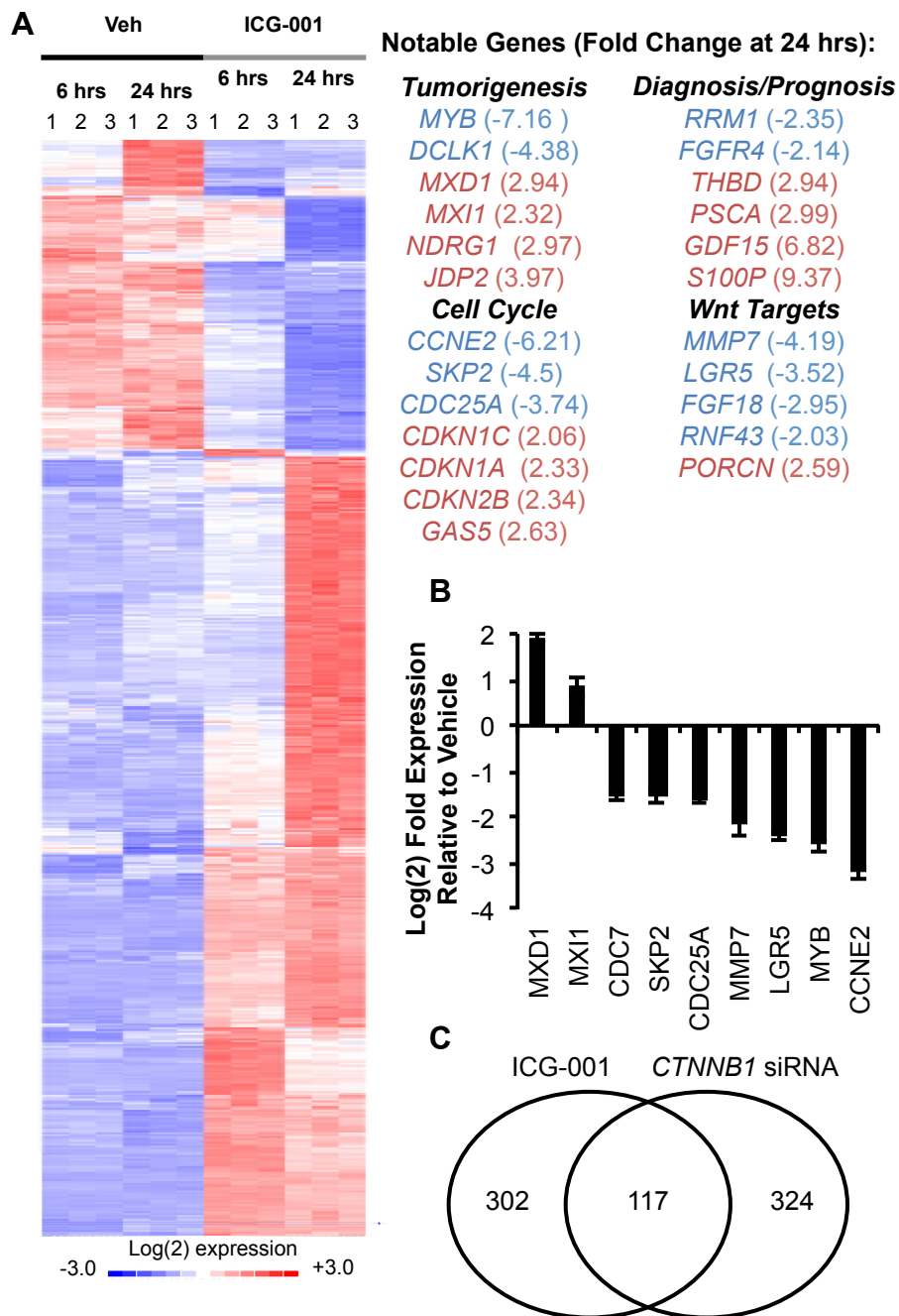


Figure 3-5. ICG-001 alters the expression of cell cycle genes. (A) Gene expression was determined with Affymetrix Human U133 plus 2.0 arrays using RNA collected from AsPC1 following 6 or 24 hour treatment with vehicle or 10 μ M ICG-001. Biological replicates are shown on the heatmap of significantly regulated genes along with a

partial curated list of genes based on known roles in tumorigenesis, cell cycle control, cancer diagnosis/prognosis or Wnt/ β -catenin transcriptional targets. (B) qPCR validation was performed on selected genes in AsPC-1 cells separately treated with vehicle or 10 μ M ICG-001 for 24 hours. (C) Venn diagram comparing AsPC-1 gene expression microarray changes observed with either 10 μ M ICG-001 treatment or *CTNNB1* siRNA transfection.

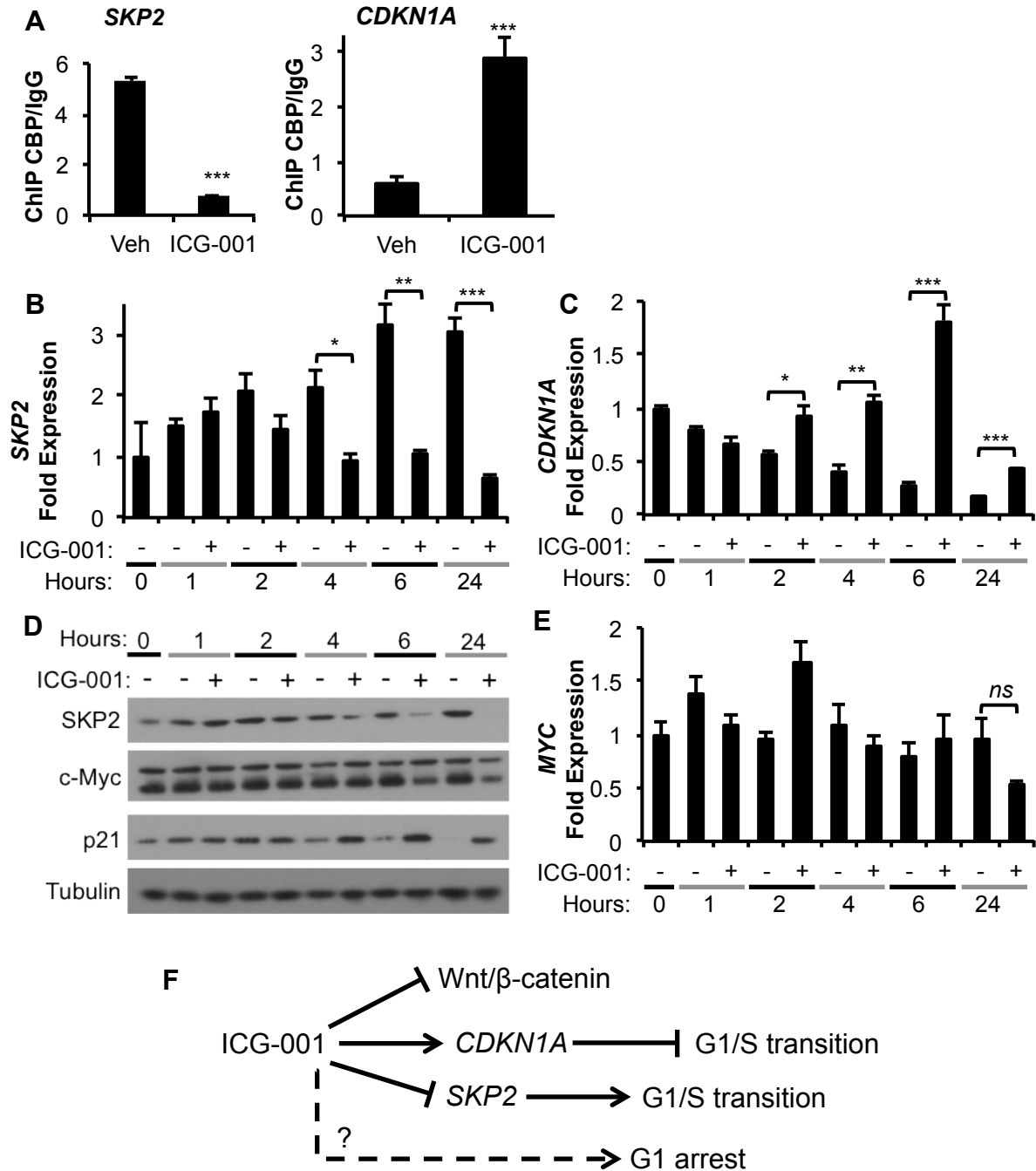


Figure 3-6. ICG-001 directly regulates change in *SKP2* and *CDKN1A* expression.

(A) Chromatin immunoprecipitation results for CBP association with *SKP2* and *CDKN1A* regulatory elements in AsPC-1 cells following 1 hour treatment with vehicle or 10 μ M ICG-001. Data were normalized to input DNA and are presented as fold change of

CBP/IgG. Representative experiments of three replicates are shown. (B-E) Time course study following treatment of AsPC-1 cells with vehicle or 10 μ M ICG-001 measuring (B) *SKP2* and (C) *CDKN1A* gene expression by qPCR, (D) SKP2, MYC, p21 and tubulin (loading control) protein expression by western blot and (E) *MYC* gene expression by qPCR. All qPCR values were normalized to ACTB and shown relative to 0 hour. (F) Schematic illustrating ICG-001 effects on cell cycle. Representative experiments from at least two replicates are shown for all qPCR and western blots. *P<0.05, **P<0.01, ***P<0.001, ns=not significant.

CHAPTER 4: CALCIPOTRIOL TARGETS LRP6 TO INHIBIT WNT SIGNALING IN PANCREATIC CANCER

Abstract

Pancreatic ductal adenocarcinoma (PDAC) is an aggressive malignancy in need of more effective therapeutic approaches. One potential therapeutic target is Wnt/ β -catenin signaling, which plays important roles in PDAC tumor initiation and progression. Among Wnt inhibitors with suitable *in vivo* biological activity is vitamin D, which has been shown to antagonize Wnt/ β -catenin signaling in colorectal cancer and has been documented to have anti-tumor activity in PDAC. For this study the relationship between vitamin D signaling, Wnt/ β -catenin activity and tumor cell growth in PDAC was investigated through the use of calcipotriol, a potent non-hypercalcemic vitamin D analog. PDAC tumor cell growth inhibition by calcipotriol was positively correlated with vitamin D receptor (VDR) expression and Wnt/ β -catenin activity. Furthermore, vitamin D and Wnt signaling activity were functionally linked to one another through reciprocal feedback regulation. Furthermore, calcipotriol was found to inhibit Wnt/ β -catenin signaling in PDAC cell lines through a novel mechanism involving transcriptional upregulation of low-density lipoprotein receptor adaptor protein 1 (LDLRAP1). Calcipotriol-mediated induction of LDLRAP1 inhibited autocrine Wnt/ β -catenin signaling in PDAC lines by decreasing levels of low-density lipoprotein receptor-related protein 6 (LRP6), a requisite co-receptor for ligand-dependent canonical Wnt signaling.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is a highly recalcitrant malignancy with an overall five-year survival rate of 6%(15). Its poor prognosis is linked to its typical advanced stage at diagnosis, rapid disease progression and resistance to cytotoxic chemotherapy(19). Improved therapeutic approaches based on a detailed understanding of the molecular and cellular basis of PDAC are needed. One therapeutic target now receiving attention is Wnt/ β -catenin signaling, one of twelve core pathways and processes genetically altered in most PDAC(4).

Wnt signaling plays pivotal roles in embryogenesis and adult tissue homeostasis through its regulation of diverse biological functions, including cell fate specification, proliferation and cell migration. Wnt ligands are secreted glycoproteins that signal through Frizzled (FZD) receptor and low-density lipoprotein receptor-related protein 5 or 6 (LRP5 or LRP6) co-receptor. Activation of canonical Wnt signaling (or Wnt/ β -catenin-dependent signaling) culminates in the cytoplasmic accumulation of β -catenin and its translocation to the nucleus where it forms complexes with transcription factors and co-activators to drive context-dependent target gene expression(113). Deregulation of Wnt/ β -catenin signaling occurs in many types of cancer, typically through key driver mutations or increased Wnt ligand-mediated receptor activation. In PDAC, Wnt/ β -catenin signaling is required for tumor initiation(13) and confers traits associated with “stemness” in susceptible cell lines(49). Furthermore, Wnt/ β -catenin signaling supports various pro-tumorigenic phenotypes in established PDAC cell lines and correlates with aggressive clinical behavior in primary PDAC tumor samples(11, 13, 39).

Several Wnt inhibitors are now at various stages of preclinical and early clinical development(80). They include small molecule inhibitors, blocking antibodies, inhibitory fusion proteins, peptide antagonists and naturally occurring compounds, including vitamin D. Calcitriol, the biologically active metabolite of vitamin D and ligand of the vitamin D receptor, has been shown to disrupt Wnt/ β -catenin signaling through various mechanisms. In colon cancer lines, calcitriol induces the vitamin D receptor (VDR) to directly bind β -catenin and increases E-cadherin expression to retain β -catenin at adherens junctions, ultimately resulting in reduced β -catenin/TCF interaction and target gene expression(114). Calcitriol also indirectly increases the expression of DKK1, a secreted protein antagonist of Wnt/ β -catenin signaling(115).

Vitamin D is acquired through dietary intake and ultraviolet-light mediated metabolism of its precursors in the skin. Vitamin D metabolites bind to VDR, a transcription factor which functions as an obligate heterodimer with the retinoid X receptor (RXR) to regulate the expression of target genes with vitamin D response elements. In addition to regulating calcium homeostasis, vitamin D exhibits anti-cancer action through a variety of anti-proliferative, pro-apoptotic and differentiation effects. Accordingly, vitamin D and its analogs are promising chemopreventative and chemotherapeutic agents for numerous malignancies(116). In relation to PDAC, certain epidemiologic studies show higher intake or increased plasma levels of vitamin D correlates with decreased incidence of PDAC(117, 118). Although a published meta-analysis concludes there is no significant association between vitamin D levels and PDAC

risk(119), a recent study detected a high prevalence of vitamin D insufficiency in patients with advanced pancreatic cancer(120). Published studies show vitamin D and its synthetic analogs inhibit the *in vitro* and *in vivo* growth of some but not all PDAC cell lines through pro-apoptotic and anti-proliferative actions (121-123). A more recent study by Sherman, et al., finds VDR serves as a master transcriptional regulator of pancreatic stellate cell quiescence with the vitamin D analog calcipotriol able to suppress pancreatitis and mediate stromal remodeling to improve chemotherapeutic drug delivery and survival in a *Kras*-driven mouse model of PDAC(124).

Here we examined potential interplay between Wnt/ β -catenin and vitamin D signaling in PDAC. The ability of calcipotriol to inhibit PDAC growth was strongly correlated with expression of VDR, which was correlated with and regulated by Wnt signaling. We further show calcipotriol induces expression of low-density lipoprotein receptor adaptor protein 1 (LDLRAP1) that, in turn, mediates rapid reduction in LRP6 protein and corresponding inhibition of Wnt signaling, offering a novel mechanism by which vitamin D is able to negatively regulate Wnt signaling activity. Our data indicate that, in addition to their use as stromal targeting agents, vitamin D analogs may be suitable direct anti-cancer agents for subsets of PDAC tumors and precursor lesions with higher levels of Wnt-dependent growth activity and VDR expression.

Results

PDAC growth inhibition by calcipotriol is linked to VDR expression and Wnt signaling

In previous work, we have described a distinct subset of PDAC cell lines with high levels of autocrine Wnt/ β -catenin signaling and whose tumor growth is distinctly responsive to genetic or pharmacologic inhibition of Wnt(39). Given that vitamin D inhibits Wnt signaling in colon cancer(114, 115), we examined the correlation between the growth-inhibitory effects of calcipotriol and variable autocrine Wnt/ β -catenin signaling activity across a panel of PDAC cell lines. Baseline Wnt/ β -catenin signaling was measured in dual luciferase promoter-reporter assays by determining the ratio of BAR (beta-catenin activated reporter, 12 TCF response elements driving luciferase expression) to fuBAR (found unresponsive BAR, contains mutated TCF response elements)(36). AsPC1, HPAF-2, YAPC and Suit2 each had higher levels of autocrine Wnt activity (High-BAR), while MiaPaCa-2, PANC-1 and HCG25 each had lower or absent levels of autocrine Wnt activity (Low-BAR)(Figure 4-1A). Interestingly, these levels of autocrine Wnt activity paralleled VDR protein expression with western blots of High-BAR lines demonstrating higher levels of VDR expression (High-VDR) and Low-BAR lines showing low or undetectable levels of VDR (Low-VDR) (Figure 4-1A). Accordingly, calcipotriol inhibited anchorage-dependent growth of High-BAR/High-VDR cell lines in a dose-dependent manner at concentrations ranging from 10 nM to 1 μ M, while calcipotriol at concentrations up to 10 μ M was unable to inhibit the growth of Low-BAR/Low-VDR cell lines (Figure 4-1B). Calcipotriol also inhibited anchorage-independent growth of High-BAR/High-VDR cell lines in soft agar assays (Figure 4-1C), a phenotype we had previously shown to be specifically tied to Wnt signaling in these

High-BAR PDAC lines(39). These results correlate growth inhibition by calcipotriol to VDR expression status and suggest variations in vitamin D response in PDAC may be linked to Wnt activity, whereby Wnt/ β -catenin signaling may serve as a mediator and/or target of vitamin D action.

To further explore possible mechanistic links between vitamin D and Wnt signaling in PDAC, AsPC-1 cells stably transduced with BAR were treated with increasing concentrations of calcipotriol. Calcipotriol significantly inhibited Wnt/ β -catenin activity in a dose-dependent manner as measured by BAR reporter (Figure 4-2A). Calcipotriol also significantly inhibited expression of the endogenous Wnt/ β -catenin transcriptional target *AXIN2* across the panel of High-BAR/High-VDR cell lines (Figure 4-2B). To confirm calcipotriol inhibited Wnt signaling in a VDR-dependent manner, calcipotriol effects on BAR reporter was determined in the context of siRNA-mediated *VDR* knockdown. *VDR* knockdown fully rescued inhibition of reporter seen with calcipotriol treatment (Figure 4-2C-D), indicating that Wnt inhibition by calcipotriol was VDR-dependent and not mediated through off-target or receptor-independent actions.

We next explored the mechanism by which calcipotriol inhibits Wnt activity in PDAC. In colon cancer cell lines, ligand-activated VDR directly interacts with β -catenin, leading to cytoplasmic/plasma membrane sequestration of β -catenin and inhibition of β -catenin/TCF mediated transcription(114). Addressing this possibility in PDAC, cell extracts of AsPC-1 treated with either vehicle or calcipotriol were immunoprecipitated with anti- β -catenin antibody. While E-cadherin co-immunoprecipitated with β -catenin as

expected, VDR did not co-immunoprecipitate with β -catenin either in the presence of absence of calcipotriol (Figure 4-3A). Reverse co-immunoprecipitation with anti-VDR antibody also failed to demonstrate an interaction between VDR and β -catenin (data not shown), indicating Wnt inhibition by calcipotriol in PDAC does not occur through ligand-activated sequestration of β -catenin by VDR. Calcitriol has also been proposed to increase expression of the secreted Wnt inhibitor DKK1 through an indirect transcriptional mechanism(115). While calcipotriol increased DKK1 protein levels in AsPC-1, the increase in DKK1 appeared to be functionally decoupled from inhibition of Wnt signaling by calcipotriol, as calcipotriol-mediated inhibition of BAR activity was not rescued by concurrent siRNA-mediated knockdown of *DKK1* (Figure 4-3B-C). Together, these results indicate that calcipotriol inhibits Wnt signaling in PDAC through an alternative mechanism.

Calcipotriol reduces LRP6 protein levels

LRP6 is a requisite co-receptor for Wnt ligand, and mediates canonical signaling. Notably, LRP6 protein levels decreased within 6 hours of calcipotriol treatment in all High-BAR/High-VDR PDAC lines (Figure 4-4A-B), but not in Low-BAR/Low-VDR PDAC lines (Figure 4-4C). In addition, transient overexpression of LRP6 in AsPC-1 partially but significantly abrogated the inhibition of BAR activity by calcipotriol (Figure 4-4D), leading us to hypothesize that LRP6 might be a key target for the Wnt inhibitory effects of calcipotriol. Further supporting this hypothesis, siRNA knockdown of VDR blocked the decrease in LRP6 protein observed with calcipotriol treatment (Figure 4-2C).

To discriminate between genomic versus potential non-genomic mechanisms of action of VDR on LRP6, the VDR transcriptional co-activators RXR α and RXR β were concurrently knocked down by siRNA transfection. Combined RXR α /RXR β knockdown also blocked the decrease in LRP6 seen with calcipotriol, arguing in favor of a VDR/RXR-dependent transcriptional mechanism (Figure 4-4E). However, while calcipotriol decreased LRP6 protein levels as early as 6 hours in each of the High-BAR/High-VDR PDAC lines, *LRP6* transcript levels were found to only be decreased after 24 hours in AsPC-1, HPAF-2 and YAPC (Figure 4-4F-G) and not at all in Suit2 (Figure 4-4G). These data suggest calcipotriol inhibits LRP6 expression and Wnt signaling in PDAC through an indirect VDR/RXR-dependent transcriptional mechanism given that LRP6 protein decreases prior to any effect on *LRP6* transcript levels.

LDLRAP1 mediates reduced LRP6 protein levels in response to calcipotriol

To further explore the mechanism by which LRP6 protein decreases in response to calcipotriol, RNA sequencing (RNA-seq) was performed on AsPC-1 cells. Comparison of transcriptomes following calcipotriol treatment confirmed expression of known vitamin D transcriptional targets, including *CYP24A1* as the most highly upregulated gene (Figure 4-5A). A total of 325 genes (220 upregulated and 105 downregulated) showed at least 1.5-fold change in expression at 6 hours ($P < 0.00001$). Top gene ontology terms enriched in the subset of the top 220 upregulated genes included regulation of transcription from RNA polymerase II promoter, osteoblast differentiation, positive regulation of cellular biosynthetic processes and response to steroid hormone stimulus ($P < 0.0005$). Additionally, for the top 100 calcipotriol-regulated genes at 6 hours we

noted enrichment of categories such as the endomembrane system (i.e., *LDLRAP1*, *ULK1*), wound response (i.e., *CEBPB*, *PDGFA*, *SHH*, *THBD*), and cellular homeostasis (i.e., *CLDN1*, *CSF1*), as well as several genes with established roles in BMP and Wnt signaling (Figure 4-5A).

In relation to the endomembrane system, the cytoplasmic adapter protein *LDLRAP1* was highly upregulated at both 2 and 6 hours post calcipotriol treatment (Figure 4-5A). *LDLRAP1* is required for efficient endocytosis of LDL receptors in polarized cells and has been shown to interact with LRP1 and LRP2 to facilitate LDL receptor clustering into clathrin-coated pits(125-127). Notably, internalization of LRP6 via clathrin antagonizes Wnt signaling(128, 129) and promotes LRP6 degradation through endosomal trafficking to lysosomes(130-132). We speculated that *LDLRAP1* could decrease LRP6 protein levels through a similar mechanism involving receptor internalization and degradation. To examine this further, we first established whether lysosomal activity was needed to decrease LRP6 protein levels in response to calcipotriol treatment. Treatment with Bafilomycin A1, a specific inhibitor of vacuolar-type H(+)-ATPase that blocks acidification and protein degradation in lysosomes, abrogated the decrease of LRP6 protein levels seen with calcipotriol treatment (Figure 4-5B). Bafilomycin also increased LRP6 protein levels in the absence of calcipotriol, suggesting a role for lysosomal protein degradation in regulating steady-state levels of LRP6 in PDAC lines. We then separately validated RNA-seq data by qPCR, confirming *LDLRAP1* gene expression was significantly upregulated in AsPC-1 cells within 4 hours of calcipotriol treatment (Figure 4-5C). Western blot analysis confirmed that *LDLRAP1*

protein was also increased with calcipotriol (Figure 4-5D). *In silico* analysis of the *LDLRAP1* locus identified a predicted vitamin D response element (VDRE) at the 3' end of the *LDLRAP1* gene. Evaluation of published ChIP-seq data generated on hepatic stellate cells (133) clearly demonstrated VDR occupancy of the site encompassing this VDRE, raising the possibility that VDR directly binds to and activates transcription of the *LDLRAP1* gene in PDAC cells. Together, these data indicate that *LDLRAP1* is a direct target of VDR-mediated transcription. To link calcipotriol-induced *LDLRAP1* expression to changes in LRP6 protein and Wnt signaling, siRNA-mediated knockdown of *LDLRAP1* was performed. Knockdown of *LDLRAP1* partially rescued the reduction in LRP6 protein (Figure 4-5D) and inhibition of Wnt reporter activity (Figure 4-5E) seen with calcipotriol treatment. These results indicate *LDLRAP1* is a transcriptional target of ligand-activated VDR and can mediate reduction in LRP6 protein and Wnt pathway activity seen with calcipotriol in these PDAC cell lines.

VDR is regulated by Wnt signaling

Given the observed correlation between VDR expression and autocrine Wnt signaling activity across a panel of PDAC cell lines (Figure 4-1A), we addressed whether a similar correlation exists in clinical specimens. Gene set enrichment analysis (GSEA) of a previously published cohort of 25 primary PDAC tumors(134) confirmed positive enrichment of a PDAC-specific Wnt/ β -catenin target gene set in those tumors with higher *VDR* expression (Figure 4-6A, normalized enrichment score = 1.39, false discovery rate q value = 0.08). In order to address whether *VDR* is directly regulated by Wnt/ β -catenin signaling in PDAC, AsPC-1 cells with stable BAR reporter were

transfected with siRNA against *WNT7B*, previously shown to be essential for autocrine Wnt signaling in this cell line(39). *WNT7B* knockdown significantly inhibited BAR activity and also decreased *VDR* expression by ~70% (Figure 4-6B). Separately, the selective GSK3 β inhibitor CHIR99021 was used to activate Wnt/ β -catenin signaling in both High-BAR/High-VDR and Low-BAR/Low-VDR cell lines. CHIR99021 increased Wnt reporter activity as expected, and also increased *VDR* expression (Figure 4-6C), indicating Wnt/ β -catenin signaling increases *VDR* expression in PDAC. These data suggest the potential existence of context-dependent Wnt feedback mechanism regulated through *VDR* when sufficient levels of vitamin D are available to engage *VDR*.

Discussion

Diverse anti-tumorigenic actions of vitamin D have been shown in numerous *in vitro* and *in vivo* preclinical studies of pancreatic cancer(121-124, 135-138). In particular, non-hypercalcemic analogs of vitamin D that circumvent dose-limiting side effects of hypercalcemia and hypercalciuria represent especially attractive chemotherapeutic agents for PDAC and other malignancies. However, to date there are only limited published data addressing the clinical efficacy of vitamin D in PDAC. In a non-randomized phase II clinical trial of advanced stage PDAC the vitamin D analog seocalcitol (EB1089) was well tolerated but did not improve patient survival as a single agent(139). However, this study involved only a small cohort of patients with rapid disease progression and patients were not selected based on tumor *VDR* levels or Wnt signaling activity. It has been observed in colon cancer that *VDR* expression decreases

in late-stage tumors(140) and VDR expression in PDAC is lower in poorly differentiated tumors(141, 142), suggesting that the patients in this phase II study may be lacking VDR and could explain why they were unresponsive. Another phase II trial of advanced PDAC involving oral vitamin D (calcitriol) in combination with docetaxel demonstrated prolonged time to disease progression relative to docetaxel alone(143). Offering a potential explanation for this promising result, VDR was recently shown to be a master regulator of PDAC stromal activation with calcipotriol able to inhibit tumor stroma and enhance delivery of gemcitabine chemotherapy in a genetically engineered mouse model of PDAC(124). Clinical trials focused on this exciting result are now underway (NCT#02030860). While stromal targeting is a key functional activity to be considered in the design of an effective vitamin-D-based therapy in PDAC, any stromal effects will also have to be interpreted in relation to potential actions of vitamin D directly on PDAC tumor cells themselves. For instance, calcitriol has been shown directly to potentiate the cytotoxic activity of gemcitabine in PDAC cell lines both *in vitro* and *in vivo*(122), and additional studies have clearly shown VDR expression in pancreatic tumor tissue(141, 142, 144). Our findings here also indicate interaction between VDR and Wnt/ β -catenin signaling in PDAC tumor cells that could dictate their response to vitamin D analogs. Pancreatic stromal cells are also influenced by paracrine Wnt signaling(78), so effects on Wnt pathway activity could have significant bearing on the clinical and biological response of subsets of PDAC tumors to vitamin D analogs. Importantly, newer synthetic vitamin D analogs (i.e., calcipotriol) are resistant to degradation by the product of the VDR target gene *CYP24A1*, which is responsible for rapid feedback inhibition of naturally occurring vitamin D (i.e., calcitriol).

Our calcipotriol results are in line with previous studies showing vitamin D and its analogs inhibit the growth of certain PDAC cell lines (i.e., AsPC-1), while others (i.e., MiaPaCa-2 and PANC-1) are largely unresponsive(123). Our study expands this observation to additional PDAC cell lines and indicates growth inhibitory activity is dependent on the relative levels of VDR expression, which in turn is positively regulated by Wnt/ β -catenin signaling. This bidirectional relationship between Wnt and vitamin D signaling could have significant bearing on identifying patients most likely to respond to vitamin D-based therapy and raises a potential confounding issue related to the use of vitamin D analogs either alone or in combination with other therapeutic Wnt inhibitors. Namely, inhibition of Wnt activity by vitamin D will also reduce Wnt/ β -catenin-mediated expression of *VDR*, which the potential to blunt the Wnt inhibitory response to vitamin D. While positive feed-forward mechanisms (i.e., *VDR* itself is a direct transcriptional target of ligand-activated VDR) may compensate for loss of Wnt signaling-driven *VDR* expression, careful consideration needs to be given to the combination of and order in which various targeted agents (i.e., a second Wnt inhibitor drug) are used in combination with vitamin D analogs.

Calcipotriol inhibited anchorage-independent growth in cell lines with high endogenous Wnt activity, a phenotype we previously attributed to autocrine Wnt signaling in these cell lines(39). However, calcipotriol also inhibited anchorage-dependent growth, a phenotype we previously found to be decoupled from Wnt signaling in these cell lines(39). These divergent results are not surprising given known pleiotropic anti-tumor

effects attributed to vitamin D that extend beyond its actions as an inhibitor of Wnt signaling. Vitamin D-mediated growth inhibitory effects have previously been attributed to cell cycle arrest occurring via upregulation of key regulators of cell cycle such as p21 and p27(121, 123). Indeed, our RNA-seq analysis of calcipotriol treated AsPC-1 cells revealed altered expression of several key mediators of cell cycle (i.e., *SKP2* and *CDKN1A*) as well as apoptosis (i.e., *BIRC5* and *BCL10*).

The effects of vitamin D/calcipotriol could extend beyond inhibition of growth. It has been shown that the vitamin D analog EB1089 inhibits migration, invasion and stemness in PDAC lines(141). Accordingly, our RNAseq revealed significant upregulation of several genes involved in wound response and negative regulation of differentiation. These effects could be attributed to vitamin D inhibition of Wnt signaling as PDAC tumors with high Wnt/ β -catenin transcriptional signature have increased lymphovascular invasion and Wnt signaling mediates stemness in a subset of cell lines(8, 39).

Calcipotriol significantly inhibited Wnt/ β -catenin transcriptional activity in a subset of PDAC cell lines characterized by higher levels of Wnt ligand-mediated autocrine signaling and VDR expression. Vitamin D was previously shown to inhibit Wnt/ β -catenin signaling in colon cancer cell lines by inducing an interaction between VDR and β -catenin that disrupts β -catenin/TCF mediated transcription(114) and by indirectly increasing expression of the secreted Wnt antagonist DKK1(115). We were unable to detect a physical interaction between VDR and β -catenin in PDAC lines either in the

presence or absence of calcipotriol. While calcipotriol did induce the expression of DKK1 in AsPC-1 cells, this could not be functionally linked to its inhibition of Wnt signaling, as DKK1 knockdown did not rescue the inhibition of Wnt signaling seen with calcipotriol. However, our results do not exclude other proposed mechanisms of VDR and β -catenin cross-regulation such as the possibility that calcipotriol could alter the distribution of β -catenin across the genome(145, 146).

Our study reveals a novel mechanism by which vitamin D signaling negatively regulates Wnt signaling. Protein levels of the Wnt co-receptor LRP6 were reduced as early as 6 hours post calcipotriol treatment. This reduction was dependent upon VDR transcriptional activity because knockdown of the VDR co-transcriptional activator RXR also rescued the reduction of LRP6 protein seen with calcipotriol treatment. Rapid reduction of LRP6 protein was not the result of reduced *LRP6* transcript levels, which were only altered 24 hours after calcipotriol treatment, a time point in which *LRP6* transcript levels actually increased in vehicle control and not in calcipotriol treated cells. We speculate that the dramatic increase in *LRP6* transcript seen at 24 hours in vehicle-treated cell lines may be tied to cell cycle-dependent regulation of LRP6 expression(147, 148), while calcipotriol treated cells remained in cell cycle arrest with lower baseline levels of *LRP6* transcript.

Although LRP6 is a critical mediator of Wnt/ β -catenin signaling, overexpression of LRP6 did not completely rescue Wnt activity in the presence of calcipotriol, indicating calcipotriol may inhibit Wnt signaling through additional, undefined mechanisms. RNA-

seq analysis does provide additional potential regulators of Wnt signaling that could be mediated by calcipotriol, including *WNT10A* and *BAMBI*. *WNT10A* expression was decreased by calcipotriol but this gene was not pursued because we previously demonstrated that knockdown of *WNT10A* had no effect on canonical Wnt signaling in AsPC-1 cells(39). *BAMBI* (BMP and activin membrane bound inhibitor) expression was significantly decreased within 2 hours of calcipotriol treatment. *BAMBI* is a known Wnt/ β -catenin agonist that promotes the interaction between Fzd and Dvl(149). In future work we will explore *BAMBI* as an additional potential mechanism of vitamin D-mediated Wnt inhibition.

In this study we focus on calcipotriol induction of *LDLRAP1* and the role of *LDLRAP1* as a regulator of *LRP6* protein levels. *LDLRAP1*, also known as autosomal recessive hypercholesteremia (ARH), is an adapter protein that binds to tyrosine motifs in the cytoplasmic tail of LDL receptors, which couples them to the endocytic machinery of the clathrin coated pit(127, 150). *LDLRAP1* promotes clathrin-mediated endocytosis of *LRP1* and *LRP2*(125-127). Similarly, tyrosine motifs in the cytoplasmic tail of LDL receptor family member *LRP6* are critical for its clathrin-mediated internalization(151). Our data expand the regulatory role of *LDLRAP1* to an additional LDL receptor family member, *LRP6*, and we speculate that *LDLRAP1* facilitates endocytosis and lysosome-mediated degradation of *LRP6*.

In summary, we have elucidated a novel feedback loop between Wnt and vitamin D signaling (Figure 4-6D). Upon activation, Wnt signaling induces expression of *VDR*. In

turn, ligand-activated VDR positively regulates the transcription of *LDLRAP1*, which results in reduced LRP6 that is partially responsible for the inhibition of Wnt/ β -catenin signaling by calcipotriol. Activation of Wnt signaling induces the expression of VDR, rendering cells susceptible to Wnt inhibition and other anti-tumorigenic effects linked to vitamin D. By offering further mechanistic insight into the bidirectional regulation of vitamin D and Wnt signaling, this work informs the use of vitamin D as a therapeutic Wnt inhibitor and provides putative biomarkers that may be effective in predicting or response to calcipotriol or other vitamin D analogs.

Materials and Methods

Cell lines and reagents

All cell lines were cultured as previously described(39). AsPC-1, HPAF-2, MiaPaCa-2 and PANC-1 were purchased from the American Type Culture Collection (Rockville, MD) in 2005. YAPC, Suit and HCG25 were kindly provided by Dr. Eric Collisson (University of California, San Francisco) in 2012. All cell lines were determined to be mycoplasma free using the MycoAlert PLUS mycoplasma detection kit (Lonza, Basel, Switzerland). Calcipotriol, Bafilomycin A1 and CHIR99021 were purchased from Tocris (Minneapolis, MN).

Quantitative real-time PCR

RNA extraction, cDNA synthesis and SYBR green qPCR were performed as previously described(39). All values were normalized to *ACTB* housekeeping gene. Primers used are *ACTB* Forward 5'- CCAACCGCGAGAAGATGA and Reverse 5'-

CCAGAGGCGTACAGGGATAG, *AXIN2* Forward 5'-
GAAGCTGGAGTTGGAGAGCCGCC and Reverse 5'-
TCTCTCTTCATCCTCTCGGATCTGC, *LRP6* Forward 5'-
TACAAGGTGTTCTTGCCCCA and Reverse 5'- TGTGAGGTTCTCCACATGAT,
LDLRAP1 Forward 5'- AGAGCCAGCACAACCAGA and Reverse 5'-
CTTGGACACCTGCCAAAA, and *VDR* Forward 5'- ACTTGCATGAGGAGGAGCAT and
Reverse 5'- TCGGCTAGCTTCTGGATCAT.

Gene Knockdown

AsPC-1 cells were transfected using Lipofectamine 2000 (Life Technologies, Grand Island, NY) per manufacturer's instructions using 20nM of siRNAs purchased from GE Dharmacon (Lafayette, CO), including control (D-001810-10-05), *VDR* (L-003448-00-0005), *LDLRAP1* (L-013025-00-0005), *DKK1* (L-003843-01-0005) or combined 10nM of *RXRA* (L-003448-00-0005) and 10 nM *RXRB* (L-003444-00-0005).

Western blot and immunoprecipitation

SDS-PAGE and immunoblots were performed as previously described(56) using antibodies for β -catenin (C2206; Sigma, St. Louis, MO), tubulin (sc-5546; Santa Cruz Biotechnology, Santa Cruz, CA), *VDR* (sc-13133), E-cadherin (sc-21791), *DKK-1* (GTX62902; GeneTex, Irvine, CA), *LRP6* (CST3395; Cell Signaling Technology, Danvers, MA), *RXR α* (CST3085), *RXR β* (CST8715), *LDLRAP1* (C20125; LSBio, Seattle, WA) or *LC3B* (ab48394; Abcam, Cambridge, MA). For *LRP6* and *LC3B* immunoblots, lysates were resolved on 4-20% gradient gels. Co-immunoprecipitations

were performed as previously described(152). Briefly, AsPC-1 lysates were pre-cleared with A/G-PLUS agarose beads (Santa Cruz; sc-2003) and immunoprecipitated using 1 µg antibody to β-catenin (BD Transduction Laboratories; 610153), VDR (Santa Cruz; sc-1008) or control isotype-matched IgG (Santa Cruz; sc-2025 or Abcam ab46540). After multiple washes, immune complexes were boiled in 6x SDS-load dye, resolved by SDS-PAGE and transferred to nitrocellulose membranes for immunoblotting.

Cell growth assays

MTT assays (ATCC) were carried out per manufacturer's instructions in 96-well plates with initial plating of 500 (Suit2), 1,000 (Tu8988t) or 2,000 (HPAF-2, YAPC, MiaPaCa-2, AsPC-1, PANC-1) cells per well. Cells were allowed to adhere overnight and then treated with calcipotriol. Soft agar assays were performed as previously described(56).

Wnt reporter assays

Baseline Wnt reporter activity was measured in dual luciferase assays (Promega, Madison, WI) as previously described(39) following transient co-transfection with control plasmid with constitutive EF1α promoter driving Renilla expression (serving as a normalization control) and either BAR (beta-catenin activated reporter, 12 TCF response elements driving luciferase expression) or fuBAR (found unresponsive BAR, contains mutated TCF response elements). In other experiments, Wnt reporter activity was measured in AsPC-1 stably transduced with the BAR reporter and Renilla control as previously described(56).

LRP6 overexpression

LRP6 or GFP (control) expression constructs in pCS2 vector have been previously described(153) and were kindly provided by Edward De Robertis (University of California, Los Angeles). Transfections were performed with X-tremeGENE9 (Roche, Indianapolis, IN) per manufacturer's instructions.

RNA isolation, library generation, sequencing and analysis

Total RNA was isolated from human pancreatic cancer cell lines as previously described(124). Sequencing libraries were prepared from 100–500 ng of total RNA using the TruSeq RNA sample preparation kit v2 (Illumina, San Diego, CA) according to the manufacturer's protocol. Briefly, mRNA was purified, fragmented and used for first- and second-strand cDNA synthesis followed by adenylation of 3' ends. Samples were ligated to unique adaptors and subjected to PCR amplification. Libraries were validated using the 2100 BioAnalyzer (Agilent, Santa Clara, CA), normalized and pooled for sequencing. RNA-seq libraries from three biological replicates for each experimental condition were sequenced on the Illumina HiSeq 2000 using barcoded multiplexing and a 100-bp read length. Image analysis and base calling were done with Illumina CASAVA-1.8.2. This yielded a median of 12.4 million usable reads per sample. Short read sequences were mapped to a UCSC hg19 reference sequence using the RNA-seq aligner STAR(154). Known splice junctions from hg19 were supplied to the aligner and *de novo* junction discovery was also permitted. Differential gene expression analysis, statistical testing and annotation were performed using Cuffdiff 2(155). Transcript expression was calculated as gene-level relative abundance in fragments per kilobase

of exon model per million mapped fragments and employed correction for transcript abundance bias (156). RNAseq data can be found on the NCBI Sequence Read Archive (SRP057571). Functional enrichment was limited to analysis of gene ontology terms and examined using default parameters in the web-based Database for Annotation, Visualization and Integrated Discovery (DAVID, v6.7; <http://david.abcc.ncifcrf.gov>)(112).

Microarray data analysis

Gene set enrichment analysis (GSEA) method (<http://www.broadinstitute.org/gsea>)(157) was used to determine whether a defined pancreatic-specific Wnt/ β -catenin target gene set showed statistically significant concordance with VDR expression in a previously published gene expression microarray dataset of 25 primary human PDAC samples (deposited in NCBI GEO under accession number GSE32688). GSEA default parameter settings and the Pearson metric for ranking genes were used with VDR expression as a continuous phenotypic variable. The pancreatic-specific Wnt/ β -catenin target gene set represented the overlap of downregulated transcripts observed in AsPC-1 in response to either Wnt inhibitor ICG-001 or *CTNNB1* siRNA transfection that we previously published(56).

Statistical methods

Statistical analyses were performed in GraphPad Prism (ver 5.04, La Jolla, CA). Student *t* tests were used to compare continuous variables. The level of significance for all statistical tests was defined as $\alpha=0.05$.

Figures

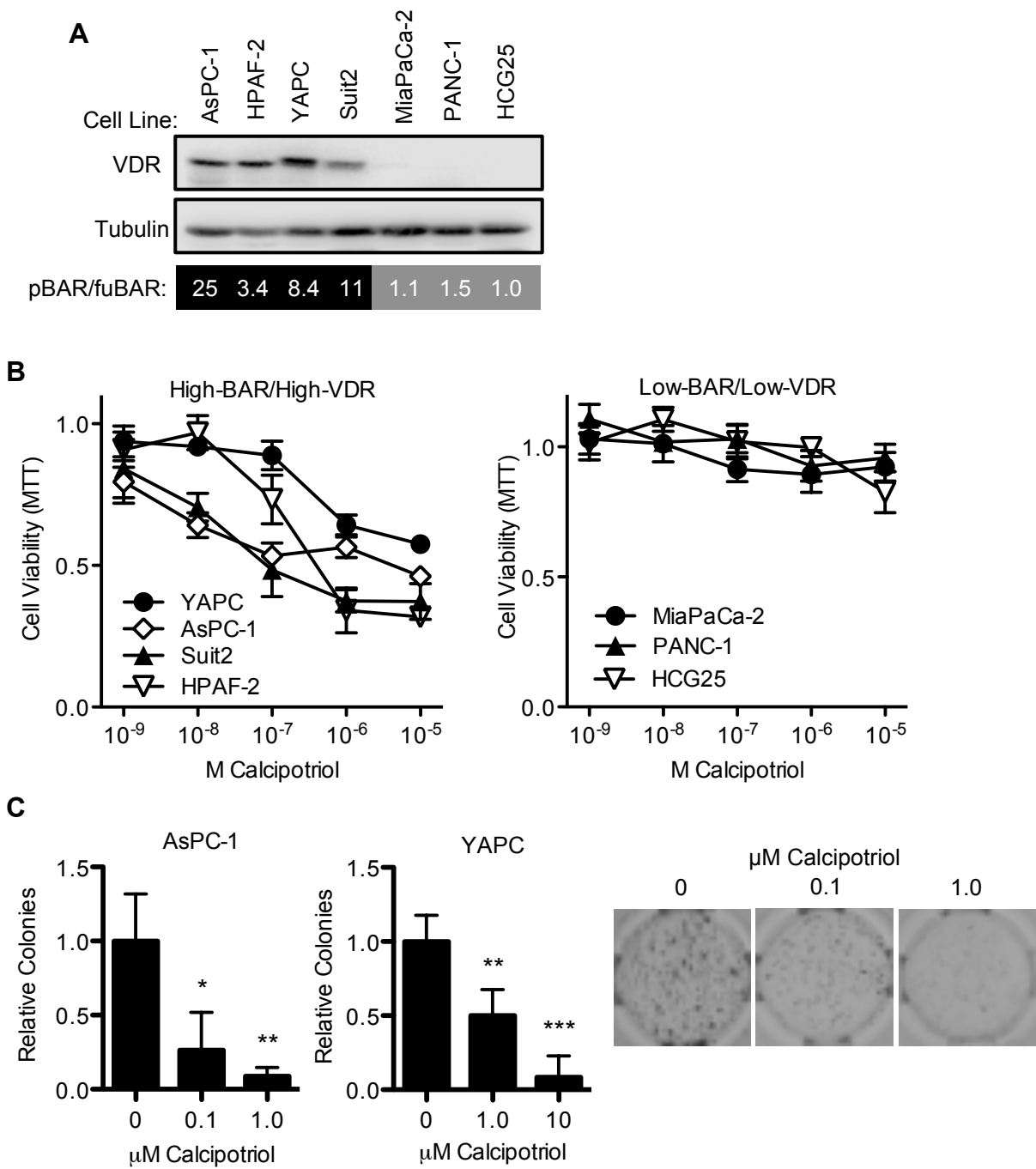


Figure 4-1. Inhibition of pancreatic cancer growth by calcipotriol correlates with levels of VDR expression and autocrine Wnt/ β -catenin signaling. (A) Western blot for VDR and tubulin (loading control) and mean Wnt reporter activity as indicated by

BAR/fuBAR ratios and measured by dual luciferase assays 48 hours after co-transfection with control Renilla and either BAR-luciferase or fuBAR-luciferase reporter constructs in indicated PDAC cell lines ($n=3$ biological replicates). (B) MTT growth assays (measured at 72 hours) and (C) soft agar colony formation at indicated concentrations of calcipotriol. Representative images of AsPC-1 soft agar colony formation are also shown. Data are normalized to vehicle only and reported as mean \pm standard deviation (SD) with one representative experiment of 3-5 biological repeats shown. * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

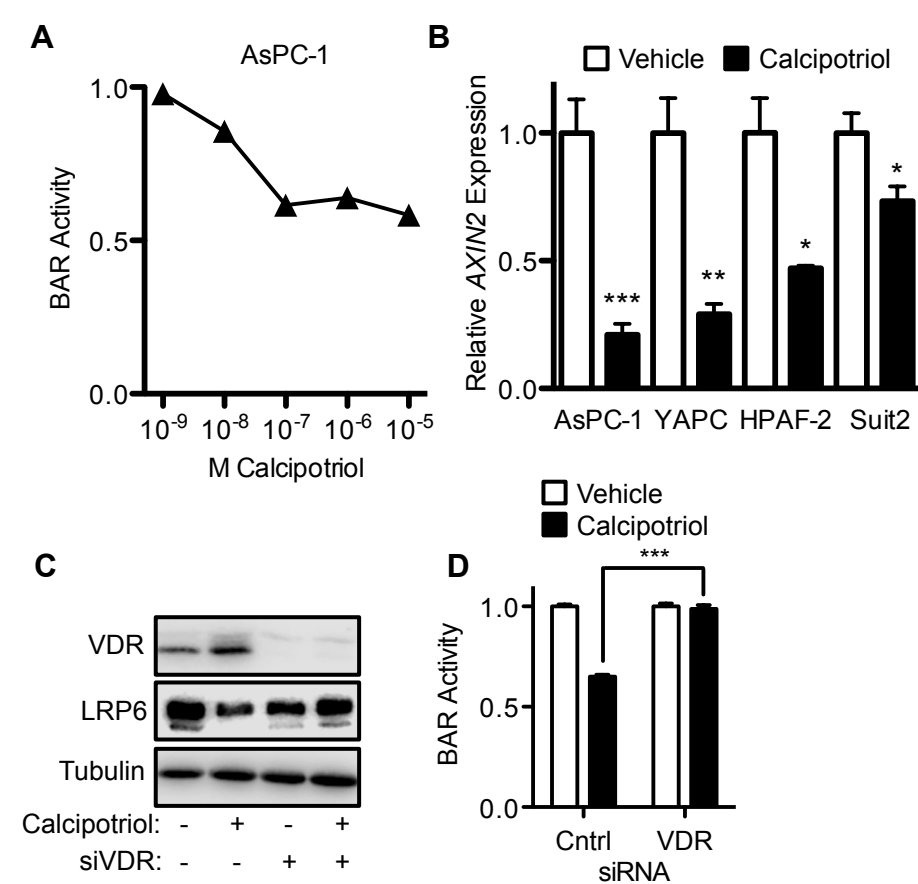


Figure 4-2. Calcipotriol mediates inhibition of Wnt signaling through VDR. (A)

Dose response curve for calcipotriol on Wnt signaling as measured at 24 hours in

AsPC-1 cells with stable BAR-luciferase reporter. (B) *AXIN2* expression measured by qPCR at 24 hours following treatment with vehicle or 100 nM (AsPC-1), 5 μ M (YAPC) or 1 μ M (HPAF-2 and Suit2) calcipotriol. (C) VDR, LRP6 and tubulin (loading control) western blots and (D) BAR-luciferase activity for AsPC-1 transfected with either control or VDR siRNA for 48 hours and an additional 24 hour treatment with vehicle or 100 nM calcipotriol. All values were normalized to respective controls, with BAR reported as mean \pm SD and qPCR reported as mean \pm SEM. One representative experiment from at least three biological repeats is shown. * P <0.05, ** P <0.01, *** P <0.001.

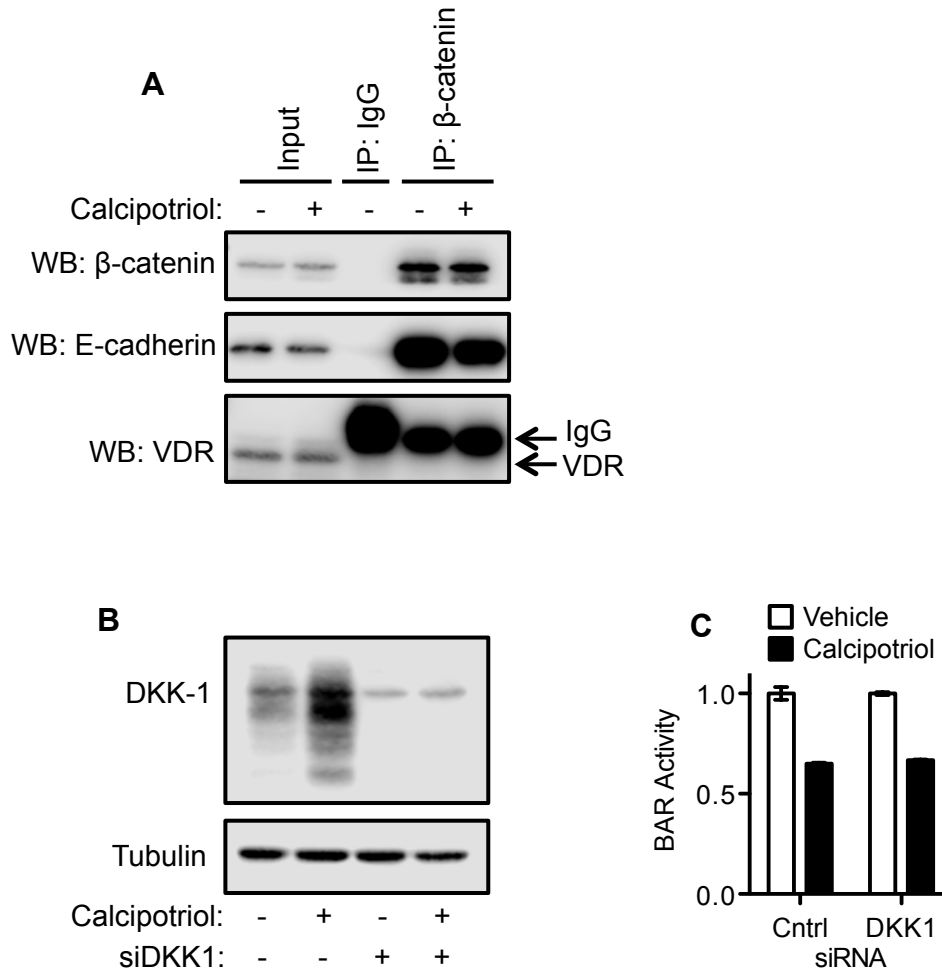


Figure 4-3. Calcipotriol does not inhibit Wnt signaling through VDR sequestration of β -catenin or induction of DKK1. (A) AsPC-1 were treated with 100 nM calcipotriol for 4 hours and lysates were immunoprecipitated with anti- β -catenin or isotype control antibodies followed by western blots for β -catenin, E-cadherin or VDR. (B) Western blot for DKK-1 and tubulin (loading control) and (C) BAR reporter activity for indicated conditions. AsPC-1 were transfected with control or *DKK1* siRNA for 48 hours and treated with vehicle or 100 nM calcipotriol for an additional 24 hours. BAR was normalized to vehicle and siRNA control and is reported as mean \pm SD, with one representative experiment of 3 biological repeats shown.

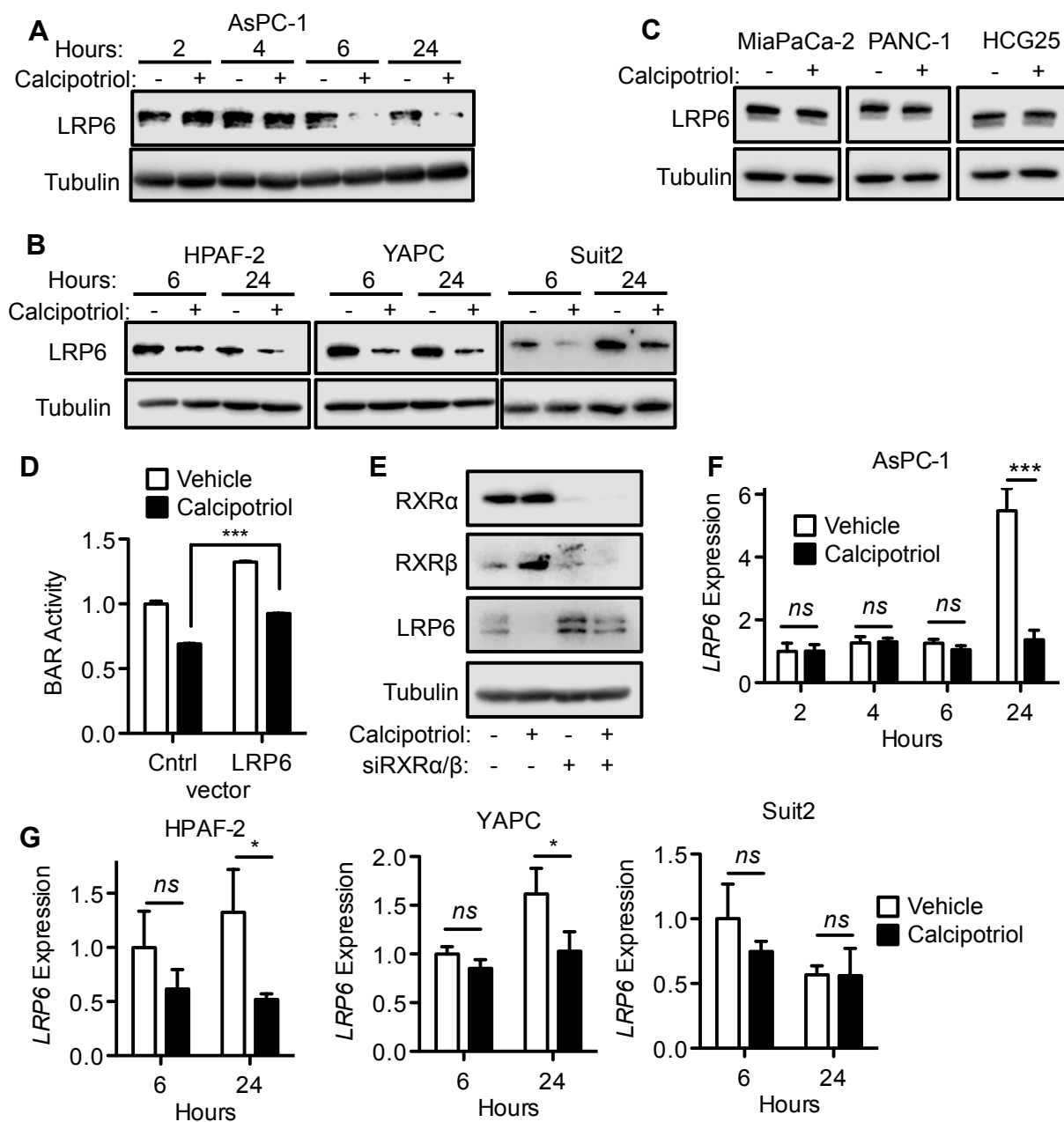


Figure 4-4. Calcipotriol treatment reduces LRP6 expression in PDAC. (A-C)

Western blots for LRP6 or tubulin (loading control) were performed on whole cell lysates at indicated time points (24 hours for MiaPaCa-2, PANC-1 and HCG25) after cell line treatment with vehicle or 100 nM (AsPC-1), 5 μ M (YAPC), 1 μ M (HPAF-2 and Suit2) or 10 μ M (MiaPaCa-2, PANC-1 and HCG25) calcipotriol. (D) BAR-luciferase activity for

AsPC-1 transfected with full-length LRP6 expression vector or control GFP vector for 48 hours and measured after a further 24 hour treatment with vehicle or 100 nM calcipotriol. (E) RXR α , RXR β , LRP6 and tubulin western blots on AsPC-1 whole cell lysates after control or tandem *RXRA* and *RXRB* siRNA transfection for 48 hours and further treatment with vehicle or 100 nM calcipotriol for 24 hours. (F-G) *LRP6* gene expression measured by qPCR after 100 nM calcipotriol treatment for indicated times. Cells were synchronized by serum-starvation prior to calcipotriol treatment. All values were normalized to respective controls, with BAR reported as mean \pm SD and qPCR reported as mean \pm SEM. One representative experiment of 2-3 biological repeats is shown. * P <0.05, ** P <0.01, *** P <0.001, *ns* = not significant.

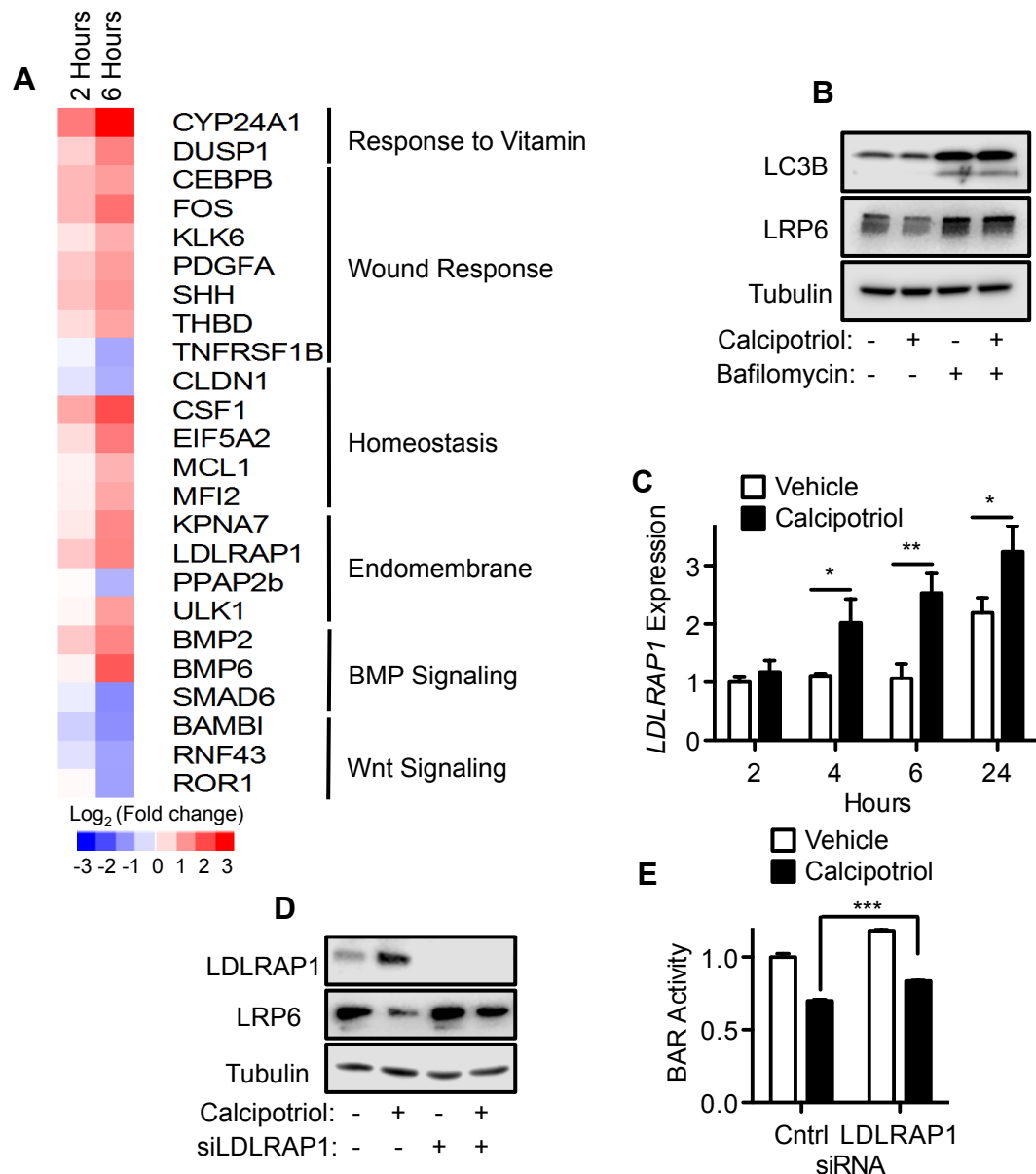


Figure 4-5. Calcipotriol induces *LDLRAP1* to regulate LRP6 protein levels. (A)

Heat map of selected genes from RNA-seq after 2 or 6 hours treatment of AsPC-1 cells with vehicle or 100 nM calcipotriol. (B) Western blot for LC3B, LRP6 and tubulin (loading control) after AsPC-1 cells were treated with vehicle, 100 nM Calcipotriol, 100 nM Bafilomycin A1 or a combination of the two for 6 hours. (C) *LDLRAP1* expression by qPCR after treatments described in Figure 4-4A. (D) Western blot for LDLRAP1,

LRP6 and tubulin (loading control). AsPC-1 cells were transfected with control or LDLRAP1 siRNA for 48 hours and then treated with vehicle or 100 nM calcipotriol for 24 hours. (E) BAR-luciferase activity in AsPC-1 cells treated as in (D). Values are shown normalized to respective controls, with BAR reported as mean \pm SD and qPCR reported as mean \pm SEM. One representative experiment of 3 biological repeats is shown.

P*<0.05, *P*<0.01, ****P*<0.001.

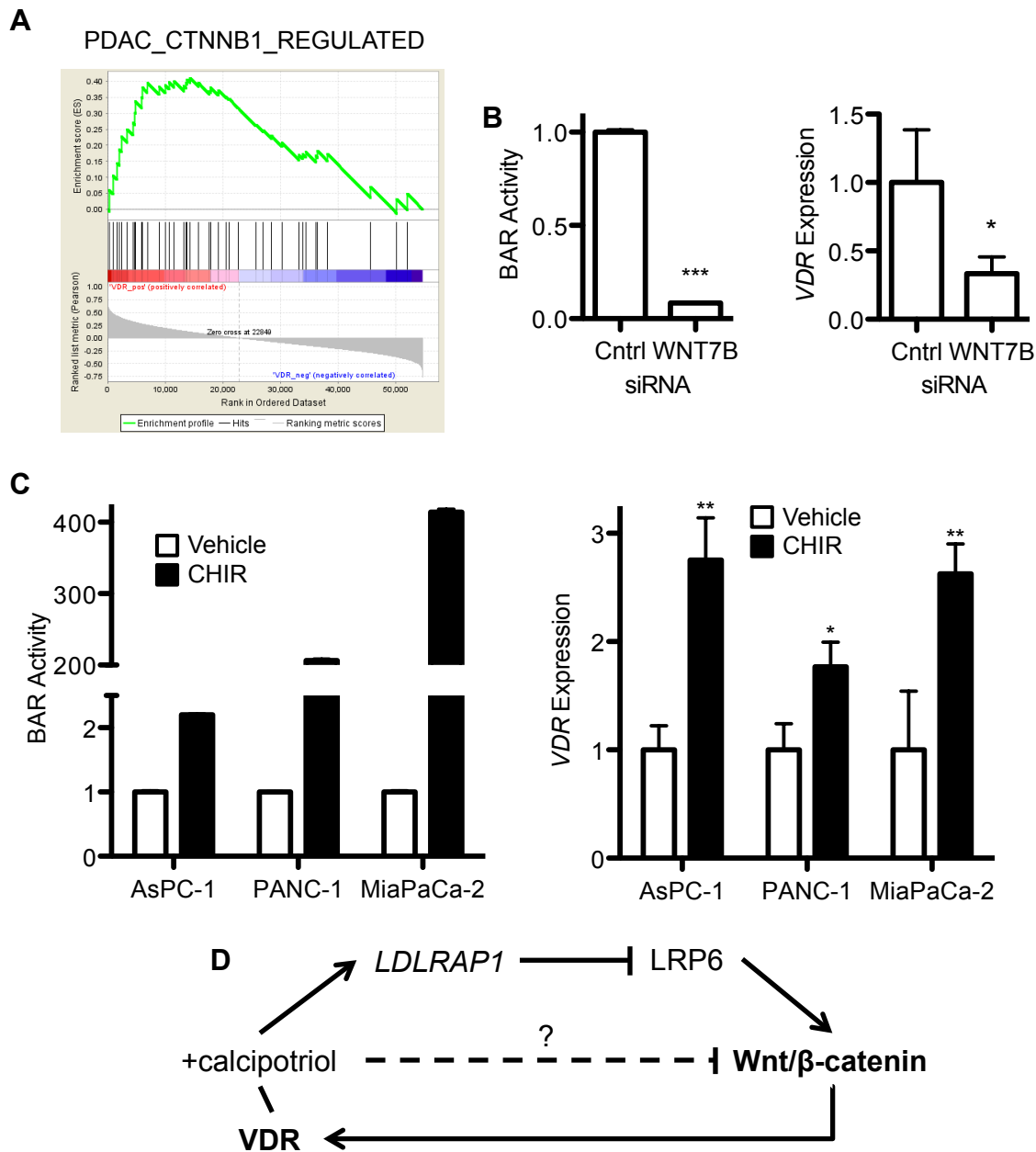


Figure 4-6. VDR is regulated by Wnt signaling in PDAC. (A) Gene set enrichment analysis enrichment plot for PDAC-specific Wnt/ β -catenin target gene set determined relative to VDR expression phenotype using a published gene expression microarray dataset of 25 primary PDAC tumor samples. The top curve shows a running

enrichment score for the target gene set, while the middle portion shows a heatmap of VDR expression (red – high, blue – low) and bottom portion shows the value of the ranking metric down the list of genes. (B) BAR luciferase activity and *VDR* expression by qPCR 48 hours after transfection of AsPC-1 cells with control or WNT7B siRNA. (C) BAR luciferase activity and *VDR* expression by qPCR 24 hours after treatment of indicated PDAC cell lines with vehicle or 5 μ M CHIR99021 (CHIR). (D) Schematic for proposed model of VDR/calcipotriol and Wnt/ β -catenin feedback loop. Values are shown normalized to respective controls, with BAR reported as mean \pm SD and qPCR reported as mean \pm SEM. One representative experiment of 3 biological repeats is shown. * P <0.05, ** P <0.01, *** P <0.001.

FUTURE DIRECTIONS

This dissertation has identified regulators of Wnt/ β -catenin signaling and has characterized the ability of several compounds to inhibit this pathway in pancreatic cancer. Sumoylation involving SUMO2 was found to act as a Wnt pathway inhibitor, as disruption of the sumoylation cascade resulted in increased Wnt activity. Wnt7B was identified as the key Wnt ligand driving pathway activation and as a critical mediator of anchorage-independent growth. The CBP inhibitor ICG-001, while a potent inhibitor of tumor growth, was found to act outside the Wnt signaling pathway through direct modulation of key mediators of cell cycle arrest. The vitamin D analog calcipotriol was also found to suppress PDAC growth and inhibited Wnt signaling through a novel mechanism involving the destabilization of LRP6. While this work has significantly contributed to our understanding of the driving mechanisms and druggability of PDAC, it also opens the door for additional avenues of investigation.

This dissertation has focused on cell-autonomous effects of Wnt signaling. However, it is becoming increasingly clear that the stroma plays important and diverse roles in PDAC. For example, inhibition of sonic hedgehog signaling in the stroma has been shown to both shorten and prolong survival depending on temporal context(158, 159). The Wnt signaling pathway appears to be at play in the stroma, as pancreatic stellate cells have been documented to secrete factors that could modulate Wnt signaling in the tumor, such as sFRPs(78) and Wnts(124), but it remains unclear whether this paracrine signaling is pro- or anti-tumorigenic. It is therefore critical to understand the consequences of manipulating developmental signaling pathways, such as Wnt/ β -

catenin, on the overall tumor microenvironment, so that we avoid inadvertently disrupting anti-tumorigenic factors. A better understanding of the stroma may also yield additional soluble factors that may be therapeutically targetable.

The data presented in this thesis will allow us to transition from *in vitro* and animal models to analysis of human tissues and application of our therapeutics towards patients. Our study found that vitamin D receptor (VDR) expression in tumors predicts response to vitamin D analogs and inhibits Wnt/ β -catenin signaling. A clinical trial using paricalcitol (a vitamin D analog similar to calcipotriol) in the neoadjuvant setting is currently underway (NCT#02030860). While this trial focuses on the ability of paricalcitol to deplete the stroma and facilitate gemcitabine delivery(124), it will be in our best interest to assess VDR status in these tumors during treatment, as paricalcitol may be less effective in tumors lacking VDR. We can also utilize this clinical trial to validate our *in vitro* findings by assessing paricalcitol effects on the Wnt pathway in patient tumors.

Wnt signaling has been previously shown to be a driver of PDAC(12, 13), but methods to assess pathway activity in patient tumors were lacking(11). To address this need we developed a Wnt/ β -catenin transcriptional signature to monitor pathway activation based on expression of genes controlled by β -catenin transcriptional activity. While this revealed that Wnt signaling mediates an aggressive form of PDAC, we are currently pursuing ways to improve upon this transcriptional signature. We have acquired additional PDAC lines and are compiling gene sets enriched in lines with either high or

low endogenous Wnt activity in order to encompass a broader range of PDAC tumor subtypes. This revamped transcriptional signature will reveal additional key mediators and targets of Wnt signaling and may also be used to predict response to Wnt-targeted therapeutics when applied to patient tumors.

An additional component to Wnt signaling that has recently come to light is β -catenin independent canonical Wnt signaling, also known as Wnt-dependent stabilization of proteins (Wnt/STOP). Wnt/STOP is the result of ligand-activated Wnt receptor complex inhibition of GSK3 kinase activity, which stabilizes potentially thousands of proteins. This is critical during G2/M transition in order to maintain cell size and promote growth (160), implicating WNT/STOP in tumor proliferation as well. This largely unexplored GSK3 phosphoproteome could explain why knockdown of *CTNNB1* results in different phenotypes than inhibition of Wnt at the ligand/receptor level, and may also explain difficulties experienced in correlating changes in Wnt transcriptional targets to changes in phenotypes. Wnt/STOP is an exciting new aspect of the Wnt cascade that may bridge the gap between Wnt ligand inhibition and downstream phenotypic effects, and it will be important to consider when evaluating therapeutics targeting the Wnt pathway at the level of the receptor or ligand. Wnt/STOP may also reveal novel downstream mediators and druggable targets of Wnt signaling.

While there is still much to be learned about the Wnt signaling pathway alone and in the context of pancreatic cancer, this dissertation has helped guide the development of Wnt/ β -catenin pathway inhibitors for the treatment of PDAC by revealing key regulators

and targets of the pathway, as well as by characterizing existing drugs. Promising compounds that specifically target this pathway, such as tankyrase inhibitors(10) and function-blocking Fzd antibodies(53), are currently under clinical and pre-clinical development, and our work may lead to the identification of additional such pharmacologic agents. Future work will lead to a better understanding of pancreatic cancer so that we will be able to target specific pathways in individual patients resulting in more precise and effective treatment.

BIBLIOGRAPHY

1. Singh M, Maitra A. Precursor lesions of pancreatic cancer: molecular pathology and clinical implications. *Pancreatology*. 2007;7:9-19.
2. Maitra A, Hruban RH. Pancreatic cancer. *Annu Rev Pathol*. 2008;3:157-88.
3. Koorstra JB, Hustinx SR, Offerhaus GJ, Maitra A. Pancreatic carcinogenesis. *Pancreatology*. 2008;8:110-25.
4. Jones S, Zhang X, Parsons DW, Lin JC, Leary RJ, Angenendt P, et al. Core signaling pathways in human pancreatic cancers revealed by global genomic analyses. *Science*. 2008;321:1801-6.
5. Collisson EA, Sadanandam A, Olson P, Gibb WJ, Truitt M, Gu S, et al. Subtypes of pancreatic ductal adenocarcinoma and their differing responses to therapy. *Nat Med*. 2011;17:500-3.
6. Reya T, Clevers H. Wnt signalling in stem cells and cancer. *Nature*. 2005;434:843-50.
7. Clevers H. Wnt/beta-catenin signaling in development and disease. *Cell*. 2006;127:469-80.
8. Ilmer M, Boiles AR, Regel I, Yokoi K, Michalski CW, Wistuba II, et al. RSPO2 Enhances Canonical Wnt Signaling to Confer Stemness-Associated Traits to Susceptible Pancreatic Cancer Cells. *Cancer Res*. 2015.
9. Wu C, Qiu S, Lu L, Zou J, Li WF, Wang O, et al. RSPO2-LGR5 signaling has tumour-suppressive activity in colorectal cancer. *Nat Commun*. 2014;5:3149.

10. Jiang X, Hao HX, Growney JD, Woolfenden S, Bottiglio C, Ng N, et al. Inactivating mutations of RNF43 confer Wnt dependency in pancreatic ductal adenocarcinoma. *Proc Natl Acad Sci U S A*. 2013;110:12649-54.
11. White BD, Chien AJ, Dawson DW. Dysregulation of Wnt/beta-catenin signaling in gastrointestinal cancers. *Gastroenterology*. 2012;142:219-32.
12. Pasca di Magliano M, Biankin AV, Heiser PW, Cano DA, Gutierrez PJ, Deramaudt T, et al. Common activation of canonical Wnt signaling in pancreatic adenocarcinoma. *PLoS One*. 2007;2:e1155.
13. Zhang Y, Morris JPt, Yan W, Schofield HK, Gurney A, Simeone DM, et al. Canonical Wnt signaling is required for pancreatic carcinogenesis. *Cancer Res*. 2013.
14. Stathis A, Moore MJ. Advanced pancreatic carcinoma: current treatment and future challenges. *Nat Rev Clin Oncol*. 2010;7:163-72.
15. Rahib L, Smith BD, Aizenberg R, Rosenzweig AB, Fleshman JM, Matrisian LM. Projecting cancer incidence and deaths to 2030: the unexpected burden of thyroid, liver, and pancreas cancers in the United States. *Cancer Res*. 2014;74:2913-21.
16. Burris HA, 3rd, Moore MJ, Andersen J, Green MR, Rothenberg ML, Modiano MR, et al. Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: a randomized trial. *J Clin Oncol*. 1997;15:2403-13.
17. Paulson AS, Tran Cao HS, Tempero MA, Lowy AM. Therapeutic advances in pancreatic cancer. *Gastroenterology*. 2013;144:1316-26.

18. Gysin S, Salt M, Young A, McCormick F. Therapeutic strategies for targeting ras proteins. *Genes Cancer*. 2011;2:359-72.
19. Vincent A, Herman J, Schulick R, Hruban RH, Goggins M. Pancreatic cancer. *Lancet*. 2011;378:607-20.
20. Hong SM, Park JY, Hruban RH, Goggins M. Molecular signatures of pancreatic cancer. *Arch Pathol Lab Med*. 2011;135:716-27.
21. American_Cancer_Society. Cancer Facts & Figures 2012. Cancer. New York: American Cancer Society; 2012. p. 1-66.
22. Donahue T, Tran L, Hill R, Li Y, Kovochich A, Calvopina J, et al. Integrative survival-based molecular profiling of human pancreatic cancer. *Clinical Cancer Research*. 2012;18:1352-63.
23. Vincent A, Omura N, Hong SM, Jaffe A, Eshleman J, Goggins M. Genome-wide analysis of promoter methylation associated with gene expression profile in pancreatic adenocarcinoma. *Clin Cancer Res*. 2011;17:4341-54.
24. Clevers H, Nusse R. Wnt/beta-catenin signaling and disease. *Cell*. 2012;149:1192-205.
25. Angers S, Moon RT. Proximal events in Wnt signal transduction. *Nat Rev Mol Cell Biol*. 2009;10:468-77.
26. Chien AJ, Conrad WH, Moon RT. A Wnt survival guide: from flies to human disease. *J Invest Dermatol*. 2009;129:1614-27.
27. Lucero OM, Dawson DW, Moon RT, Chien AJ. A re-evaluation of the "oncogenic" nature of Wnt/beta-catenin signaling in melanoma and other cancers. *Curr Oncol Rep*. 2010;12:314-8.

28. Caca K, Kolligs FT, Ji X, Hayes M, Qian J, Yahanda A, et al. Beta- and gamma-catenin mutations, but not E-cadherin inactivation, underlie T-cell factor/lymphoid enhancer factor transcriptional deregulation in gastric and pancreatic cancer. *Cell Growth Differ.* 1999;10:369-76.
29. Gerdes B, Ramaswamy A, Simon B, Pietsch T, Bastian D, Kersting M, et al. Analysis of beta-catenin gene mutations in pancreatic tumors. *Digestion.* 1999;60:544-8.
30. Biankin AV, Waddell N, Kassahn KS, Gingras MC, Muthuswamy LB, Johns AL, et al. Pancreatic cancer genomes reveal aberrations in axon guidance pathway genes. *Nature.* 2012;491:399-405.
31. Zeng G, Germinaro M, Micsenyi A, Monga NK, Bell A, Sood A, et al. Aberrant Wnt/beta-catenin signaling in pancreatic adenocarcinoma. *Neoplasia.* 2006;8:279-89.
32. Nawroth R, van Zante A, Cervantes S, McManus M, Hebrok M, Rosen SD. Extracellular sulfatases, elements of the Wnt signaling pathway, positively regulate growth and tumorigenicity of human pancreatic cancer cells. *PLoS One.* 2007;2:e392.
33. Wang L, Heidt DG, Lee CJ, Yang H, Logsdon CD, Zhang L, et al. Oncogenic function of ATDC in pancreatic cancer through Wnt pathway activation and beta-catenin stabilization. *Cancer Cell.* 2009;15:207-19.
34. Zhong Y, Wang Z, Fu B, Pan F, Yachida S, Dhara M, et al. GATA6 activates Wnt signaling in pancreatic cancer by negatively regulating the Wnt antagonist Dickkopf-1. *PLoS One.* 2011;6:e22129.

35. Morris JPt, Wang SC, Hebrok M. KRAS, Hedgehog, Wnt and the twisted developmental biology of pancreatic ductal adenocarcinoma. *Nat Rev Cancer*. 2010;10:683-95.
36. Biechele TL, Moon RT. Assaying beta-catenin/TCF transcription with beta-catenin/TCF transcription-based reporter constructs. *Methods Mol Biol*. 2008;468:99-110.
37. Jho EH, Zhang T, Domon C, Joo CK, Freund JN, Costantini F. Wnt/beta-catenin/Tcf signaling induces the transcription of Axin2, a negative regulator of the signaling pathway. *Mol Cell Biol*. 2002;22:1172-83.
38. Chang JT, Gatza ML, Lucas JE, Barry WT, Vaughn P, Nevins JR. SIGNATURE: a workbench for gene expression signature analysis. *BMC Bioinformatics*. 2011;12:443.
39. Arensman MD, Kovochich AN, Kulikauskas RM, Lay AR, Yang PT, Li X, et al. WNT7B mediates autocrine Wnt/beta-catenin signaling and anchorage-independent growth in pancreatic adenocarcinoma. *Oncogene*. 2014;33:899-908.
40. Chen B, Dodge ME, Tang W, Lu J, Ma Z, Fan CW, et al. Small molecule-mediated disruption of Wnt-dependent signaling in tissue regeneration and cancer. *Nat Chem Biol*. 2009;5:100-7.
41. Huang SM, Mishina YM, Liu S, Cheung A, Stegmeier F, Michaud GA, et al. Tankyrase inhibition stabilizes axin and antagonizes Wnt signalling. *Nature*. 2009;461:614-20.
42. Port F, Basler K. Wnt trafficking: new insights into Wnt maturation, secretion and spreading. *Traffic*. 2010;11:1265-71.

43. Yu M, Ting DT, Stott SL, Wittner BS, Ozsolak F, Paul S, et al. RNA sequencing of pancreatic circulating tumour cells implicates WNT signalling in metastasis. *Nature*. 2012;487:510-3.
44. Lowe AW, Olsen M, Hao Y, Lee SP, Taek Lee K, Chen X, et al. Gene expression patterns in pancreatic tumors, cells and tissues. *PLoS ONE*. 2007;2:e323.
45. Heiser PW, Cano DA, Landsman L, Kim GE, Kench JG, Klimstra DS, et al. Stabilization of beta-catenin induces pancreas tumor formation. *Gastroenterology*. 2008;135:1288-300.
46. Morris JPt, Cano DA, Sekine S, Wang SC, Hebrok M. beta-catenin blocks Kras-dependent reprogramming of acini into pancreatic cancer precursor lesions in mice. *J Clin Invest*. 2010.
47. Froeling FE, Feig C, Chelala C, Dobson R, Mein CE, Tuveson DA, et al. Retinoic Acid-Induced Pancreatic Stellate Cell Quiescence Reduces Paracrine Wnt-beta-Catenin Signaling to Slow Tumor Progression. *Gastroenterology*. 2011.
48. Froeling FE, Mirza TA, Feakins RM, Seedhar A, Elia G, Hart IR, et al. Organotypic culture model of pancreatic cancer demonstrates that stromal cells modulate E-cadherin, beta-catenin, and Ezrin expression in tumor cells. *Am J Pathol*. 2009;175:636-48.
49. Ilmer M, Recio Boiles A, Regel I, Yokoi K, Michalski CW, Wistuba, II, et al. RSpO2 enhances canonical Wnt signaling to confer stemness-associated traits to susceptible pancreatic cancer cells. *Cancer Res*. 2015.
50. Chien AJ, Moon RT. WNTs and WNT receptors as therapeutic tools and targets in human disease processes. *Front Biosci*. 2007;12:448-57.

51. Yu M, Ting DT, Stott SL, Wittner BS, Ozsolak F, Paul S, et al. RNA sequencing of pancreatic circulating tumour cells implicates WNT signalling in metastasis. *Nature*. 2012.
52. Jiang H, Li F, He C, Wang X, Li Q, Gao H. Expression of Gli1 and Wnt2B correlates with progression and clinical outcome of pancreatic cancer. *Int J Clin Exp Pathol*. 2014;7:4531-8.
53. Gurney A, Axelrod F, Bond CJ, Cain J, Chartier C, Donigan L, et al. Wnt pathway inhibition via the targeting of Frizzled receptors results in decreased growth and tumorigenicity of human tumors. *Proc Natl Acad Sci U S A*. 2012;109:11717-22.
54. Liu J, Pan S, Hsieh MH, Ng N, Sun F, Wang T, et al. Targeting Wnt-driven cancer through the inhibition of Porcupine by LGK974. *Proc Natl Acad Sci U S A*. 2013;110:20224-9.
55. Nguyen Kovochich A, Arensman M, Lay AR, Rao NP, Donahue T, Li X, et al. HOXB7 promotes invasion and predicts survival in pancreatic adenocarcinoma. *Cancer*. 2013;119:529-39.
56. Arensman MD, Telesca D, Lay AR, Kershaw KM, Wu N, Donahue TR, et al. The CREB-binding protein inhibitor ICG-001 suppresses pancreatic cancer growth. *Mol Cancer Ther*. 2014;13:2303-14.
57. Gao C, Xiao G, Hu J. Regulation of Wnt/beta-catenin signaling by posttranslational modifications. *Cell Biosci*. 2014;4:13.
58. Dohmen RJ. SUMO protein modification. *Biochim Biophys Acta*. 2004;1695:113-31.

59. Gareau JR, Lima CD. The SUMO pathway: emerging mechanisms that shape specificity, conjugation and recognition. *Nat Rev Mol Cell Biol.* 2010;11:861-71.
60. Kerscher O. SUMO junction-what's your function? New insights through SUMO-interacting motifs. *EMBO Rep.* 2007;8:550-5.
61. Sachdev S, Bruhn L, Sieber H, Pichler A, Melchior F, Grosschedl R. PIASy, a nuclear matrix-associated SUMO E3 ligase, represses LEF1 activity by sequestration into nuclear bodies. *Genes Dev.* 2001;15:3088-103.
62. Yamamoto H, Ihara M, Matsuura Y, Kikuchi A. Sumoylation is involved in beta-catenin-dependent activation of Tcf-4. *EMBO J.* 2003;22:2047-59.
63. Choi HK, Choi KC, Yoo JY, Song M, Ko SJ, Kim CH, et al. Reversible SUMOylation of TBL1-TBLR1 regulates beta-catenin-mediated Wnt signaling. *Mol Cell.* 2011;43:203-16.
64. Huang HJ, Zhou LL, Fu WJ, Zhang CY, Jiang H, Du J, et al. beta-catenin SUMOylation is involved in the dysregulated proliferation of myeloma cells. *Am J Cancer Res.* 2015;5:309-20.
65. Rui HL, Fan E, Zhou HM, Xu Z, Zhang Y, Lin SC. SUMO-1 modification of the C-terminal KVEKVD of Axin is required for JNK activation but has no effect on Wnt signaling. *J Biol Chem.* 2002;277:42981-6.
66. Eun Jeoung L, Sung Hee H, Jaesun C, Sung Hwa S, Kwang Hum Y, Min Kyoung K, et al. Regulation of glycogen synthase kinase 3beta functions by modification of the small ubiquitin-like modifier. *Open Biochem J.* 2008;2:67-76.

67. Ihara M, Yamamoto H, Kikuchi A. SUMO-1 modification of PIASy, an E3 ligase, is necessary for PIASy-dependent activation of Tcf-4. *Mol Cell Biol.* 2005;25:3506-18.
68. Shitashige M, Satow R, Honda K, Ono M, Hirohashi S, Yamada T. Regulation of Wnt signaling by the nuclear pore complex. *Gastroenterology.* 2008;134:1961-71, 71 e1-4.
69. Li J, Wang CY. TBL1-TBLR1 and beta-catenin recruit each other to Wnt target-gene promoter for transcription activation and oncogenesis. *Nat Cell Biol.* 2008;10:160-9.
70. Kim MJ, Chia IV, Costantini F. SUMOylation target sites at the C terminus protect Axin from ubiquitination and confer protein stability. *FASEB J.* 2008;22:3785-94.
71. Johnson ES. Protein modification by SUMO. *Annu Rev Biochem.* 2004;73:355-82.
72. Yeh ET. SUMOylation and De-SUMOylation: wrestling with life's processes. *J Biol Chem.* 2009;284:8223-7.
73. Lallemand-Breitenbach V, de The H. PML nuclear bodies. *Cold Spring Harb Perspect Biol.* 2010;2:a000661.
74. Kim WJ, Wittner BS, Amzallag A, Brannigan BW, Ting DT, Ramaswamy S, et al. The WTX Tumor Suppressor Interacts with the Transcriptional Corepressor TRIM28. *J Biol Chem.* 2015.
75. Chu Y, Yang X. SUMO E3 ligase activity of TRIM proteins. *Oncogene.* 2011;30:1108-16.

76. Seeler JS, Marchio A, Sitterlin D, Transy C, Dejean A. Interaction of SP100 with HP1 proteins: a link between the promyelocytic leukemia-associated nuclear bodies and the chromatin compartment. *Proc Natl Acad Sci U S A*. 1998;95:7316-21.
77. Siegel R, Naishadham D, Jemal A. Cancer statistics, 2012. *CA Cancer J Clin*. 2012;62:10-29.
78. Froeling FE, Feig C, Chelala C, Dobson R, Mein CE, Tuveson DA, et al. Retinoic acid-induced pancreatic stellate cell quiescence reduces paracrine Wnt-beta-catenin signaling to slow tumor progression. *Gastroenterology*. 2011;141:1486-97, 97 e1-14.
79. Voronkov A, Krauss S. Wnt/beta-catenin signaling and small molecule inhibitors. *Curr Pharm Des*. 2013;19:634-64.
80. Anastas JN, Moon RT. WNT signalling pathways as therapeutic targets in cancer. *Nat Rev Cancer*. 2013;13:11-26.
81. Emami KH, Nguyen C, Ma H, Kim DH, Jeong KW, Eguchi M, et al. A small molecule inhibitor of beta-catenin/CREB-binding protein transcription [corrected]. *Proc Natl Acad Sci U S A*. 2004;101:12682-7.
82. Lazarova DL, Chiaro C, Wong T, Drago E, Rainey A, O'Malley S, et al. CBP Activity Mediates Effects of the Histone Deacetylase Inhibitor Butyrate on WNT Activity and Apoptosis in Colon Cancer Cells. *J Cancer*. 2013;4:481-90.
83. Lazarova DL, Wong T, Chiaro C, Drago E, Bordonaro M. p300 Influences Butyrate-Mediated WNT Hyperactivation In Colorectal Cancer Cells. *J Cancer*. 2013;4:491-501.

84. Ma H, Nguyen C, Lee KS, Kahn M. Differential roles for the coactivators CBP and p300 on TCF/beta-catenin-mediated survivin gene expression. *Oncogene*. 2005;24:3619-31.
85. Henderson WR, Jr., Chi EY, Ye X, Nguyen C, Tien YT, Zhou B, et al. Inhibition of Wnt/beta-catenin/CREB binding protein (CBP) signaling reverses pulmonary fibrosis. *Proc Natl Acad Sci U S A*. 2010;107:14309-14.
86. Hao S, He W, Li Y, Ding H, Hou Y, Nie J, et al. Targeted inhibition of beta-catenin/CBP signaling ameliorates renal interstitial fibrosis. *J Am Soc Nephrol*. 2011;22:1642-53.
87. Gang EJ, Hsieh YT, Pham J, Zhao Y, Nguyen C, Huantes S, et al. Small-molecule inhibition of CBP/catenin interactions eliminates drug-resistant clones in acute lymphoblastic leukemia. *Oncogene*. 2014;33:2169-78.
88. Sasaki T, Hwang H, Nguyen C, Kloner RA, Kahn M. The small molecule Wnt signaling modulator ICG-001 improves contractile function in chronically infarcted rat myocardium. *PLoS One*. 2013;8:e75010.
89. Beyer C, Reichert H, Akan H, Mallano T, Schramm A, Dees C, et al. Blockade of canonical Wnt signalling ameliorates experimental dermal fibrosis. *Ann Rheum Dis*. 2013;72:1255-8.
90. Wend P, Fang L, Zhu Q, Schipper JH, Loddenkemper C, Kosel F, et al. Wnt/beta-catenin signalling induces MLL to create epigenetic changes in salivary gland tumours. *EMBO J*. 2013;32:1977-89.
91. Takahashi-Yanaga F, Kahn M. Targeting Wnt signaling: can we safely eradicate cancer stem cells? *Clin Cancer Res*. 2010;16:3153-62.

92. Hotz HG, Reber HA, Hotz B, Yu T, Foitzik T, Buhr HJ, et al. An orthotopic nude mouse model for evaluating pathophysiology and therapy of pancreatic cancer. *Pancreas*. 2003;26:e89-98.
93. Dickson MA. Molecular pathways: CDK4 inhibitors for cancer therapy. *Clin Cancer Res*. 2014;20:3379-83.
94. Mini E, Nobili S, Caciagli B, Landini I, Mazzei T. Cellular pharmacology of gemcitabine. *Ann Oncol*. 2006;17 Suppl 5:v7-12.
95. Xie H, Jiang W, Jiang J, Wang Y, Kim R, Liu X, et al. Predictive and prognostic roles of ribonucleotide reductase M1 in resectable pancreatic adenocarcinoma. *Cancer*. 2013;119:173-81.
96. Wei CH, Gorgan TR, Elashoff DA, Hines OJ, Farrell JJ, Donahue TR. A meta-analysis of gemcitabine biomarkers in patients with pancreaticobiliary cancers. *Pancreas*. 2013;42:1303-10.
97. McMillan M, Kahn M. Investigating Wnt signaling: a chemogenomic safari. *Drug Discovery Today*. 2005;10:1467-74.
98. Kahn M. Symmetric division versus asymmetric division: a tale of two coactivators. *Future Med Chem*. 2011;3:1745-63.
99. Miyabayashi T, Teo JL, Yamamoto M, McMillan M, Nguyen C, Kahn M. Wnt/beta-catenin/CBP signaling maintains long-term murine embryonic stem cell pluripotency. *Proc Natl Acad Sci U S A*. 2007;104:5668-73.
100. Bailey JM, Alsina J, Rasheed ZA, McAllister FM, Fu YY, Plentz R, et al. DCLK1 marks a morphologically distinct subpopulation of cells with stem cell properties in preinvasive pancreatic cancer. *Gastroenterology*. 2014;146:245-56.

101. Goodman RH, Smolik S. CBP/p300 in cell growth, transformation, and development. *Genes Dev.* 2000;14:1553-77.
102. Kim YM, Ma H, Oehler VG, Gang EJ, Nguyen C, Masiello D, et al. The gamma catenin/CBP complex maintains survivin transcription in beta-catenin deficient/depleted cancer cells. *Curr Cancer Drug Targets.* 2011;11:213-25.
103. Ait-Si-Ali S, Polesskaya A, Filleur S, Ferreira R, Duquet A, Robin P, et al. CBP/p300 histone acetyl-transferase activity is important for the G1/S transition. *Oncogene.* 2000;19:2430-7.
104. Chan CH, Lee SW, Wang J, Lin HK. Regulation of Skp2 expression and activity and its role in cancer progression. *ScientificWorldJournal.* 2010;10:1001-15.
105. Hurlin PJ, Huang J. The MAX-interacting transcription factor network. *Semin Cancer Biol.* 2006;16:265-74.
106. Faiola F, Liu X, Lo S, Pan S, Zhang K, Lyman E, et al. Dual regulation of c-Myc by p300 via acetylation-dependent control of Myc protein turnover and coactivation of Myc-induced transcription. *Mol Cell Biol.* 2005;25:10220-34.
107. Jin J, Harper JW. A license to kill: transcriptional activation and enhanced turnover of Myc by the SCF(kp2) ubiquitin ligase. *Cancer Cell.* 2003;3:517-8.
108. Kim MP, Evans DB, Wang H, Abbruzzese JL, Fleming JB, Gallick GE. Generation of orthotopic and heterotopic human pancreatic cancer xenografts in immunodeficient mice. *Nat Protoc.* 2009;4:1670-80.
109. Burkholder T, Foltz C, Karlsson E, Linton CG, Smith JM. Health Evaluation of Experimental Laboratory Mice. *Curr Protoc Mouse Biol.* 2012;2:145-65.

110. Guo R, Xu D, Wang W. Identification and analysis of new proteins involved in the DNA damage response network of Fanconi anemia and Bloom syndrome. *Methods*. 2009;48:72-9.
111. Li C, Wong WH. Model-based analysis of oligonucleotide arrays: expression index computation and outlier detection. *Proc Natl Acad Sci U S A*. 2001;98:31-6.
112. Huang da W, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc*. 2009;4:44-57.
113. MacDonald BT, Tamai K, He X. Wnt/beta-catenin signaling: components, mechanisms, and diseases. *Dev Cell*. 2009;17:9-26.
114. Palmer HG. Vitamin D3 promotes the differentiation of colon carcinoma cells by the induction of E-cadherin and the inhibition of beta-catenin signaling. *J Cell Biol*. 2001;154:369-88.
115. Aguilera O, Pena C, Garcia JM, Larriba MJ, Ordonez-Moran P, Navarro D, et al. The Wnt antagonist DICKKOPF-1 gene is induced by 1alpha,25-dihydroxyvitamin D3 associated to the differentiation of human colon cancer cells. *Carcinogenesis*. 2007;28:1877-84.
116. Moukayed M, Grant WB. Molecular link between vitamin D and cancer prevention. *Nutrients*. 2013;5:3993-4021.
117. Skinner HG, Michaud DS, Giovannucci E, Willett WC, Colditz GA, Fuchs CS. Vitamin D intake and the risk for pancreatic cancer in two cohort studies. *Cancer Epidemiol Biomarkers Prev*. 2006;15:1688-95.

118. Wolpin BM, Ng K, Bao Y, Kraft P, Stampfer MJ, Michaud DS, et al. Plasma 25-hydroxyvitamin D and risk of pancreatic cancer. *Cancer Epidemiol Biomarkers Prev.* 2012;21:82-91.
119. Liu SL, Zhao YP, Dai MH, You L, Wen Z, Xu JW. Vitamin D status and the risk of pancreatic cancer: a meta-analysis. *Chin Med J (Engl).* 2013;126:3356-9.
120. Van Loon K, Owzar K, Jiang C, Kindler HL, Mulcahy MF, Niedzwiecki D, et al. 25-Hydroxyvitamin D levels and survival in advanced pancreatic cancer: findings from CALGB 80303 (Alliance). *J Natl Cancer Inst.* 2014;106.
121. Kawa S, Nikaido T, Aoki Y, Zhai Y, Kumagai T, Furihata K, et al. Vitamin D analogues up-regulate p21 and p27 during growth inhibition of pancreatic cancer cell lines. *Br J Cancer.* 1997;76:884-9.
122. Yu WD, Ma Y, Flynn G, Muindi JR, Kong RX, Trump DL, et al. Calcitriol enhances gemcitabine anti-tumor activity in vitro and in vivo by promoting apoptosis in a human pancreatic carcinoma model system. *Cell Cycle.* 2010;9:3022-9.
123. Kawa S, Yoshizawa K, Nikaido T, Kiyosawa K. Inhibitory effect of 22-oxa-1,25-dihydroxyvitamin D₃, maxacalcitol, on the proliferation of pancreatic cancer cell lines. *J Steroid Biochem Mol Biol.* 2005;97:173-7.
124. Sherman MH, Yu RT, Engle DD, Ding N, Atkins AR, Tiriack H, et al. Vitamin D receptor-mediated stromal reprogramming suppresses pancreatitis and enhances pancreatic cancer therapy. *Cell.* 2014;159:80-93.
125. Nagai M, Meerloo T, Takeda T, Farquhar MG. The adaptor protein ARH escorts megalin to and through endosomes. *Mol Biol Cell.* 2003;14:4984-96.

126. Mameza MG, Lockard JM, Zamora E, Hillefors M, Lavina ZS, Kaplan BB. Characterization of the adaptor protein ARH expression in the brain and ARH molecular interactions. *J Neurochem.* 2007;103:927-41.
127. Garuti R, Jones C, Li WP, Michaely P, Herz J, Gerard RD, et al. The modular adaptor protein autosomal recessive hypercholesterolemia (ARH) promotes low density lipoprotein receptor clustering into clathrin-coated pits. *J Biol Chem.* 2005;280:40996-1004.
128. Yamamoto H, Sakane H, Yamamoto H, Michiue T, Kikuchi A. Wnt3a and Dkk1 regulate distinct internalization pathways of LRP6 to tune the activation of beta-catenin signaling. *Dev Cell.* 2008;15:37-48.
129. Jiang Y, He X, Howe PH. Disabled-2 (Dab2) inhibits Wnt/beta-catenin signalling by binding LRP6 and promoting its internalization through clathrin. *EMBO J.* 2012;31:2336-49.
130. Mao B, Wu W, Davidson G, Marhold J, Li M, Mechler BM, et al. Kremen proteins are Dickkopf receptors that regulate Wnt/beta-catenin signalling. *Nature.* 2002;417:664-7.
131. Maharzi N, Parietti V, Nelson E, Denti S, Robledo-Sarmiento M, Setterblad N, et al. Identification of TMEM131L as a novel regulator of thymocyte proliferation in humans. *J Immunol.* 2013;190:6187-97.
132. Rives AF, Rochlin KM, Wehrli M, Schwartz SL, DiNardo S. Endocytic trafficking of Wingless and its receptors, Arrow and DFrizzled-2, in the *Drosophila* wing. *Dev Biol.* 2006;293:268-83.

133. Ding N, Yu RT, Subramaniam N, Sherman MH, Wilson C, Rao R, et al. A vitamin D receptor/SMAD genomic circuit gates hepatic fibrotic response. *Cell*. 2013;153:601-13.
134. Donahue TR, Tran LM, Hill R, Li Y, Kovochich A, Calvopina JH, et al. Integrative survival-based molecular profiling of human pancreatic cancer. *Clin Cancer Res*. 2012;18:1352-63.
135. Zugmaier G, Jager R, Grage B, Gottardis MM, Havemann K, Knabbe C. Growth-inhibitory effects of vitamin D analogues and retinoids on human pancreatic cancer cells. *Br J Cancer*. 1996;73:1341-6.
136. Colston KW, James SY, Ofori-Kuragu EA, Binderup L, Grant AG. Vitamin D receptors and anti-proliferative effects of vitamin D derivatives in human pancreatic carcinoma cells in vivo and in vitro. *Br J Cancer*. 1997;76:1017-20.
137. Pettersson F, Colston KW, Dalglish AG. Differential and antagonistic effects of 9-cis-retinoic acid and vitamin D analogues on pancreatic cancer cells in vitro. *Br J Cancer*. 2000;83:239-45.
138. Schwartz GG, Eads D, Rao A, Cramer SD, Willingham MC, Chen TC, et al. Pancreatic cancer cells express 25-hydroxyvitamin D-1 alpha-hydroxylase and their proliferation is inhibited by the prohormone 25-hydroxyvitamin D3. *Carcinogenesis*. 2004;25:1015-26.
139. Evans TR, Colston KW, Lofts FJ, Cunningham D, Anthoney DA, Gogas H, et al. A phase II trial of the vitamin D analogue Seocalcitol (EB1089) in patients with inoperable pancreatic cancer. *Br J Cancer*. 2002;86:680-5.

140. Sheinin Y, Kaserer K, Wrba F, Wenzl E, Kriwanek S, Peterlik M, et al. In situ mRNA hybridization analysis and immunolocalization of the vitamin D receptor in normal and carcinomatous human colonic mucosa: relation to epidermal growth factor receptor expression. *Virchows Arch.* 2000;437:501-7.
141. Li Z, Jia Z, Gao Y, Xie D, Wei D, Cui J, et al. Activation of vitamin D receptor signaling downregulates the expression of nuclear FOXM1 protein and suppresses pancreatic cancer cell stemness. *Clin Cancer Res.* 2015;21:844-53.
142. Wang K, Dong M, Sheng W, Liu Q, Yu D, Dong Q, et al. Expression of vitamin D receptor as a potential prognostic factor and therapeutic target in pancreatic cancer. *Histopathology.* 2015.
143. Blanke CD, Beer TM, Todd K, Mori M, Stone M, Lopez C. Phase II study of calcitriol-enhanced docetaxel in patients with previously untreated metastatic or locally advanced pancreatic cancer. *Invest New Drugs.* 2009;27:374-8.
144. Hummel D, Aggarwal A, Borka K, Bajna E, Kallay E, Horvath HC. The vitamin D system is deregulated in pancreatic diseases. *J Steroid Biochem Mol Biol.* 2014;144 Pt B:402-9.
145. Meyer MB, Goetsch PD, Pike JW. VDR/RXR and TCF4/beta-catenin cistromes in colonic cells of colorectal tumor origin: impact on c-FOS and c-MYC gene expression. *Mol Endocrinol.* 2012;26:37-51.
146. Shah S, Islam MN, Dakshanamurthy S, Rizvi I, Rao M, Herrell R, et al. The molecular basis of vitamin D receptor and beta-catenin crossregulation. *Mol Cell.* 2006;21:799-809.

147. Olmeda D, Castel S, Vilaro S, Cano A. Beta-catenin regulation during the cell cycle: implications in G2/M and apoptosis. *Mol Biol Cell*. 2003;14:2844-60.
148. Davidson G, Shen J, Huang YL, Su Y, Karaulanov E, Bartscherer K, et al. Cell cycle control of wnt receptor activation. *Dev Cell*. 2009;17:788-99.
149. Lin Z, Gao C, Ning Y, He X, Wu W, Chen YG. The pseudoreceptor BMP and activin membrane-bound inhibitor positively modulates Wnt/beta-catenin signaling. *J Biol Chem*. 2008;283:33053-8.
150. He G, Gupta S, Yi M, Michaely P, Hobbs HH, Cohen JC. ARH is a modular adaptor protein that interacts with the LDL receptor, clathrin, and AP-2. *J Biol Chem*. 2002;277:44044-9.
151. Liu CC, Kanekiyo T, Roth B, Bu G. Tyrosine-based signal mediates LRP6 receptor endocytosis and desensitization of Wnt/beta-catenin pathway signaling. *J Biol Chem*. 2014;289:27562-70.
152. Lozano E, Cano A. Cadherin/catenin complexes in murine epidermal keratinocytes: E-cadherin complexes containing either beta-catenin or plakoglobin contribute to stable cell-cell contacts. *Cell Adhes Commun*. 1998;6:51-67.
153. Fuentealba LC, Eivers E, Ikeda A, Hurtado C, Kuroda H, Pera EM, et al. Integrating patterning signals: Wnt/GSK3 regulates the duration of the BMP/Smad1 signal. *Cell*. 2007;131:980-93.
154. Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, et al. STAR: ultrafast universal RNA-seq aligner. *Bioinformatics*. 2013;29:15-21.

155. Trapnell C, Hendrickson DG, Sauvageau M, Goff L, Rinn JL, Pachter L.
Differential analysis of gene regulation at transcript resolution with RNA-seq. *Nat Biotechnol.* 2013;31:46-53.
156. Roberts A, Pimentel H, Trapnell C, Pachter L. Identification of novel transcripts in annotated genomes using RNA-Seq. *Bioinformatics.* 2011;27:2325-9.
157. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci U S A.* 2005;102:15545-50.
158. Rhim AD, Oberstein PE, Thomas DH, Mirek ET, Palermo CF, Sastra SA, et al. Stromal elements act to restrain, rather than support, pancreatic ductal adenocarcinoma. *Cancer Cell.* 2014;25:735-47.
159. Olive KP, Jacobetz MA, Davidson CJ, Gopinathan A, McIntyre D, Honess D, et al. Inhibition of Hedgehog signaling enhances delivery of chemotherapy in a mouse model of pancreatic cancer. *Science.* 2009;324:1457-61.
160. Acebron SP, Karaulanov E, Berger BS, Huang YL, Niehrs C. Mitotic wnt signaling promotes protein stabilization and regulates cell size. *Mol Cell.* 2014;54:663-74.