DISCOVERY AND CHARACTERIZATION OF NOVEL CHEMICAL INHIBITORS OF WNT LIGAND PRODUCTION WITH IMPLICATIONS FOR ANTI-CANCER THERAPY

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In dedication to my family, teachers, and friends, whose support and guidance throughout the years created a foundation for the following effort

DISCOVERY AND CHARACTERIZATION OF NOVEL CHEMICAL INHIBITORS OF WNT LIGAND PRODUCTION WITH IMPLICATIONS FOR ANTI-CANCER THERAPY

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Discovery and characterization of novel chemical inhibitors of
Wnt ligand production with implications for anti-cancer therapy

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The widespread and indispensable nature of Wnt morphogens during early development has long been a focal point of embryogenesis research. More recently, pathway activation has been uncovered in adult stem cell niches and cancers arising from associated tissues. Consequently, interest has shifted towards achieving chemically based control over aberrant pathway responses, with the ultimate goal of creating therapeutic utility. To this end our laboratory screened a diverse 200K compound library using cell based transcriptional reporters and uncovered several classes of small molecules that modulate distinct Wnt regulatory nodes. One class in particular, termed IWP (Inhibitor of Wnt Production), represents the first specific chemical inhibitors of a Membrane Bound O-Acyltransferase family member: Porcupine. Herein we postulate that IWPs function by binding to and disrupting Porcupine's active site, preventing subsequent acylation of Wnt ligands necessary for downstream pathway activation. Further SAR studies reveal first generation IWP lead structures to be superior at this task *in vitro*, with even modest structural changes negatively impacting compound activity. Finally, we propose that due to Porcupine's upstream position within the Wnt pathway, IWP administration has the potential to disrupt oncogenic contributions made by recently uncovered non-canonical Wnt signaling events. Together these findings advance our understanding of Wnt chemical tractability with long-term implications for targeted therapies.

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Prior Publications

1. Tang W, Dodge M, Gundapaneni D, Michnoff C, Roth M, Lum L. A genome-wide RNAi screen for Wnt-beta-catenin pathway components identifies unexpected roles for TCF transcription factors in cancer. *PNAS*. (2008) 105: 9697-702.
2. Chen B, Dodge M (Co-first author), Tang W, Lu J, Ma Z Fan C, Wei S, Hao W, Kilgore J, Williams N, Roth M, Amatruda J, Chen C, Lum L. Small molecule-mediated disruption of Wnt-dependent signaling in tissue regeneration and cancer. *Nat Chem Biol.* (2009) 5: 100-7
3. Karner C, Merkel C, Dodge M, Ma Z, Lu J, Chen C, Lum L, Carroll T. Tankyrase is necessary for canonical Wnt signaling during kidney development. *Dev. Dyn*. (2010) 239: 2014-23.
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6. Dodge M, Lum L. Drugging the cancer stem cell compartment: Lessons learned from the Hedgehog and Wnt signal transduction pathways. *Annu. Rev. Pharmacol. Toxicol*. (2011). 10: 289-310.

List of Figures and Tables

Abbreviations

aa: Amino acid
ABC: ATP-binding cassette
ACAT: Acetyl-CoA acetyltransferase
ACC: Acetyl-CoA carboxylase
ACP: Acyl-carrier protein
AKT: RAC-alpha serine/threonine-protein kinase
ALDH: Aldehyde dehydrogenase
AML: Acute myeloid leukemia
ATC: Anatomical therapeutic chemical
ATCC: American tissue culture collection
BA2: BODIPY-aminoacetate
BA3: BODIPY-aminoacetaldehyde
BODIPY: Boron-dipyrromethene
bp: Base pair
β-TRCP: Beta-transducin repeat containing protein
APC: Adenomatous polyposis coli
Cadherin: Calcium dependent adhesion protein
cAMP: Cyclic adenosine monophosphate
CD: Cluster of differentiation
CK1α: Casein kinase 1 alpha
CoA: Coenzyme A
CM: Conditioned media
CML: Chronic myelogenous leukemia
CMV: Cytomegalovirus
ConA: Concanavalin A
COX: Cyclooxygenase
CSC: Cancer stem cell
CTNNB1: β-Catenin
Cy3: Cyanine dye 3
DEAB: 4-Diethylamino-benzaldehyde
DGAT: Diacylglycerol O-acyltransferase
DHFR: Dihydrofolate reductase
DIX: Disheveled-Axin

DKK: Dickkopf
EGF(R): Epidermal growth factor (receptor)
EmpV: pcDNA3.1 empty vector
EMS: Ethyl methanesulfonate
ER: Endoplasmic reticulum
ERK/MAPK: Extracellular signal related kinase/mitogen-activated protein kinase
FACS: Fluorescence activated cell sorting
FAP: Familial adenomatous polyposis
Fc: Fragment crystallizable region
FITC: Fluorescein isothiocyanate
5-FU: Fluorouracil
GOF: Gain-of-function
GPCR: G-protein coupled receptor
GPI: Glycosyl phosphatidylinositol
GSK3β: Glycogen synthase kinase beta
GST: Glutathione S-transferase
HDL: High-density lipoprotein
HEK: Human embryonic kidney
HMG: High mobility group
HSC: Hematopoietic stem cell
HSP: Heat shock protein
HSPG: Heparan sulfate proteoglycan
HTS: High throughput screening
IC50: Half maximal inhibitory concentration
IF: Immunofluorescence
IL: Interleukin
IP: Immunoprecipitation
IWP: Inhibitor of wnt production
IWP-PB: Inhibitor of wnt production-polyethylene glycol- biotin derivative
IWR: Inhibitor of wnt response
JAK/STAT: Janus kinase/ signal transducer and activator of transcription
LCSC: Lung cancer stem cell
LEF: Lymphoid enhancer binding factor
LGR: Leucine-rich repeat-containing G protein coupled receptor
LOF: Loss of function

LRP: Low density lipoprotein receptor-related protein
MCR: Mutation cluster region
MBOAT: Membrane bound O-acyltransferase
Min: Multiple intestinal neoplasia
MMTV: Mouse mammary tumor virus
mTor: Mammalian target of rapamycin
NOD: Non-obese diabetic
OST: Oligosaccaharyl transferase complex
PCP: Planer cell polarity
PDGFR: Platelet derived growth factor receptor
PEG: Polyethylene glycol
PL: Pre-lyses
PP2: Protein phosphatase 2
PTEN: Phosphatase and tensin homolog
Rb: Retinoblastoma protein
RGS: Regulator of G protein signaling
RT: Room temperature
SAR: Structure-activity relationship
SCD: Stearoyl-CoA desaturase
SCID: Severe combined immunodeficiency
SLB: Sample loading buffer
SOST: Sclerostin
STF: Super-top-flash
Snx: Sorting nexin
TCF: T-cell specific transcription factor
TGF: Transforming growth factor
TOFA: 5-(Tetradecyloxy)-2-furoic acid
USC: United States code
Vps: Vacuolar protein sorting
WHO: World health organization

An introduction to Wnt mediated signaling

Metazoan development and subsequent tissue homeostasis arise from complex interplays between multiple intercellular signaling networks. The establishment of protein based morphogenic gradients represents a common means of facilitating such communication and is mechanistically effectuated by only a handful of highly conserved pathways [1]. Among them, the Wnt family of secreted ligands provides a nearly universal means of directing cell orientation, migration and differentiation in a variety of tissue types [2]. Owing to the pathways profound global effects on cellular function, co-option of Wnt signaling frequently occurs in disease states, including cancers closely associated with normal pathway processes [3].

Our present understanding of mammalian Wnt signaling began in the early 1980s with efforts to elucidate the oncogenic properties of the first known mammalian retrovirus, Mouse Mammary Tumor Virus (MMTV). Although MMTV strains were hypothesized to be a causative tumorigenesis factor in RIII/C3H mice as far back as the 1940s, the genetic mechanisms of induction remained elusive until the advent of southern blotting and advance cloning techniques [4]. Utilizing these technologies, Harold Varmus and Roel Nusse identified the preferred integration site of MMTV as a 35 kb region termed *int-1* (Integration site 1) [5]. Follow up sequencing efforts published five years later found that a gene within Int-1 was homologous to a previously characterized drosophila ortholog, *wingless* (Wg), suspected to encode a segment polarity factor (Figure 1) [6] [7]. Consequently the mammalian gene was renamed *Wnt1* (a

portmanteau of Wg and Int) [8], the first of what was to become a family of nineteen proteins (Figure 2A) [9].

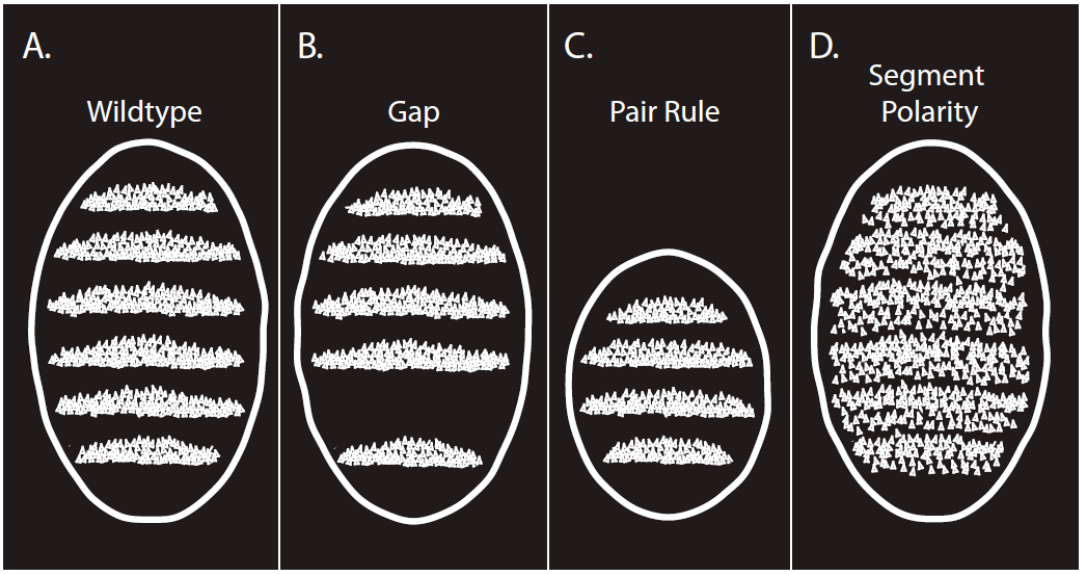


Figure 1: Classic *Drosophila* denticular phenotypes. **A**) Beginning ~4 hours post gastrulation wildtype cuticle (a chitinous structure homologous to the adult exoskeleton) produced by the ventral epidermis becomes elaborated by alternating bands of secreted denticles (white). Formation of this pattern is a complex process controlled by a variety of extracellular communication pathways and represents a convenient means of studying early segmentation. **B)** The initial expression of 'gap' genes establishes the anterior/posterior axis and includes members such as *button head, hunchback*, and *tailless*. Disruptions to this gene family often result in increased segment spacing correlating with the absence of one or more denticle belts, leading to eventual loss of contiguous body segments. **C)** 'Pair rule' genes (e.g. *even-skipped, paired, runt*) promote proper segment number and spacing. A symmetrical reduction in the number of alliterating denticle belts is frequently observed in corresponding mutants, resulting in

varying degrees of embryo length reduction. **D)** Order within denticle belts (via underlying parasegments) is controlled by 'segment polarity' genes (e.g. *wg, hedgehog, engrailed*), arguably the most phenotypically diverse of the three denticular gene groups presented here. Mutations can result in patterns duplicating, deleting, or changing anterior/posterior orientation. In the case of significant *wg* disruption, this manifests as an extension of the denticle belts (as pictured). Conversely, milder *wingless* phenotypes, as the name implies, were originally attributed to wing and haltere developmental processes, with little to no impact observed on earlier segmentation until the creation of a complete knockout[10]. Although exceptions exist, segment polarity genes are often controlled by the pair rule family, which are in turn regulated by the gap genes. (The number of denticle belts is reduced in all cases for clarity).

With the exception of Wnts 2B, 10A &10B, which are slightly larger, remaining Wnt proteins are ~40 kDa and contain 23-26 highly conserved cysteine residues, two of which bracket the families signature (C-K-C-H-G-X-S-G-X-C) motif [11]. Signaling competence is conferred by several posttranslational modifications in the Endoplasmic Reticulum (ER), beginning with oligosaccharyl transferase complex (OST) dependant N-linked glycosylation (Figure 2B) [12] [13]. Although dispensable for downstream receptor activation, loss of this adduct has been shown to impair Wnt ligand secretion from producing cells, instead favoring ER retention [14]. As efforts to quantitate Wnt binding to known chaperone proteins, such as the Hsp70 member BiP [15, 16], failed to demonstrate glycosylation dependent affinity, the addition of glycans were thought to instead facilitate subsequent ligand processing events [14, 17]. For this to occur, it has

been proposed that localization of the OST complex and its substrate, dolichol pyrophosphate, to the ER membrane sequesters Wnts into two dimensions, favoring interactions between the ligand and additional membrane associated proteins [18]. Evidence for this hypothesis comes from mutational analysis of glycosylated asparagines in select Wnts, demonstrating a loss of hydrophobicity attributed to a reduction of Wnt's second modification: palmitoylation [17]. Unlike the addition of polysaccharides, the function of Wnt acylation has been characterized to a greater extent, owing to the indispensable nature of this modification for almost all subsequent aspects of Wnt production.

Protein palmitoylation is the covalent addition of a saturated 16-carbon (C16:0) fatty acid to the side chain of an amino acid, most commonly cysteine (Figure 2C). Due to the utilization of a thioester linkage, this is specifically known as S-palmitoylation, although it is important to note that alternative reactions are possible. Attachment via hydroxyl groups (O-palmitoylation) such as on serine or threonine also occur, as well the ε-amino group of lysine (N-palmitoylation) in the case of some bacterial toxins [19]. Corresponding amine linkages in eukaryotes are comparatively rare, and are primarily restricted to the backbone of the N-terminus as classically observed with hedgehog ligands [20]. Regardless of the modified residue, palmitoylation serves to generally increase the hydrophobicity of target proteins with diverse consequences for function and stability [21-23]. Specific implications for Wnt signaling will be reviewed below at the appropriate points in the pathway.

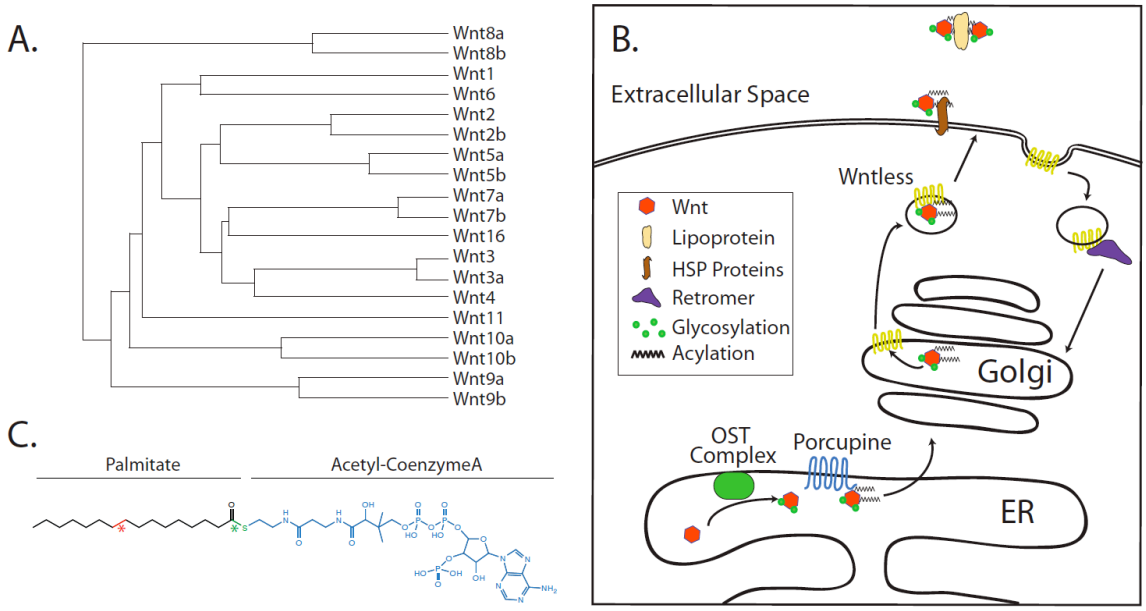


Figure 2: Wnt ligand production. **A)** Dendrogram of all 19 mammalian Wnts grouped by sequence homology (murine Wnts, modified from [24]. Human variants similarly cluster). Wnts were historically numbered based on the order of discovery, with several proteins subsequently renumbered to reflect interrelatedness based upon sequence, with the lone exception of Wnt16. A moderate but poorly understood correlation exists between structure and function. **B)** Secretion of Wnts from producing cells. Wnts are glycosylated and palmitoylated in the ER by the OST complex and Porcupine respectively. Hydrophobicity imparted by lipidation facilitates interactions with the chaperoning protein Wls, packaging/transport at the cell surface, and receiving cell receptor activation. Loss of palmitoylation prevents Wnt/Wg secretion. **C)** Structure of Palmitoyl-CoA. The leaving group during palmitoylation is speculated to be acetyl-CoA, with the thioester linkage (green) converting to an oxyester linkage upon palmitate addition to serines. Structurally related palmitoleic-CoA (Z-9-hexadecenoic acid; a

monounsaturated bond occurs at the position highlighted in red) may also be utilized in this reaction after conversion by SCD1.

Lipidation of Wnt proteins was suspected soon after their initial discovery, as biochemical studies revealed a highly hydrophobic character not apparent in the primary sequence [25]. Immunofluorescence further supported this assessment by demonstrating tight ligand association with the outer membrane of producing cells, with only a small percentage of Wnts present in the extracellular space [25, 26]. Ironically, such properties confounded the isolation of fully processed Wnts until 2003 when a mass spec of Wnt3a confirmed palmitoylation on C77 [27]. Subsequent assaying of additional Wnts found the equivalent residue to be conserved and palmitoylated/palmitoleoylated (a mono unsaturated derivative of palmitate- for simplicity, both types of fatty acylation will subsequently be referred to as 'palmitoylation' unless a distinction is explicitly required) across the family, with an additional downstream serine (S209-murine Wnt3a) similarly modified [18]. Since considerable divergence in sequence has been noted around these residues, there are no known recognition elements for Wnt palmitoylation. This leaves the extent of this modification and its impact on Wnt pathway activation relatively unknown.

As with many Wnt pathway components, the genetic underpinnings of Wnt palmitoylation were first uncovered by a *drosophila* EMS screen [28]. A search for X-linked zygotic lethal genes revealed a locus from 16E-17B that produced an expanded denticular phenotype when disrupted, similar to that previously observed in *wg* null flies (Figure 1). The studies authors called this region "Porcupine" (*porc*, *porcn* in

mammals), adding to a list of similarly manifesting Wg –like segment polarity genes including “dishevelled”, “frizzled”, “shaggy” and “armadillo”. Epigenetic mapping would later place Porcupine upstream of almost all known pathway components, functioning specifically within the ER of Wg producing cells [29]. Loss of *porc* in knockout flies resulted in Wg retention within this compartment, suggesting a role in facilitating ligand production/transport [30]. Kyte-Doolittle hydrophobicity plots indicated *porc* likely encoded a multipass membrane protein with a minimum of eight transmembrane domains, leading to some speculation that they folded to create a pore (although there was no experimental evidence to support this assertion) [30]. Questions regarding Porcupine’s actual mechanism would remain unresolved until 2000 when Kay Hofmann aligned *porcn’s* sequence to the acyltransferases ACAT, DGAT, and DltB using a bioinformatics based approach [31]. Because these other proteins acylated hydroxyl groups on lipids and lipopolysaccharides, the superfamily of Membrane Bound O-Acyltransferases (MBOAT) was created to describe Porcn activity. Unfortunately, since none of the other family members were known to target proteins directly, saccharides on Wg/Wnts, and not the protein itself, were believed to be the most likely lipid acceptors.

Following modification by Porcupine, Wnts are transported to the Golgi where they associate with the GPCR-like protein Wntless (Wls) [32, 33]. Binding is dependent upon ligand palmitoylation and serves to facilitate chaperoning of Wnts to the cell surface [34]. While the exact mechanism of this process remains to be elucidated, early studies indicate Wls possesses a high degree of specificity for Wnt transport. Secretion of additional ligands including TGF, CD2, and Shh appears unaffected, and exogenous

expression of Wls driven specifically within Wg producing cells is sufficient to rescue Wls null flies [32]. Wls function is in turn intimately linked to the more general activity of the retromer complex composed of Vps subunits 26, 29, 35, and Snx3 [35, 36]. Vps 35 and 26 in particular are known to interact directly with Wls, promoting retrieval from endosomes and presumably recycling back into the golgi to bind additional Wnts [37, 38]. Loss of either Wls or the retromer complex prevents Wnt secretion from producing cells, and in the case of *Drosophila*, further manifests as a classic *wg* null phenotype (Figure 1) [39, 40].

Physiologically relevant release of Wnt from producing cells must invariably address hydrophobicity imparted by lipidation. Consequently, most models of Wnt secretion propose burying palmitate/palmitoeyl adducts into soluble micelles, exosomes, lipoproteins or even ligand multimers [41, 42]. Although several of these mechanisms may function in concert to facilitate proper gradient formation, the majority of published data points to a lipoprotein dependence. Studies in *Drosophila* demonstrated Wg co-fractionation with lipoprotein particles, particularly those containing apolipophorin II [43]. Loss of this protein in wing imaginal disks significantly reduces Wg gradient formation, and is corroborated by similar observations of mammalian Wnt3a/HDL interactions [44]. Unfortunately, these proposed mechanisms fail to completely address observed Wnt levels, particularly at short range. A second distinct secretory system has been suggested to address this discrepancy, whereby Wnts remain associated with the outer membrane and spread to neighboring cells via lateral diffusion. Possible mediators of this process include heparan sulfate proteoglycans family members (HSPGs, e.g. Dally and Dally-like), and the microdomain protein reggie1/flotillin2. Both of these groups

have been shown to associate with Wnt proteins and promote cell surface localization [45, 46].

Wnt pathway responses elicited by ligand exposure represent a classic case of effector de-repression; in the absence of Wnt, receptive cells rely upon constitutive destruction of the pathways principle transcription factor, β-Catenin (CTNNB1) [47]. Mediating this process is a cytosolic "destruction complex" consisting of the scaffolding proteins APC, Axin, and several associated kinases (Figure 3A) [48]. Phosphorylation of β-Catenin on serine 45 by CK1α primes sequential phosphorylations on threonine 41 and serines 33/37 by GSK3β [49]. The modification of these final two residues is of particular importance and serves to promote ubiquitination by the F-Box E3 ligase β-TRCP [50]. CTNNB1 is then degraded in a proteasome dependent manner, leading to a default "pathway off" state [51]. It should be noted that this sequence of events belies considerable additional interplay between components of the destruction complex, leading to further complexities beyond simple protein turnover. Similar to CTNNB1, both Axin and APC are subject to multiple phosphorylation events by CK1α and GSK3β, leading to modulation of their respective activities [52-54]. Binding between Axin's RGS domain and APC consequently increases significantly, with additional Axin subunits possibly recruited to the complex via their DIX domains. APC responds with increased affinity for CTNNB1 on an interface common with Axin, perhaps serving to facilitate loading/unloading of CTNNB1 onto the scaffolding [55]. Alternatively, increased APC association may prevent dephosphorylation of processed CTNNB1 by shielding the transcription factor from its primary phosphatase, PP2A [56].

Ironically, the same kinase activity responsible for destruction complex assembly proves ultimately to be its undoing. Wnts binding to one of ten Frizzled cell surface receptors in conjunction with an LRP5/6 co-receptor induces localization of the scaffolding protein disheveled (Dvl) to the cytoplasmic face of frizzled (Figure 3B) [57-59]. This creates a binding element for Axin, whose recruitment (along with associated CK1α/GSK3β) results in the phosphorylation of five PPPSPxS repeats in LRP5/6 [60]. Destruction complex inactivation follows soon thereafter and focuses on subsequent regulation of Axin and GSK3. Both components exhibit significantly increased binding to the phosphorylated LRP5/6 sites, which in the case of GSK3β serves a sequestering role [61]. Isolation of the kinase from the destruction complex is further mediated by PP2A dependent Axin dephosphorylation, resulting in an overall relaxing of associations (see above). Finally, Axin itself is marked for degradation by the PARsylating (Poly-ADP-Ribose Polymerase) enzyme Tankyrase, modification by which promotes subsequent ubiquitination by the RING-domain E3 ubiquitin ligase RNF146 [62].

Freed from the destruction complex, accumulating cytosolic β-Catenin is shuttled into the nucleus through direct interactions with importins and nuclear pore proteins [63]. As CTNNB1 lacks a recognizable nuclear localization signal, transport is facilitated by the small GTPase Rac1 and Jun N-terminal kinase 2 (Jnk2) [64]. These interaction require Wnt stimulation, possibility induced by a secondary signaling pathway arising from Frizzled coupled G-protein signaling (discussed in chapter 3). Phosphorylation of two serine residues on CTNNB1 by Jnk2 leads to nuclear accumulation and complexing with several high mobility group (HMG) transcription factors including TCF1, LEF1, TCF3 and TCF4 [65, 66]. (Current accepted symbols. For reference: TCF1, LEF1, &

TCF4 were respectively referred to as TCF7, TCF1α & TCF7L2 in older literature) [67]. Together these proteins bind to the minor groove of Wnt responsive elements (WREs- CCTTTGWW where W is A or T) and induce localized DNA distortions, allowing chromatin remodeling and polymerase access [68, 69]. Binding may be further enhanced through interactions between commonly present distal GC rich elements (WCCG where W is A or G) and the C-clamp domain of TCF1/4 (if present).

It is important to note that not all Wnt responsive elements are created equal; considerable CTNNB1/TCF variability for target sequences has been observed following Wnt stimulation. WRE sequence variation aside, a host of contributing factors, including posttranslational modification of transcriptional complex components, additional co-activator/repressor recruitment and alternative TCF splice variants, combine to produce numerous output perturbations. Ultimately however, the absolute level of nuclear CTNNB1 serves as the most reliable correlate to target gene expression [70]. Because proteasome dependent CTNNB1 turnover is relatively slow (~30 min λ,[71]), additional secondary mechanisms exist in the nucleus to further negatively regulate this transcriptional node. Transducin-like enhancer of split (TLE) family members (i.e. Groucho) are particularly important in this regard and function by competing for TCF binding [72]. At the same time, other proteins (e.g. Chibby, ICAT, Sox9) interact directly with CTNNB1s C-terminus to buffer against pathway activation that may result from transient CTNNB1 nuclear localization [73, 74]. Even APC, in addition to its more widely recognized destruction complex functions, has been observed to localize (via Chip) to known WREs, seemingly in direct association with the negative transcriptional regulator CtBP [75]. While this may serve to directly prevent CTNNB1 from binding to

promoters, the further exclusion of TCF/CTNNB1 interactions when CtBP/APC are present may indicate active cytoplasmic shuttling [76, 77].

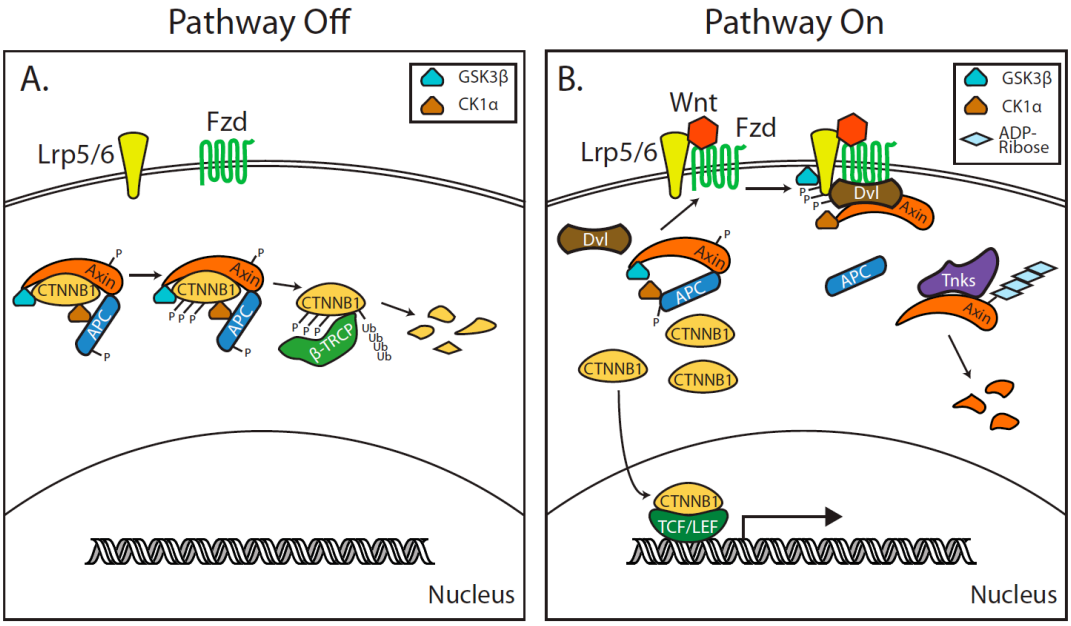


Figure 3: Canonical Wnt pathway response. **A)** In the absence of Wnt, β-Catenin (CTNNB1) is bound by a 'destruction complex' consisting of Axin, APC, GSK3β, and CK1α. The addition of multiple phosphates to CTNNB1 creates a recognition element for βTRCP which subsequently ubiquitinates CTNNB1, leading to its proteasome mediated degradation. **B)** In the presence of Wnts, Lrp5/6 and Frizzled cell surface receptors form a trimer that recruits the destruction complex and the scaffolding protein Disheveled to the cytoplasmic face of the membrane. Phosphorylation of Lrp5/6 results in increased association of GSKβ with the receptor, correlating with dephosphorylation and disassembly of the destruction complex/CTNNB1. Axin is marked for degradation by Tankyrase mediated poly ADP-ribosylation, followed by RNF146 mediated ubiquitination and degradation (whether this process is directly controlled by receptor

activation or serves a more general housekeeping function remains to be determined). CTNNB1 levels begin to increase and the protein translocates to the nucleus where it associates with TCF/LEF transcription factors, promoting target gene expression.

The first evidence of Wnts modulating transcriptional output came from a 1988 study of drosophila *engrailed* (en) expression [78]. Early embryos lacking Wg showed a significant reduction in the intensity of en-lacZ staining, suggesting receiving cells responded transcriptionally to *wg* secretion. While technically correct, the situation proved significantly more complex when engrailed was found to be a Hedgehog target gene [79], ultimately influenced by crosstalk between the pathways (within this context). These so called secondary and tertiary effects, characterized by Wnt target genes modulating additional networks, would prove to be the most visible aspect of Wnt signaling found in future studies. EGF, ERK, JAK/STAT, PTEN, AKT, IL, TGF, p53, p38 and Rb transduction (to name a few) are all impacted by Wnt, and in many cases, feed back to regulate several nodes within the CTNNB1 signaling cascade. Such far-reaching effects made the identification of true Wnt target genes difficult, and it wasn't until 1996 that drosophila *ultrabithorax* (ubx) was observed to recruit LEF1 to its promoter, making it the first *bona fide* target gene [80]. Since that time several hundred additional genes have been identified, many consisting of transcription factors (e.g. c-Myc, c-Jun, Oct4, Nanog), secreted ligands (e.g VEGF, BMPs, FGFs) or pathway intermediaries (e.g.Nemo, Cox2, CD44). Consequently, Wnts are capable of inducing complex systemic responses with profound implications for development and survival [81, 82].

Assignment of Wnt signaling to vertebrate embryogenesis arose from exploration of tumorigenicity previously associated with Int-1 (Wnt1). To this end, Andrew McMahon and Randall Moon injected Xenopus eggs with mouse Int-1 RNA, inducing significant embryonic anterior bifurcation [83]. A corresponding loss-of-function experiment in mice several years later was similarly striking, producing animals with hypoplastic midbrain and cerebrum structures [84]. (Such anterior phenotypes would ultimately prove so common upon Wnt modulation that several pathway components would take their names from them. Perhaps best known is the destruction complex scaffolding protein Axin, short for 'axis inhibition'[85].) The eventual generation of a transgenic reporter mouse (BAT-Gal) confirmed significant and complex pathway transcription within the developing brain, heart, blood vessels, lungs, kidneys, intestines, and hair follicles [86]. Numerous follow-up studies in corresponding tissues indicated widespread pathway utilization during both organogenesis and subsequent tissue homeostasis. Loss of Wnt signaling these contexts often resulted in gross histological abnormalities with ensuing embryonic or early postnatal lethality (Table 1). Unfortunately, such outcomes were so profound as to make them inaccessible to current treatment modalities. More promising are therapeutic interventions focused on gain-of-function pathway activation in adults, a frequent occurrence in a variety of diseases and cancers.

Gene	Knockout defects
Wnt1	Midbrain and hindbrain
Wnt2	Placental
Wnt2b	Wildtype
Wnt3	A/P axis fails to form
Wnt3a	A/P axis truncation, CNS dysmorphism
Wnt4	Absence of renal tubes and mullerian ducts (kidney)
Wnt5a	A/P axis truncation, distal lung, pancreas formation
Wnt5b	Wildtype
Wnt6	Wildtype
Wnt7a	A/P axis truncation, reproductive structures, synaptic maturation
Wnt7b	Placental, hypoplastic lung mesenchyme
Wnt8a	Wildtype
Wnt8b	Wildtype
Wnt9a	Skeletal/synovial malformations
Wnt9b	Kidney/reproductive defects
Wnt10a	Unknown
Wnt10b	Accelerated myoblast differentiation
Wnt11	Kidney/reproductive defects
Wnt16	Wildtype
Fzd1	Wildtype
Fzd2	Wildtype
Fzd3	Axon tract formation
Fzd4	Cerebellar/auditory dysfunction, Infertility
Fzd5	Placental angiogenesis, yolk sac
Fzd6	Hair patterning abnormalities
Fzd7	Wildtype
Fzd8	Wildtype
Fzd9	B-cell development, hippocampal defects
Fzd10	Unknown

Gene	Knockout defects
Lrp5	Pancreas defects, low bone mass
Lrp6	Truncated A/P axis, CNS defects
Dvl1	Abnormal social interaction
Dvl2	Cardiac neural crest development, neural tube closure
Dvl3	Unknown
Axin1	A/P axis duplication, Neuroectodermal and cardiac defects
Axin2	Increased osteoblast proliferation and differentiation
Apc1	Failure of ectoderm to form, A/P axis duplication, anterior truncation
Apc2	Unknown
CTNNB1	A/P axis fails to form
Lef1	B-cell development, hippocampal defects, hair formation
Tcf1	Thymocyte development
Tcf3	A/P axis fails to form
Tcf4	Intestine epithelial stem cell loss

Table 1: Summary of murine Wnt pathway component knockouts. 'Wildtype' indicates that the studies authors noted no aberrant phenotype- future crosses may reveal component redundancy in these instances (e.g. Wnts 8a/b). Components are grouped based upon general function. Red: Ligand. Blue: Receptor. Yellow: Intracellular mediator. Green: Transcription. Referenced from: [87, 88]

As with earlier MMTV integration studies, a mouse model for colon cancer would ultimately provide the first breakthroughs into Wnt driven diseases. In 1992 a min (multiple intestinal neoplasia) mouse produced from a ethylnitrosourea screen [89] was found via linkage analysis to harbor a C-terminal truncating mutation in APC (Figure 4). This by itself was not completely novel; nonsense mutations in APC (Adenomatous Polyposis Coli) had been associated with spontaneous and familial colon cancer for some time [90]. However, since these earlier observations were corollary in nature, APC loss was often viewed as secondary lesion induced by better-known oncogenes. With the discovery that APC alone (and by extension Wnt signaling) was permissive for intestinal polyp formation, loss of this gene in human cancers took on new significance. Widespread follow-up sequencing efforts uncovered rampant APC loss-of-function mutations in over 90% of colon cancers (all types) [91, 92]. More recently, adenomas have been cultured *in vitro* by isolating putative Lgr5+ intestinal stem cells and knocking out APC [93, 94]. The resulting single cells successfully reconstituted hyperplasic crypt/villa architecture as they grew, confirming that aberrant Wnt signaling alone could reliably induce structures akin to pre-adenomas. In the clinic, such 'Aberrant Crypt Foci' (ACF) represent the first stage of what can become full blown carcinomas following additional somatic hits [95]. APC thus serves as a principle 'gatekeeper' in this context, the loss of which primes subsequent disruptions to K-Ras, TGFβ (via SMAD2/4) and p53 signaling to further disease progression [96].

While the aforementioned link between APC and colon cancer represents the best-known example of Wnt driven disease, other pathway components are similarly vulnerable to appropriation. Many Wnt associated cancer types subject Exon 3 of

CTNNB1 to frequent mutations, particularly at those residues comprising CK1α/GSK3β phosphorylation recognition sequences (Figure 4) [97, 98]. The resultant uncoupling from destruction complex activity is sufficient to phenocopy APC loss in mice, although the equivalent human *in situ* occurrence is seldom observed in colorectal cancers (~1% of cases), being more prevalently in breast cancers [99]. Destruction complex activity may also be circumvented by disrupting Axin scaffolding proteins, although such cases remain considerably limited when compared to APC/CTNNB1 disruption (observed mainly in liver cancers and medulloblastomas).

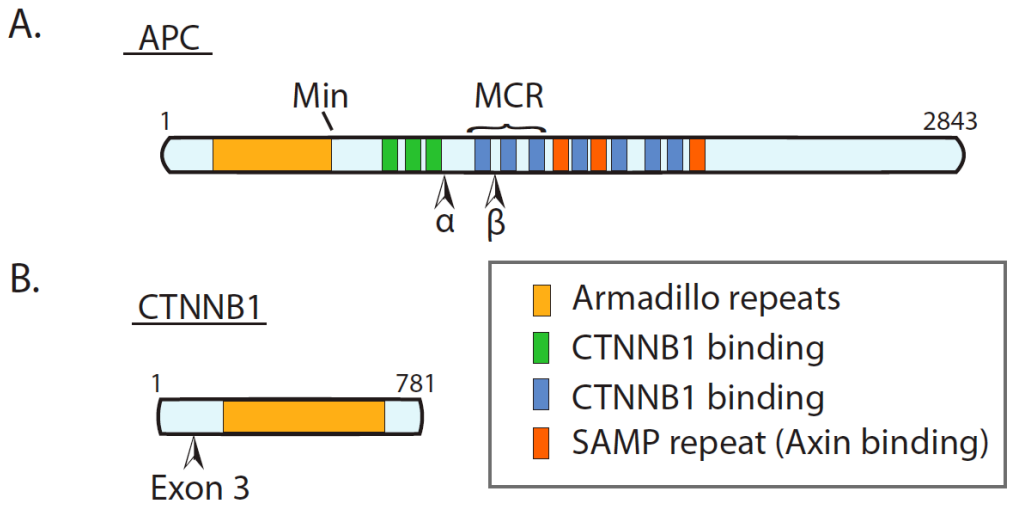


Figure 4: Common mutations found in human APC and CTNNB1. **A)** Twenty five and forty percent of familial colon cancer cases contain APC mutations at positions α (1061aa) and β (1309aa) respectively. Somatic mutations as well as FAP secondary mutations (on the remaining WT APC allele) further localize to the mutation cluster region (MCR) spanning the first three CTNNB1 binding domains (~1200-1600 aa). Mutations are primarily nonsense/frameshift in nature, effectively truncating APC and disrupting Axin/CTNNB1 interactions. The mutation corresponding to the Min mouse

model is shown for reference (aa 850). **B)** While infrequently encountered in colon cancer cases, CTNNB1 disruptions are relatively common in other cancer types. Most often these mutations center on CNNB1's third exon, interfering with negative regulation of the transcription factor. Additional domains present in both proteins not relevant to this discussion have been omitted for clarity.

Changes in absolute Wnt pathway component protein levels, as opposed to gain/loss of function mutations, are present in a variety of additional cancer types, including those arising from the skin (melanoma), lung, cervix, blood, liver, kidney (Wilms' tumor), prostate and bladder [100]. Wnt ligands, frizzled co-receptors, and disheveled family members are the most frequently upregulated candidates in these contexts, although their ultimate contribution to disease progression remains largely unknown and may in some cases be purely correlative [101]. That said, several lines of evidence support causal assignment of Wnt pathway component expression levels to disease progression. Starting with some of the earliest overexpression studies of Wnts 1,2, and 3a, a capacity to transform C57MG and CH310T1/2 cells was discovered consistent with the aforementioned retroviral MMTV1 work [102]. Other pathway components, particularly Dishevelleds 1/3 and Frizzled 7 also promote increased cell growth and metastatic properties when upregulated in lung and colon cancers [103]. Finally, loss of function studies of wildtype Wnt components, either via siRNAs or dominant negative expression, produce a reduction in cell growth and reinforce the clinical potential of Wnt pathway targeting.

Gene	Perturbation	Disease
Porcn	LOF	Focal dermal hypoplasia
Wnt3	LOF	Tetra-amelia
Wnt4	LOF	Mullerian-duct regression and virillation
Wnt4	LOF	SERKAL syndrome
Wnt5b	LOF	Type 2 diabetes
Wnt7a	LOF	Fuhrmann syndrome
Wnt10a	LOF	Odonto-onycho-dermal hypoplasia
Wnt10b	LOF	Obesity, split hand/foot malformations
Lrp5	GOF	Hyperparathyroid tumors
Lrp5	GOF	High bone mass
Lrp5	LOF	Osteoporosis-pseudoglioma
Lrp5	LOF	Familial Exudative Vitreoretnopathy
Lrp6	LOF	Coronary disease, osteoporosis
Fzd4	LOF	Familial Exudative Vitreoretnopathy
Axin1	LOF	Caudial duplication, hepatoblastomas
Axin2	LOF	Tooth agenesis
APC	LOF	Familial adenomatous polyposis, sporadic colon cancer
CTNNB1	GOF	Colon, gastric, hepatocellular, and ovarian cancers
TCF4	GOF	Type 2 diabetes

Table 2: Summary of human diseases associated with Wnt pathway signaling. The majority of listed cases are congenital and in some instances mirror observations made in mouse knockout studies (Table 1). Mutations in APC and CTNNB1 are responsible for the bulk of known Wnt associated cancers. Select disease characteristics: *Focal dermal hypoplasia;* X-linked skin, eye, teeth, skeletal, urinary, gastrointestinal, cardiovascular, and CNS defects. Also known as Goltz syndrome. *Osteoporosis-pseudoglioma*; Childhood osteoporosis and retinal detachment. *Famillial Exudative Vitreoretnopathy*; Poor retinal vascularization leading to blindness. *Terta-amelia;* Absence of all four limbs and additional bone malformations. *SERKAL syndrome (Sex Reversal and dysgenesis of Kidneys, Adrenals, and Lungs;* female to male sex reversal with renal, adrenal, and lung hypoplasia. *Fuhrmann syndrome*; Underdeveloped pelvis and finger bones. *Odonto-onycho-dermal hypoplasia;* Abnormal teeth, thickened palm skin, hair loss. GOF: Gain-of-function. LOF: Loss-of-function.

Chapter 1:

Identifying novel chemical inhibitors of Wnt ligand production

Introduction

Despite the established relevance of Wnt signaling to disease, no specific small molecules are available for therapeutic intervention [104]. Discounting recurrent pharmacology issues, (e.g. bioavailability, half-life, excretion, therapeutic window) the Wnt pathway poses a number of unique challenges for drug discovery and design. Using colon cancer as an example, arguably the most prevalent Wnt driven disease, several problems become immediately apparent. First, the nearly universal truncation of APC significantly reduces the 'drugable space' within the pathway, limiting potential targets to primarily kinases, phosphatases, ubiquitin ligases and scaffolding proteins [105, 106]. Second, all of these classes make for less than ideal regulatory nodes; pathway proteins regulating phosphorylation/ubiquitination have additional Wnt-independent functions (e.g. GSK3b also participates in BMP, TLR, hedgehog pathways) and scaffolding protein-protein interactions are notoriously difficult to specifically target (e.g. CTNNB1's TCF interface is also shared with Axin/APC). This is not to say disrupting such processes would necessarily lack Wnt therapeutic utility, but rather they would in many cases carry known off-targeting effects. Consequently, several promising chemicals targeting the Wnt pathway have been abandoned after early clinical trials, including inhibitors of GSK3 (e.g. SB-216763) and CTNNB1 complex interactions (2,4-diaminoquinazoline, ICG-001, Quercetin). What remains in the development pipeline

are almost exclusively additional GSK3 inhibitors, all of which are in the preclinical stage or on hold (as of 2011) [104].

Considering the ongoing difficulties in bringing a Wnt modulator to market, it is worth noting that some of the oldest FDA approved drugs are known to double as Wnt agonists/antagonists. Perhaps most studied is aspirin, which along with many other NSAIDs, function as cyclooxygenase (COX) inhibitors. Reduction of this enzyme family's activity blunts polyp formation in the APC Min mouse model, likely by lowering prostaglandins that are known to indirectly synergize with the Wnt pathway [107, 108]. While this mechanism would fail to provide a cure for CRC patents, the potential exists to ameliorate disease progression, particularly when coupled with additional chemotherapeutics. Consequently several studies are underway to test the efficacy of selective COX2 inhibitors and Nitric Oxide (NO) releasing aspirin with an emphasis on decreasing common side effects associated with long-term acetylsalicylic acid use [109, 110].

Although structurally divergent, both Vitamins A and D have been shown to influence Wnt signaling by activating retinoic acid receptors (RAR) and vitamin D receptors (VDR) respectively [111, 112]. Further direct interactions between these components and the CTNNB1 transcription complex have been observed and likely serve to influence promoter affinity and output. However, because numerous Wnt independent functions arise from both vitamin pathways, it becomes difficult to assign specific mechanisms to potential anticancer effects achieved by their use. The further failure of Vitamin A to improve disease progression in APC min mice casts additional doubt upon the use of retinoids to meaningfully impact Wnt signaling in cancerous contexts [113].

For its part, Vitamin D may be a more promising candidate owing to its ability to induce *in vitro* cell death and delay the onset of polyp formation in mouse models [114, 115]. Transitioning these findings to humans may require overcoming a relatively limited therapeutic window, and similar to COX inhibitors, may only yield a decrease in disease progression.

A final chemical modulator of note is lithium chloride, which has the distinction of being the first compound to produce a Wnt phenotype in a scientific context. Similar to what would be found in McMahon and Moon's Xenopus Int-1 RNA study, Thomas Morgan reported in 1902 that lithium chloride could induce secondary axis formation in developing frog embryos [116]. Follow up work by Douglas Melton almost a century later attributed these effects (in part) to lithium inhibition of GSK3, effectively preventing the phosphorylation and degradation of CTNNB1 [117]. Unfortunately, because LiCl is now known to influence many other pathways, the salt has been supplanted in contemporary Wnt studies by more specific alternatives, particularly Bio (6-bromoin-dirubin-3'-oxime) [118]. While both chemicals have gone on to advance our understanding of Wnt signaling from an academic perspective, their therapeutic utility has remained limited as most Wnt associated diseases are the result of pathway hyperactivations.

Although beyond the scope of the work to be presented, it is important to highlight several successful biologically based therapeutics. These include recently developed antibodies against the LRP5, Frizzleds, and Wnts. In all cases the mechanism of action is similar; prevention of ligand/receptor interactions and subsequent pathway activation. Success of these reagents, now in early phase I studies, will likely necessitate

targeting diseases reliant upon a subset of possible components [104]. (For example, Wnt5a can promote endothelial cell proliferation during angiogenesis in a non-redundant manner, possibly presenting a viable target in certain cancers [119]) Perhaps a more significant challenge for biologics is the perceived absence of Wnt driven diseases that would be susceptible to targeting, particularly given observed hyperactiving downstream mutations. That said, as will be explored in a latter chapter, ligand dependent signaling may still occur in cases were constitutive activation appears to predominate.

Current efforts to supplement the limited existing stock of Wnt chemical antagonists frequently involve the use of high throughput screening (HTS). While specifics vary between approaches, all HTS techniques share a number of characteristics:

1. Biological process: The starting point of screen design and the question under investigation. Possible items that can be interrogated are limited only by system complexity and in terms of Wnt signaling, have in the past included disrupting general transcription, TCF/CTNBB1 interactions, blocking kinase active sites (e.g. GSK) or modulating sub cellular localization.
2. Assay design: Careful assay design is required to insure that data collected contains all necessary controls and sufficiently addresses the problem in question. Consequently, proof of concept experiments followed by small-scale screens are a prerequisite to a full scale-up. A wide range of endpoints may be designed into the screen, including ELISA, cell death, chemiluminescence or fluorescence.
3. Library: Conceivably this may encompass a wide range of substances including peptides, small molecules, whole proteins, or genomics (e.g. siRNAs). Samples

may be arrayed individually or in pools, usually in a format amenable to the rapid processing required by subsequent steps. Libraries can range from hundreds to millions of items, and are often purchased in whole or in part from commercial sources.

4. Automation: Robotics driven by flexible programming is often used to process microplates in 96, 384, or 1536 well formats. With few exceptions, this involves the steps of fluid addition, incubation, and reading. Further manipulation (i.e. 'heterogeneous throughput') can include plate washing, filtration, or centrifugation and is avoided if possible due to the introduction of further error, signal loss and complexity. Library processing rates range from the thousands to hundreds of thousands of samples per day, depending upon the platform.
5. Multiple passes: Due to limitations in the number of endpoints that can be measured concurrently, most screens are designed to be iterative in nature. It is not uncommon for HTS approaches to contain secondary, tertiary, or even further steps to facilitate data collection. When this is done serially, the net effect is to narrow down potential hits possessing desired properties.

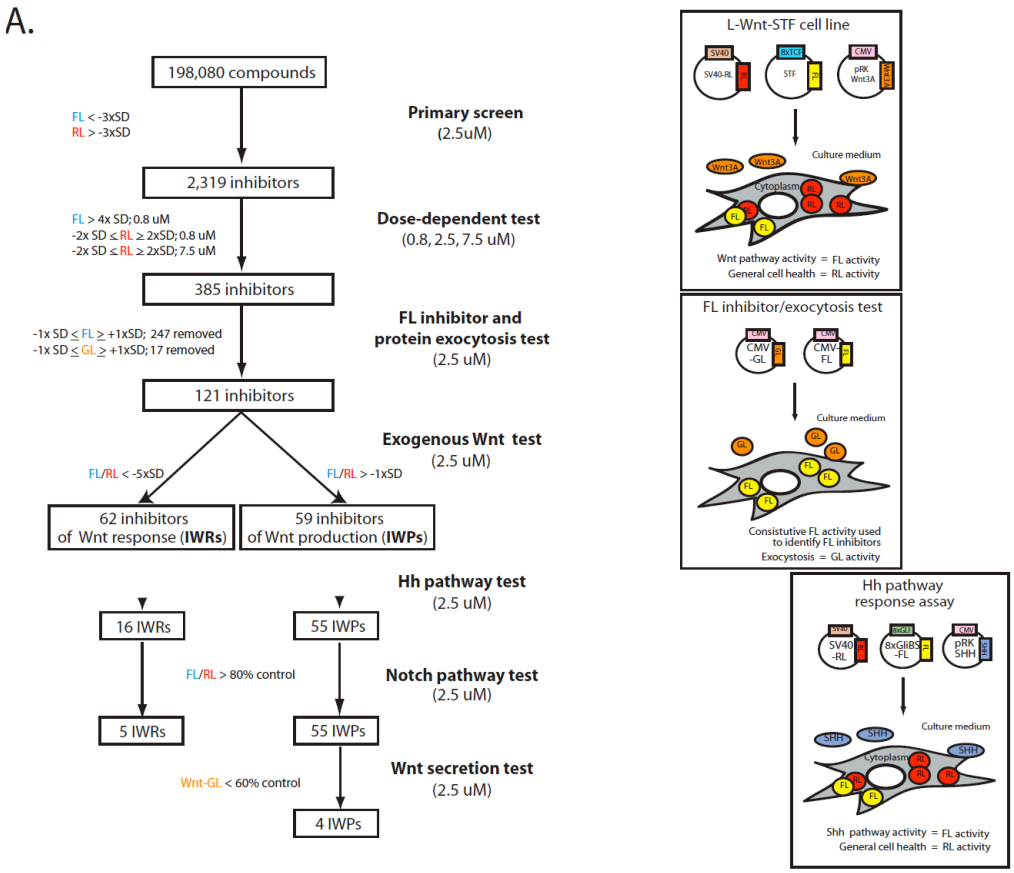
HTS based research in academia has historically been limited by the prohibitive cost of establishing required automation/libraries coupled with the inability of discoveries to recoup expenses. Intellectual property arising from federally funded research previously became public domain following any form of disclosure, creating a strong financial disincentive. This changed with the passage of the Patent and Trademark Law

Amendments Act in 1980 (35 U.S.C, §200-212), which extended patent rights to universities and the federal government prior to any third party utilization. While most researchers experienced these changes through the establishment of technology transfer offices, material transfer agreements, or industrial collaborations, the net result has been increased academic interest in HTS. Further gains in relevant expertise, robotic capabilities, and an overall lowering of associated costs have fueled the rise of the modern academic HTS core.

Despite high throughput screenings inherit flexibility, the vast majority of Wnt inhibitors are identified using fluorescent transcriptional reporters as the principle readout. Among them the SuperTopFlash (STF) reporter, consisting of eight TCF binding sites driving firefly luciferase, has become the gold standard of Wnt HTS [120]. This system allows for the simple addition of compounds to cells stably expressing the reporter, which are then measured using a luminometer following cell lyses and luciferin addition. As most cell lines have low basil levels of Wnt pathway activation, STF is often constitutively stimulated by Wnt3a conditioned media or co-expression of a Wnt construct.

Our laboratory previously utilized the STF reporter as a foundation for multiple screens, including one designed to identify novel Wnt pathway chemical antagonists (Figure 5A & methods). From this screen, 121 potential Wnt inhibitors were identified following several iterative rounds, of which 61 compounds retained activity in the presence of Wnt3a conditioned media (Figure 5B). This group's apparent dependence upon ligand source suggested an activity within Wnt receiving cells, designating the chemicals as so called Inhibitors of Wnt Response (IWRs). Conversely, the remaining 59

hits were assumed to function upstream of receptor activation (if at all), possibly by interfering with one or more aspects of ligand processing. If this were the case, such Inhibitors of Wnt Production (IWPs) would represent a completely novel means of abrogating Wnt signaling.



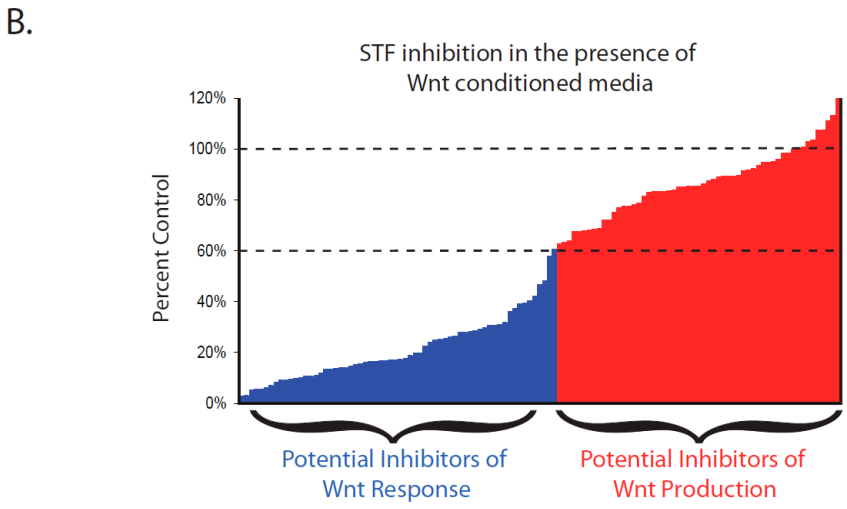


Figure 5: Novel chemical Wnt antagonists identified through HTS. **A)** The UT Southwestern 200K compound library was screened in cells constitutively expressing Wnt3a to drive a Super-Top-Flash reporter (L-Wnt STF line, upper inset panel). A SV40 driven *renilla* luciferase reporter was included as a general measure of cell health and protein production. Initial hits from the primary screen, representing approximately one percent of the library, were subsequently subjected to a 'dose dependent test' at multiple compound concentrations to verify hits with high potency and low impact on *renilla* luciferase. The resulting 385 compounds were counter screened for inhibitors of constitutively produced *gaussia* and firefly luciferases, measures of protein secretion and direct reporter inhibition respectively (middle inset). Remaining potential inhibitors were then divided into two groups based upon their ability to inhibit STF signaling in the presence of Wnt conditioned media (as opposed to cell autonomously produced Wnt). Those hits that maintained activity were assumed to function within receiving cells. Compound concentrations and cutoff values for each assay are as noted. This screen was performed by Dr. Baozhi Chen until the 121 compound stage **B)** Inhibition of exogenously mediated Wnt response. Compounds from the 121-inhibitor screen step were added L cells expressing only STF and *renilla* luciferase. Hits failing to inhibit STF response to less than 60% of DMSO control levels were tentatively referred to as inhibitors of Wnt production.

Further discussion will focus upon elucidating and applying IWPs mechanism of action to understanding compound/target interactions as they relate to cancer progression. IWR compounds were separately investigated and found to function by stabilizing Axin

proteins through disruption of Tankyrase PARsylation activity (Figure3B) [121, 122]. IWR studies are largely beyond the scope of the work to be presented, but are included as appropriate to compare and contrast with IWP findings. All error bars within figures, unless otherwise noted, are expressed as relative standard deviations.

Statement of Purpose:

Identify and characterize potential chemical inhibitors of Wnt production (IWPs) following a high throughput screening based approach. In addition to functioning as valuable tools for interrogating mechanisms underlying Wnt signal transduction, such reagents may serve as lead structures for future drug design.

Results:

The inability of potential IWPs to reduce STF activity in the presence of Wnt3a conditioned media was surprising given the multiple secondary screens passed by these compounds. In particular, failure to inhibit CMV driven *gaussia* luciferase secretion or Hh/Notch reporters suggested IWPs were disrupting Wnt specific aspects of signal generation (Figure 5A). To test this hypothesis, *in vitro* ligand secretion into surrounding media was measured using transiently expressed Wnt3a fused to *gaussia* luciferase (Figure 6A). Such an approach was necessitated by the limitations inherent to studying endogenous Wnt production; first, hydrophobicity imparted by lipidation significantly reduces Wnt release, favoring instead ligand/membrane interactions. This constrains

western blotting to cell lysate-derived fractions, which further requires Wnts to be overexpressed if commercially available antibodies are to be employed. *Gaussia* luciferase fusion circumvents these issues by both increasing Wnt solubility and providing a readily accessible enzymatic readout.

Four nearly identical compounds from the 59x subset (Figure 5A) significantly reduced extracellular Wnt3a-GL levels without affecting the cytosolic control fraction (Figure 6B). (As remaining compounds either lacked similar potency or structural homology, the IWP designation and almost all subsequent work will refer specifically to these four structures unless otherwise noted.) Further examination of journal articles, chemical databases, and patents failed to uncover any known applications for these compounds, suggesting IWPs were novel Wnt antagonists. However, because identification occurred using a single modified Wnt derivative (Wnt3a-GL), these findings were not necessarily applicable to other Wnt family members. Simply substituting other Wnt-GL luciferase constructs into the secretion assay was one possible means of exploring this issue, although this would have required additional (and time consuming) cloning steps. A faster solution was to overexpress Wnts capable of activating the STF reporter while in the presence or absence of IWPs (Figure 6C). Loss of STF response in all cases suggested that IWPs could block pathway induction by alternative Wnts, possibly through targeting of a shared production mechanism.

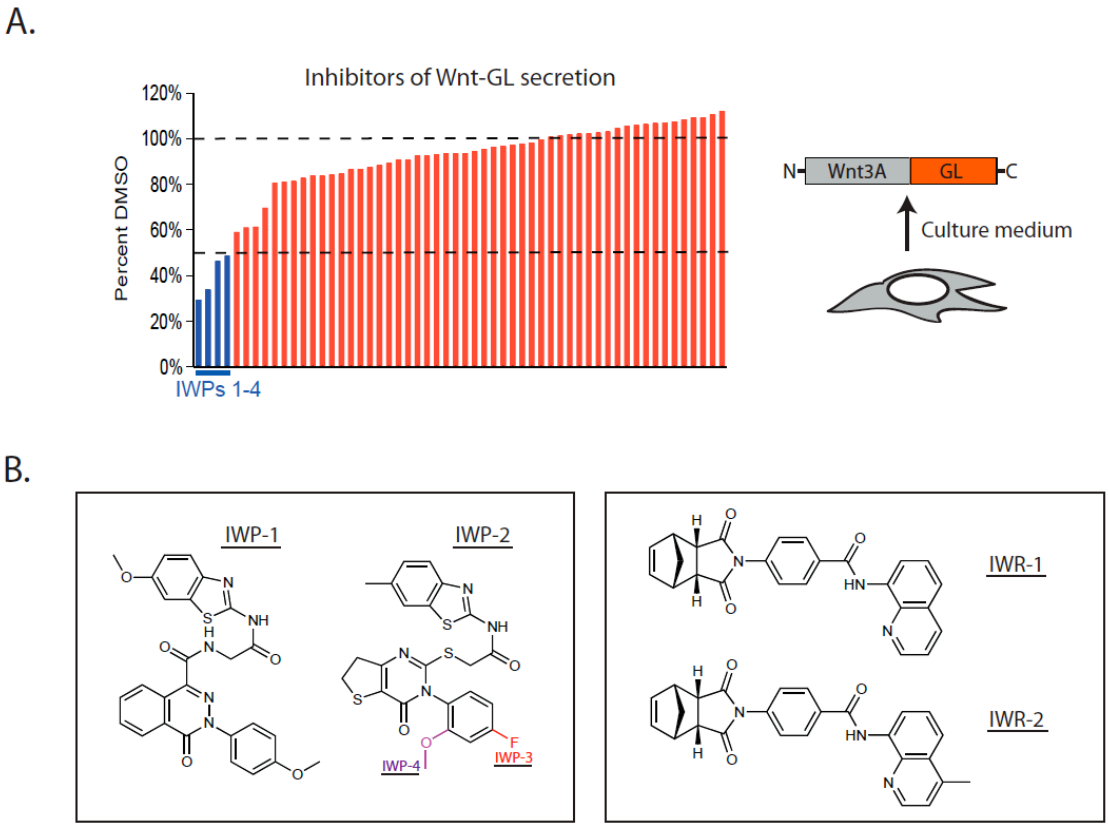


Figure 6: Inhibitors of Wnt Production. **A)** Inhibition of Wnt3a-GL secretion by potential IWPs. The 59 compounds that failed to block pathway activation in the presence of conditioned media were re-screened against cells transiently producing a Wnt3a *gaussia* luciferase C-terminal fusion protein (2.5 uM final concentrations). 4 compounds exhibited greater than 50% efficacy, and were labeled IWPs 1 through 4. **B)** Structures

of the four most potent IWPs from A). These compounds form the core of the IWP class, with IWP2 representing the lead structure. Generally speaking, the ~500 Da chemicals are composed of a dihydrothienopyrimidin/phthalazin group joined to a to a benzothiazol adduct via an acetamide linker. Additional structure/function analysis can be found in chapter 2. **C)** IWPs prevent pathway activation induced by various Wnts. L cells were transiently transfected with STF/Ren and one of several Wnts in a setup reminiscent of the primary screen design. All chemicals were tested at 2.5 uM.

IC50 values of 25-50 nM were obtained for IWPs in the cell line used for the primary screen line (Wnt3a stimulated STF, Figure 7A). This system was preferred to basing calculations upon Wnt3a-GL secretion, owing to the artificial nature of this construct, its significant overexpression, and variable transfection efficiency. Western blotting for several key Wnt pathway components further established that IWPs likely functioned at an early pathway node; membrane localized events within signal receiving cells, including the phosphorylation of Lrp6 and Disheveled, were reduced in the presence of IWPs (Figure 7B). More significantly, this translated into a downstream absence of the pathways principal effector, CTNNB1. These observations were dependent upon endogenous Wnt production and did not occur if IWP was present in conjunction with Wnt3a conditioned media (Figure 7C).

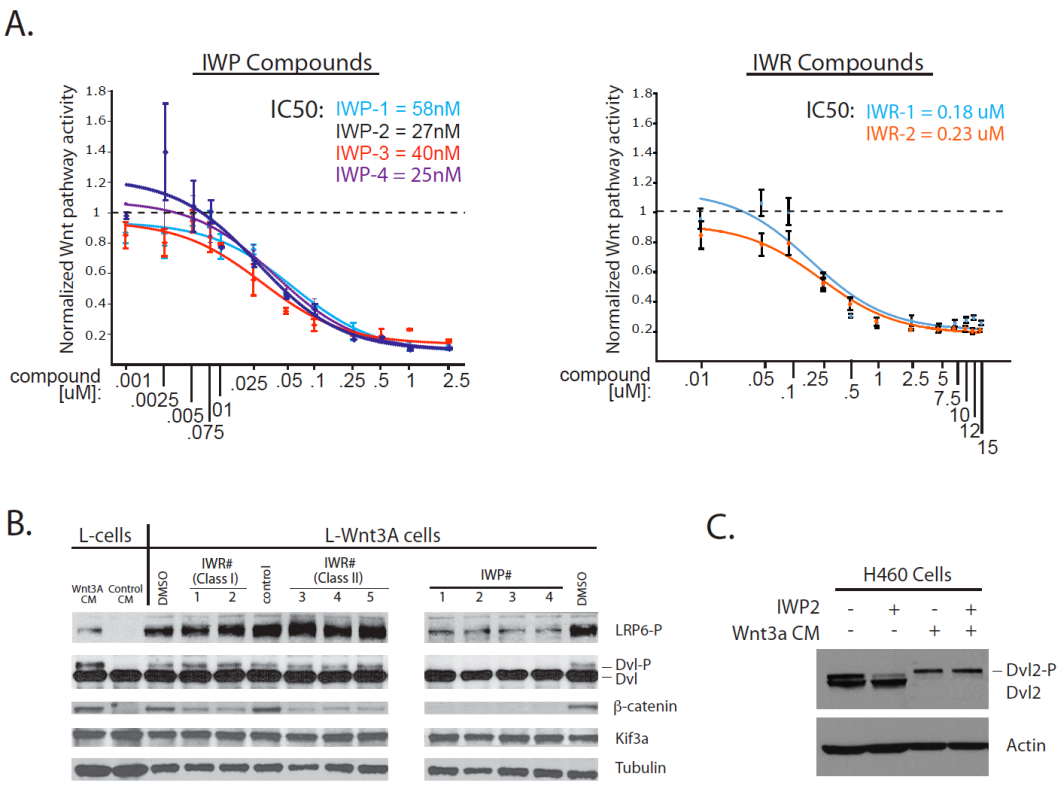


Figure 7: IWPs decrease Wnt biochemical responses in ligand receiving cells. **A)** IC50 values for IWPs. Data was collected from L-Wnt-STF cells (triplicate data points) and analyzed using Prism 5 (GraphPad). IWR compound data is shown for reference. **B)** Western blotting for select proteins within L-Wnt-STF cells implies IWP induced loss of signal transduction events at or above Fzd/Lrp. Phosphorylation of Lrp6 and Dvl2, two markers of pathway activation, decreased in the presence of IWPs (2.5 uM). This further translated to downstream reductions in CTNNB1 levels, the pathways main effector. **C)** IWPs do not function in the presence of Wnt3a conditioned media. Similar to earlier STF reporter results (Figure 5B), Dvl2 phosphorylation is insensitive to IWPs (2.5 uM) if a Wnt is externally supplied.

Determining IWPs mechanism of action via a candidate based approach appeared problematic due to an incomplete understanding of ligand production existing within the Wnt field. At the time, only Porcupine and Evenness Interrupted were believed restricted to Wnt processing, making them a reasonable starting point given the preceding data. Separate overexpression of these two genes in L cells revealed that Porcupine could rescue the inhibitory effects of IWPs on both STF and Wnt3a-GL reporters, provided low micromolar concentrations of compound were used (Figure 8A). Only Porcupine appeared to possess such activity, as no other assayed MBOAT family member reproduced these observations (Figure 8B).

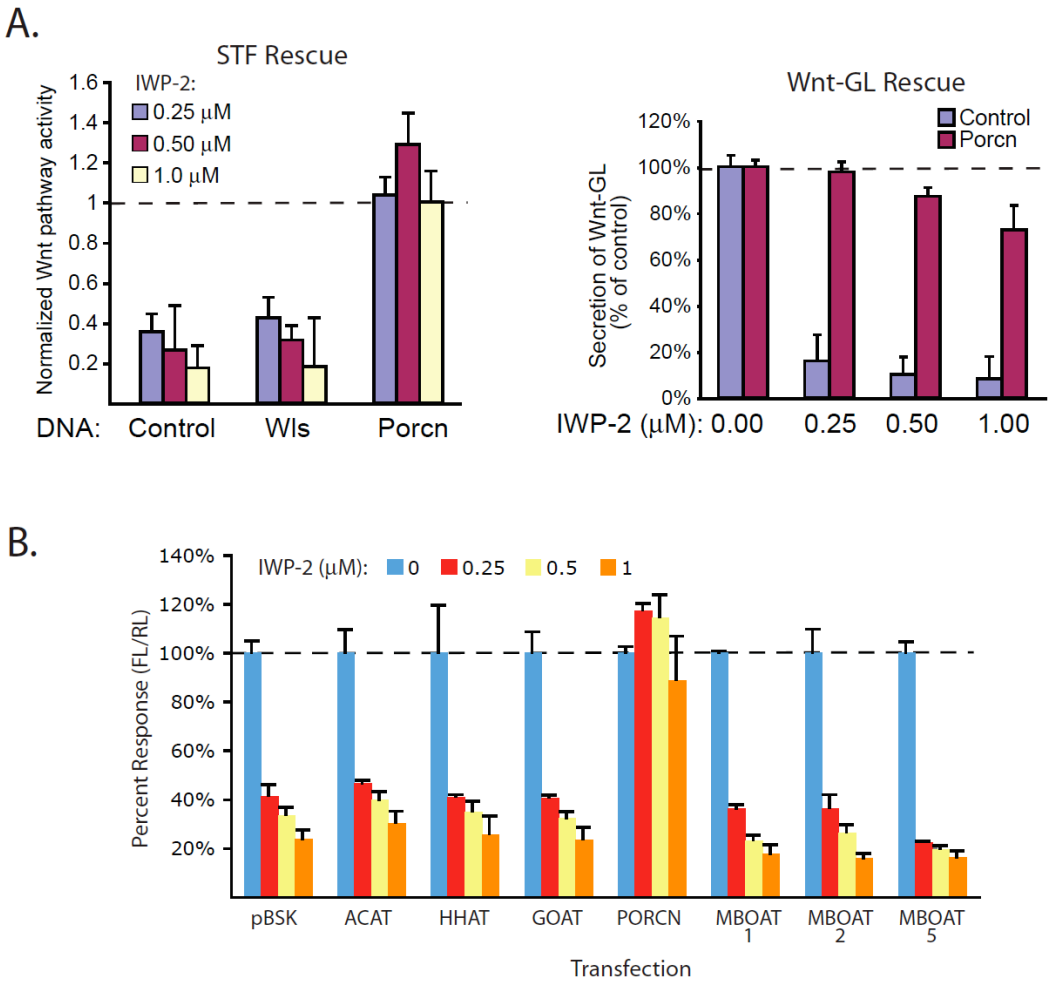


Figure 8: Porcupine overexpression reverses IWP effects. **A)** Overexpression of Porcupine rescues STF reporter response and Wnt-GL secretion as inhibited by IWPs. As shown on the right, increasing amounts of compound slowly overwhelms the ability of Porcupine to sustain Wnt-GL secretion. **B)** Select members of Porcupine's MBOAT family fail to restore Wnt transcriptional response in the presence of IWPs. Experimental setup is similar to part A), left panel. Of those shown, Porcn, Goat, and Hhat are notable for having protein-based substrates.

The hydrophobic character of palmitic acid implied that a loss of Porcupine's acyltransferase activity would manifest in increased Wnt hydrophilicity. This is indeed what was observed following a TX-114 phase assay, a commonly employed means of gauging Wnt palmitoylation status (Figures 9A/B). However, as this technique does not explicitly detect the presence of fatty acids, Wnt3a-myc incorporation of 3H-palmitoyl-CoA was additionally assayed for a more definitive result (Figure 9C). Neither experiment discounted the possibility that palmitoylation was disrupted due to glycan loss, although the banding pattern observed in the phase assay argued against this interpretation (Figure 9B). Because endogenous Wnts (and thus their glycosylation status) were difficult to detect, LRP6 glycosidase sensitivity was chosen as an alternative readout. Like Wnts, this receptor is both palmitoylated/glycosylated for function but with the added benefit of being detectable without overexpression (Figure 9D). Lrp6 PNGaseF sensitivity and EndoH resistance implied that general glycosylation could still occur in the presence of IWPs, making acylation the more likely point of disruption.

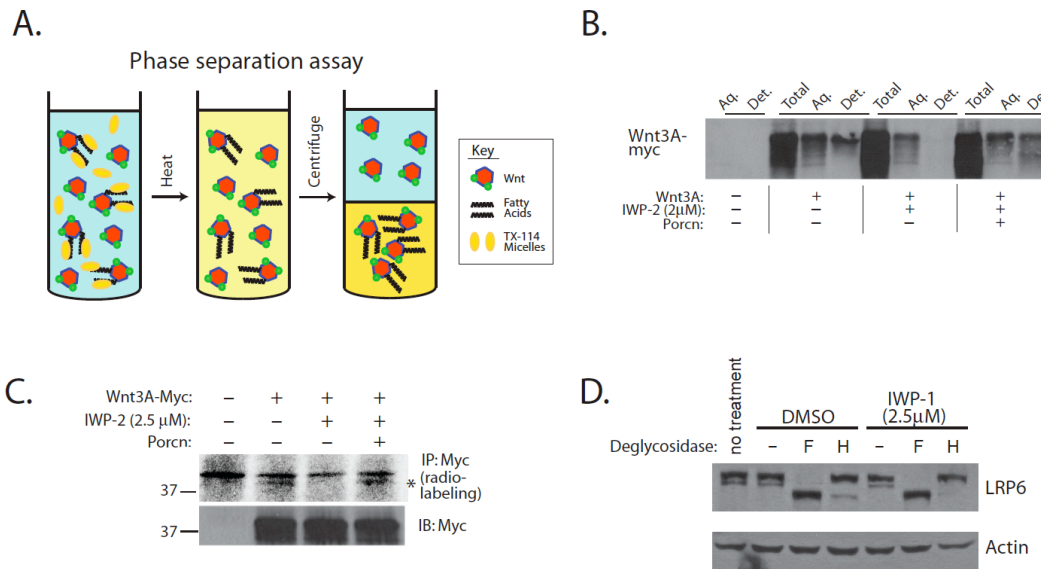


Figure 9: IWPs reduce Wnt hydrophobicity. **A)** Phase assay overview. TritonX-114 is added to sample lysates and incubated at low temperature (~4C), allowing association of hydrophobic proteins with the nonionic detergent. Heating the solution above its critical temperature (Tc ~23C at experimental concentrations, i.e. "cloud point") causes a Triton phase separation that can be pulled down along with modified Wnts following centrifugation. The upper aqueous and lower detergent phases are presumed to contain hydrophilic and hydrophobic proteins respectively. **B)** Detergent soluble Wnt3a-myc is influenced by IWPs in a TX-114 phase assay. Overexpression of Porcupine rescues this effect. The laddered appearance of the Wnt proteins, primarily in the total and aqueous fractions, is consistent with differential glycosylation states. Aq: Aqueous phase. Det: Detergent phase. **C)** Wnt incorporation of 3H-palmitate is blocked by IWPs. Similar to the phase assay, Porcn overexpression can restore lipidation. **D)** Lrp6 glycosylation is unaffected by IWPs. PNGaseF (F) sensitivity and EndoH (H) resistance following compound addition indicates the presence of mature sugars. Lrp6 was chosen based in part due to its delectability at endogenous levels when compared to Wnts.

Accounting for the data, the simplest explanation for Wnt palmitate loss was direct compound targeting of Porcupine acyltransferase activity. Other explanations were still possible, namely that Porcupine overexpression gave the appearance of a rescue by forcing Wnt production through an unknown block or that acyltransferase activity, while representing the correct node, was regulated by another factor (e.g. a porcupine-associated protein). Definitively ruling out such possibilities was viewed impractical, as even measures such as crystallizing IWP with Porcupine or reconstituting inhibition *in vitro* came with caveats. Instead, a series of somewhat unorthodox experiments were conducted that when taken together, strongly implied Porcupine targeting.

To investigate possible IWP/Porcupine interactions an affinity reagent was created with the help of Dr. Chuo Chen's lab by joining IWP2 to biotin via a PEG linker, forming IWP-PB (Figure 10A). Cell lysates containing overexpressed Porcn-myc were incubated with an IWP-PB/Avadin matrix and visualized via Western blot. Porcupine was able to associate with IWP-PB, but not with the beads themselves or the PEG linker (Figure10B). Further addition of unmodified IWP2 to the binding reaction prevented detection of Porcupine, possibly by saturating a shared binding interface.

Attempts were made to demonstrate specificity of IWP-PB for Porcupine by introducing additional peptide acyltransferases (Hhat and Goat) into the affinity assay. Unfortunately, high molecular weight aggregates were repeatedly observed for both of these MBOATs, which could only be ameliorated with harsher solubilizing conditions. Absence of binding would therefore become suspect due to the potential inability of the proteins to assume their proper native confirmations. Consequently, a cell-based approach was adopted that replaced the biotin group of IWP-PB with a Cy3 fluorophore

(IWP-Cy3, Figure 10C). Cos1 cells overexpressing Porcupine or another MBOAT family member were exposed to IWP-Cy3 and visualized using flow cytometry. It was found that only Porcupine transfected cells stained positive for Cy3, and similar to the IWP-PB binding assay, demonstrated a loss of signal in the presence of unmodified compound.

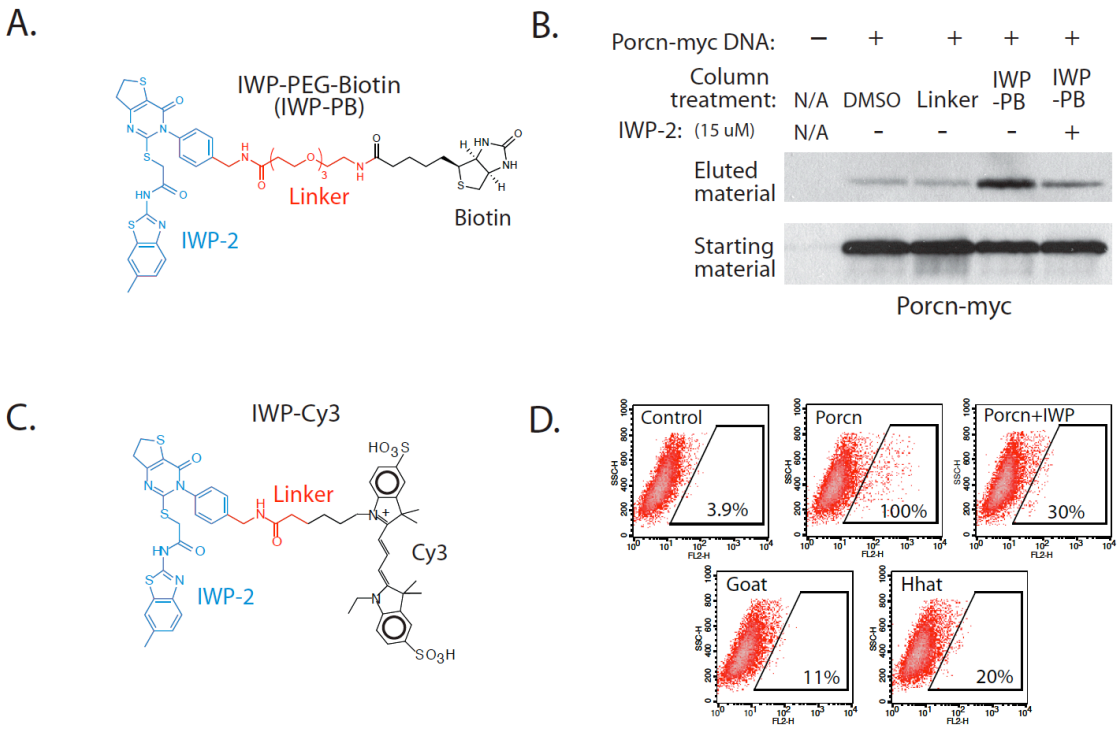


Figure 10: IWP compounds target the acyltransferase Porcupine. **A)** Structure of IWP linked to biotin (IWP-PB). Attachment of the affinity adduct to IWP via the benzyl ring's para position was chosen based upon structure-function analysis (Chapter 2). **B)** Porcupine associates with IWP-PB in an *in vitro* pull down assay. Porcn-myc derived from Cos1 cell membrane fractions was incubated with avidin beads and varying additives. Following washing, bound Porcupine was released and visualized via western blotting. Porcn-myc only associated with IWP-PB, and was competed by unmodified

IWP2. **C)** Structure of IWP-Cy3 derivative used in flow cytometry association assays. The fluorophore peak excitation and emission wavelengths are 550/570 respectively. **D)** IWP-Cy3 (0.1 uM) stains Porcupine but not GOAT or Hhat transfected Cos1 cells. The presence of unmodified IWP2 (15 uM) significantly reduces this association. Percentages shown are normalized to the Porcn sample gate (upper middle).

The final piece of evidence for IWP/Porcupine interactions came from a reconstituted *in vitro* activity assay. Porcupine-Fc was precipitated with ProteinA from Cos1 membrane fractions, combined with purified Wnt3a and incubated at 37C for 1 hour with Palmitoyl-CoA. Following a phase separation assay to partition modified Wnts, resulting products were visualized by silver staining (Figure 11A). Reminiscent of earlier cell based assays (Figure 9B), the majority of Wnt3a localized in the detergent fraction but only in the absence of IWPs (Figure 11B). Further resolving the ProteinA bead eluent revealed two protein bands migrating at ~75kDa and 200~kDa respectively that could have accounted for acyltransferase activity. Both bands were positive for human Fc via western blotting, indicating they likely originated from Porcn-Fc or its aggregates (Figure 11C).

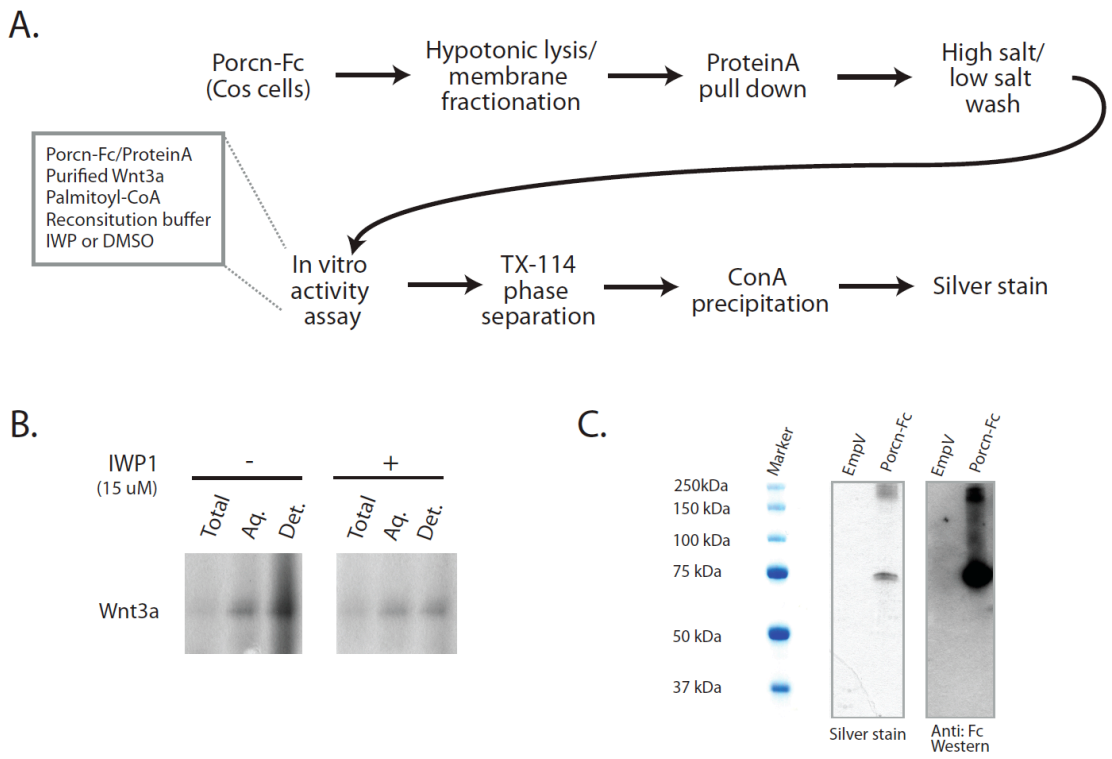


Figure 11: Porcupine acyltransferase activity can be reconstituted and inhibited *in vitro*. **A)** *In vitro* Porcn activity assay processing sequence. **B)** Wnt3a is palmitoylated by Porcn-Fc. Following incubation with Porcn-Fc, purified Wnt3a was subjected to a phase assay and silver staining to establish changes in hydrophobicity. The majority of Wnt is in the detergent soluble fraction, reversible by IWP addition to the Porcn-Fc incubation step. **C)** All discernable ProteinA silver stain bands correlate with Porcn-Fc. A portion of ProteinA beads following the wash step were heated to 95C for 2 min and eluent collected. Higher molecular weight Fc positive bands are the result of high temperature aggregate formation, and do not form under less stringent pull-down release conditions.

Discussion:

IWPs represent the first small molecules capable of specifically inhibiting Wnt ligand production. Their presumed target, the acyltransferase Porcupine, appears to be chemically tractable with profound implications for downstream pathway activity. Although preceding compound discovery efforts failed to identify IWPs despite utilizing similar libraries and reporter systems, such discrepancy is attributable to differences in Wnt ligand source. Contemporary screens more commonly employed Wnt conditioned media, which allowed reduced/uniform ligand exposure in a manner more consistent with *in vivo* conditions. Although chemicals acting upstream of receptor activation would necessarily be omitted, this was generally acceptable for two reasons. First, Wnt associated pathologies have been almost exclusively studied within receiving cells, thus narrowing the focus of corresponding drug discovery efforts. Second, most factors involved in Wnt production are not specific to the pathway, representing instead potential false positives that would latter need to be addressed. By using a cell line with autonomous Wnt production, we were able to interrogate an expanded range of pathway events.

Following counter screens to eliminate non-specific secretion inhibitors (e.g. CMV-gaussia luciferase), we identified fifty-nine chemicals absent activity when switching to Wnt conditioned media. Such dependence upon ligand source strongly favored direct disruption of Wnt production. Other interpretations were deemed less likely and involved subtle assay differences. These included the production or depletion of an unknown modulating factor in the conditioned media, or differences in signal

transduction arising from Wnts associated with the cell membrane vs. media/serum factors. Neither example could be practically addressed or otherwise falsified, leaving examination of Wnt production as the best experimental course of action. To do this, secretion of Wnt3a gaussia luciferase was assayed in the presence or absence of potential IWPs. While chemicals that impacted Wnt function but not secretion (e.g. blocking an unknown adduct) would be missed, high confidence could be assigned to positive hits since no evidence exists supporting the ability of an intracellularly retained Wnt to induce signaling.

IWP mediated loss of Wnt secretion was further corroborated by biochemical evidence. Beginning with the Wnts themselves, a TritionX-114 phase assay revealed a substantial reduction in Wnt3a-myc hydrophobicity in cells exposed to compound. Earlier chemical and genetic studies have strongly correlated this result with Wnts' palmitoylation status, although the assay could not explicitly rule out other explanations. Thus, cell based incorporation of tritium (9,10-3H) palmitic acid was undertaken but not preferred due to additional limitations. First, the chemical composition of palmitic acid most readily lends itself to 3H and C14 labeling. Both radioisotopes are relatively low beta emitters with long half lives, making for extended exposure times and low signal to noise ratios. (Iodine-125 palmitic acid could demonstrably overcome these difficulties but was not pursued for safety and availability reasons). Second, once taken up by cells, palmitic acid requires activation prior to Porcupine incorporation- *de novo* palmitate esterfication to coenzyme A via an acyl-CoA synthase occurs in multiple steps, further constraining the overall rate of radiolabeling. Attempting to bypass this bottleneck by providing cells with palmitoyl-CoA was not feasible since the conjugate cannot passively

diffuse across lipid bilayers. All told, these difficulties required several rounds of protocol optimization to overcome, ultimately confirming IWP disruption of Wnt acylation.

Earlier STF results suggested that observed losses in Wnt palmitoylation and secretion propagated down to the transcriptional level. Though previously well validated, the artificial nature of STF made it impossible to perfectly correlate reporter response with *in situ* signal transduction events. Intermediate pathway activation markers were subsequently analyzed for this purpose, namely Dvl2 and LRP6. Constitutive phosphorylation for both proteins was reduced in the presence of IWPs, consistent with an absence of ligand or disruption of Wnt/receptor interactions. Further downstream, CTNNB1 protein levels decreased significantly, considered within the field to be an unequivocal indicator of pathway deactivation. Additional cell lines consistently reproduced these findings (data not shown), provided E-Cadherin depletion (via IP) was conducted prior to western blotting. This step was needed in many cases to remove a secondary pool of CTNNB1 involved in cadherin mediated cell adhesion. (A role evident in the protein's name- 'catenin' derives from the Latin 'catena', meaning bond or restraint). While distinct from cytoplasmic CTNNB1, if present at sufficient quantity cadherin associated β-Catenins can mask detection of their Wnt transducing counterparts.

Overexpressed Porcupine's capacity to reverse IWP effects was unexpected given the relative excesses of compound present in each assay. Even by conservative estimates, the absolute concentrations of IWP should have exceeded any endogenous target by multiple orders of magnitude. Further attempts to rescue such a disparity by introducing Porcupine should have been of limited value due to transfection efficiencies and the ERs

inability to accommodate excess multipass proteins. Consequently, several models may be proposed to account for Porcupine rescue. First, the effective IWP concentration may be far lower than initially calculated due to cellular efflux, degradation or accessibility issues, making target/compound concentrations closer to parity. Second, so long as Porcupine was not undergoing suicide/irreversible inhibition, standard (competitive/noncompetitive/uncompetitive/mixed) inhibition kinetics should apply. A linear relationship would therefore exist between total enzyme concentration and reaction velocity, with additional rate limiting factors, intermediate steps, mechanism of inhibition (potentially affecting apparent KM, kcat, etc), and the cell based setting producing further complexities. All told, it is conceivable that at low but effective inhibitor concentrations, fold increases in Porcupine expression could marginally overcome IWPs effects. Further care would have to be taken under this scenario to avoid excessive inhibitor concentrations that would saturate and thereby counteract a rescue. Indeed, this negative result was observed during early rescue attempts until IWP concentrations were limited to less than 20 fold respective IC50 values. Only then was Porcupine overexpression sufficient to restore the readout in question.

Creation of several IWP derivatives (IWP-PB and IWP-Cy3) permitted indirect testing of IWP/Porcupine interactions. In the first set of experiments, the ability of Porcn-myc to associate with and be competed from IWP-PB- NeutrAvidin (using unmodified IWP) further reinforced preceding results. However, the experiment's utilization of whole cell lysates and overexpressed Porcn-myc (needed for visualization) prevented the findings from proving more definitive. For example, despite repeated column wash steps, an unknown Porcn cofactor could have served as IWPs actual target,

producing a false positive result by bridging Porcn-myc/column interactions. Likewise, an overabundance of Porcn-myc, particularly in cases where the construct misfolded, may have occasioned an interaction absent under endogenous levels. Care was taken to avoid this second problem by utilizing a buffer capable of sustaining another MBOATs (Hhat) enzymatic activity *in vitro* and presumably with it, proper folding. Additionally, observed IWP competition of Porcn-myc from IWP-PB argued for greater specificity than envisioned from aggregates.

Unlike IWP-PB, IWP-Cy3 was intended for use in live cells while concurrently avoiding *in vitro* purification problems encountered with Hhat and Goat, permitting their inclusion. However, the low number of Cy3 positive cells upon Porcn introduction was far fewer than anticipated based up previous transfection efficiencies. This argued for target accessibility issues, perhaps due to less favorable association kinetics (IWP-Cy3 possessed ~5 uM IC50s in L-Wnt cells) or compound inability to localize to the ER. Positively staining cells may therefore have represented those populations possessing exceptional Porcn expression levels, resulting in increased target availability either through mislocalization or concentration effects. Rare Cy3+ cells may also have been caused by an intrinsic limitation in experimental design; the FACSCalibur excitation laser operates at the tail end of Cy3's absorbance spectrum. This could substantially reduce fluorophore efficiency, resulting in moderately staining cells blending into the background.

In vitro reconstitution of Porcn activity arguably provided the most definitive evidence of IWP's mechanism of action. Unfortunately, Porcupine's hydrophobicity did not readily lend itself to this process; two interrelated folding issues had to first be

addressed. Buffer conditions required for a successful pull-down are often incompatible with multipass membrane proteins, resulting in active site distortions and loss of activity. Concurrent exposure of normally buried interior residues can further promote associations with a factors normally excluded under *in vivo* conditions. Both of these problems presented issue initially, and had to be countered by stringent washing (at the expense of yield) coupled with reintroduction of an activity buffer. The net result, a set of Porcn-Fc silver stain bands, was sufficient to influence Wnt hydrophobicity in an IWP dependent manner. Assuming that no faint or co-migrating contaminants account for the result, this experiment provides the first *in vitro* evidence of Porcn acyltransferase activity.

Methods:

Reagents and Equipment: University of Texas Southwestern chemical library consisting of ~200,000 compounds was assembled from ChemDiv, ChemBridge, ComGenex, Prestwick and TimTek collections according to a relaxed set of Lipinski's rules. Additional criteria and information are available at (http://www.simmonscancercenter.org/ Research/Shared+Resources/High+Throughput +Screening+HTS.html). Firefly, gaussia, and renilla luciferase activities were measured with a dual luciferase reporter system (Promega) following the manufacturer protocol. Flow cytometry experiments were carried out on a FACSCalibur (BD Biosciences) and data interpreted with CellQuest Pro (BD Biosciences). PL (pre-lyses) buffer consisted of 10 mM Tris-HCL, 150 mM NaCl, pH 7.5. Membrane fractionation buffer contained 10

mM HEPES, 10 mM KCl, 1.5 mM MgCl2, 1 mM Na-EDTA, and 250 mM sucrose at pH 7.4. Membrane solubilization buffer was made up of 100 mM MES, 20 mM NaCL, 1 mM DTT, 0.2 mM EDTA, 0.05% Tx-100, 0.2% glycerol and 0.15% octylglucoside at pH 6.5. Primary antibodies for western blotting were as follows: cMyc-9E10 (Santa Cruz), phospho-serine1490-Lrp6 (Cell Signaling), Lrp6-C5C7 (Cell Signaling), Dvl2 (Cell Signaling), Kif3a (Sigma), CTNNB1 (Sigma), Tubulin (Sigma), Actin (Sigma), and Human Fc (Bethyl Laboratories). All antibodies used at recommended concentrations (1:250-1:10,000). MBOAT constructs were a gift from the Brown and Goldstein Lab (UTSouthwestern).

Primary and secondary Wnt inhibitor screens (Performed by Baozhi Chen): 5,000 L-Wnt-STF cells were seeded into white opaque 384 well plates and individual library compounds added 24 hours later. Following an additional 24 hours of incubation, luciferase activities were measured. Potential firefly luciferase and protein secretion inhibitors were identified in a secondary screen using L cells transiently transfected with firefly (cytoplasmic) and *gaussia* (nuclear) luciferase constructs under the control of a CMV promoter. Compounds were immediately added to cells after transfection and luciferase activities measured 24 hours later. To test compound function in the presence of exogenous Wnt, Wnt3a conditioned media was added to HEK-293 cells transiently expressing the STF reporter, with luciferase activity measured 24 hours later. Hh and Notch pathway inhibition was determined by adding compounds to NIH-3T3 and L cells respectively, transiently transfected with corresponding reporters and measuring pathway response 24 hours later. See Figure 5 for relevant concentrations and cutoffs.

Wnt3a-GL secretion screen: L cells were transfected with CMV-Wnt3a-GL and CMV-FL reporters at a 5:1 ratio using Fugene6 and then incubated for 24 hours, 37C. Following trypsinization and counting, 10K cells were transferred into a 96 well opaque well format with cherry picked compounds added in triplicate, 2.5 uM, 0.5% DMSO final. After an additional 48 hours incubation, 100 uL of media was removed from each well to assay for GL activity. Remaining media was discarded and replaced with 50 uL passive lysis buffer. Cellular GL and FL activities were then read.

L-Wnt cell STF IC50s: Assayed compounds were added to L-Wnt-STF cells in a 96 opaque well format at the specified final concentrations, 0.25% DMSO. After 48 hours incubation, plates were read using the primarily screening protocol. IC50 values were derived with Prism v.5c (GraphPad) using a best-fit four parameter variable slope log(inhibitor) vs. response curve. The upper curve plateau was constrained in all cases to < 1.05, with remaining parameters determined by the program.

Porcupine STF rescue: L cells were transfected with STF-Ren, Wnt3a, and Porcupine/EmpV (1:1:1) ratio with Effectene, and seeded into 96 opaque well format, 15K cells/well. Compounds were immediately added at the stated concentrations (0.5% DMSO final) and plates incubated for 48 hrs, 37C. After media was discarded, cells were lysed with passive lysis buffer and plates read following the standard duel luciferase protocol.

Porcupine Wnt-GL rescue: L cells were transfected with CMV-FL, CMV-Wnt3a-GL, and Porcupine/EmpV (1:1:1) ratio with Effectene, and seeded into 96 opaque well format, 15K cells/well. Compounds were immediately added at the stated concentrations (0.5% DMSO final) and plates incubated for 48 hrs, 37C. 100 uL of media was collected from each well to measure GL activity (remainder discarded), cells lysed with passive lysis buffer, and plates read following the standard duel luciferase protocol.

Phase assay: Cos1 cells transfected with Wnt3a-myc (w/ Fugene6) 48 hours prior were lysed at room temperature with 300 uL 1% TX-114 in PL buffer, samples chilled on ice for 10 min, and pelleted via centrifugation. After inverting gently, supernatants were transferred to new tubes, combined with an equal volume of 3.5% TX-114 in PL buffer, and rotated at 4C for 30 min. Half of each solution was set aside representing a 'total fraction', while the remainder was heated to 37C for 5 min and then centrifuged for 8 min, 2,000 g. Detergent and aqueous phases were carefully separated, and PL buffer added in all instances to a final volume of 1 mL. Wnts were extracted via an overnight ConA pull down and visualized by anti-myc Western blotting.

Wnt radiolabeling: Cos1 cells transfected with Wnt3a-myc and/or Porcupine (Fugene6) in a 6 well format were incubated for 48 hours, the media replaced with OptiMem (no serum), and 2.5 uM IWP or DMSO control added. After 1 hour, radiolabeled palmitic acid was introduced (0.6 mCi/ well) for and additional 4 hours, the cells washed with PBS, lysed with 1% NP40 PBS, and Wnts immunoprecipitated overnight with ProteinA-anti-myc conjugated beads. Samples were run via SDS-PAGE, transferred to

nitrocellulose, and exposed to BiomaxMS film using a Transcreen LE for 3 weeks prior to development. Experiment conduced under the State of Texas Bureau of Radiation Control License #L00384, sublicense 07-12LUM.

IWP-PB membrane fractionation: Scraped Cos1 cells were resuspended in membrane fractionation buffer and passed though 21.5 and 25.5 gauge needles thirty times each. Fractions were pelleted sequentially 5x times, 1,000 g for 2 min each and the supernatants saved. Membranes were then isolated for 40 min at 20,000 g followed by resuspension in membrane solubilization buffer. Samples were vacillated 80 times with a p200 pipette tip, and placed in a sonicating water bath for 10 min. Insoluble material was pelleted 5x at 500g for 2 min each. Resulting supernatant was used immediately.

IWP-PB pull-down: NeutraAvidin beads were incubated 30 minutes with 10 mM IWP-PB, PEG linker, or DMSO (1 uL / 100 uL beads) and then washed 3x with PBS. 25 uL (bed volume) of treated Beads were combined with Porcn lysates (see IWP-PB membrane fractionation) and membrane solubilization buffer added to 300 uL with IWP2 (100 uM final) or DMSO as appropriate. Samples were rotated for 1 hr at 4C, pelleted, and washed 3 times with the binding buffer. Following SLB elution, bound Porcupine was visualized by an anti-myc Western.

IWP-Cy3 flow cytometry: 48 hours following transient transfection with a protein of interest, Cos1 cells were incubated with 0.1 uM IWP-Cy3 and 15 uM IWP2 or DMSO overnight. Cells were then washed 3x times with PBS, trypsinized, pelleted, and

resuspended in PBS for flow cytometry. Viable cells were gated on FSC vs. SSC, with Cy3 fluorescence measured on the FL2 channel. Voltages and gain adjusted as appropriate.

***In vitro* porcupine activity assay**: Cos1 cells overexpressing Porcn-Fc or empty pcDNA3.1 vector were washed 2x with RT PBS and then scraped from plates using cold membrane solubilization buffer (2 mL per 15 cm plate). Fractional lysis was completed by passing the samples though 21.5 and 25.5 gauge needles, 30 times each, followed by centrifugation to remove insoluble/nuclear material (1,000 g, 2 min, 4x times) from the supernatant. A final spin (20,000 g, 40 min) pelleted the desired membrane fraction, which was resuspended in 1% NP-40/PBS and incubated with ProteinA beads overnight at 4 C. Contaminating proteins were then removed with 5x wash steps (2x 1%NP-40/PBS, 1x 1%NP-40/PBS+ 0.5M NaCL, 1x 50% PBS/H20, and 1x PBS) and Porcn-Fc containing beads resuspended in membrane solubilization buffer. A typical *in vitro* reaction consisted of 15 uL Porcn/beads (bed volume), 60 uL purified Wnt3a (0.025 ug/uL), 2.5 uL of 5 mM Palmitoyl-CoA, 1 uL(750 uM) IWP or DMSO, and membrane solubilization buffer up to 250 uL. Samples were incubated for 1 hr at 37 C, fractions separated, and the reaction stopped by the addition of 6x SLB and incubation at 95 C for 2 min.

Silver staining: Following electrophoresis, PAGE gels were fixed in 50% MeOH/10% acetic acid for 30 min, 5% MeOH/1 % acetic acid for 15 min, and then quickly rinsed with 50% MeOH. After washing 3x, 5 min each with water, 0.2g/L Na2S2O3,5(H20)

was added for 90 seconds, the gel washed with water 4x, 5 min., incubated with $AgNO_3$ (0.2 g/100 mL) for 1 hour, and finally washed with water an additional 3 times, 1 min each. The developer consisted of Na_2CO_3 (6 g/100 mL), 2 mL $Na_2S_2O_3,5(H_2O)$ (from earlier step)/ 100 mL, and 50 uL 37% HCOH/ 100 uL, and was left standing until protein bands were satisfactorily visible. 6% acetic acid was used to stop the reaction.

Chapter 2:

The structural determinants of chemically mediated Wnt inhibition

Introduction

In contrast to their *Drosophila* ortholog, vertebrate Porcns have only been characterized within the past decade [123]. Initial clones isolated from human, mouse and *Xenopus* cDNA libraries revealed a high degree of protein conservation (Figure 12A), with the exception of a ~60 aa N-terminal leader unique to *Drosophila* Porcupine. Also of note was a short insertion sequence (NKKRKARGTMVR) found within human and mouse Porcupine consisting of two alternatively spliced exons. The four resultant mammalian isoforms appeared to have similar binding affinities for Wnts (via Co-IP), but possessed differential ability to transform P19 cells [124]. As with other proteins, Porcn splicing may ultimately regulate stability, ER sublocalization, membrane insertion, catalytic properties, ligand interactions (too subtle to be detected by previous overexpression assays) or combinations thereof [125, 126]. No functional differences between isoforms were noted in the preceding/following assays (data not shown), with all figures presented using variant C (absent exon 7). This selection was a mater of precedent; the associated construct was the first obtained and tagged.

Kyte-Doolitle and Hopp-Woods hydrophobicity plots suggest Porcupine contains 8-12 transmembrane regions, with 8 being the general consensus (Figure 12C). Detailed topology predictions have been complicated by several lengthy hydrophobic extramembrane sequences, particularly a stretch of residues preceding the 7th pass.

Rather than assuming an extended/lose conformation, this loop may fold to associate with the membrane interface residues of the adjacent 7th helix, forming an active site [31]. Both regions are highly conserved across MBOATs, with an intervening invariant polar residue characteristic of each sequence (Asn in the loop and His in the helix). Site directed mutagenesis against the histidine supports this functional assignment, corresponding to Wnt palmitoylation loss [127].

Northern blotting and RNase-protection assays revealed systemic expression of murine porcupine transcripts, particularly within the developing brain, kidney, lung, and testis [128, 129]. While most organs exhibited varying isoform combinations, certain tissues were associated with particular Porcn splice variants (e.g. Porcn D in brain/muscle, Porcn A in spleen/testis), the implications of which remain unclear. Porcn knockout mice (all isoforms) exhibited embryonic lethality and were unable to establish an anterior/posterior axis during gastrulation. A similar phenomena may occur naturally in male Porcn null humans, where the gene's hemizygous nature (from cytogenetic band Xp11.23) cannot be compensated [130]. However, even a second wildtype copy is insufficient when mosaically expressed (i.e. due to bar body formation) and is strongly associated with the development of Focal dermal hypoplasia (Goltz syndrome) in human females [131, 132]. Patients with this disease have characteristic skin lesions (atrophic macules, saccular nodules, papillomatas), hair loss, bone defects (syndactyly, ectrodactyly, osteopathia striata), eye/ear defects and renal abnormalities. Corresponding loss of function mutations typically arise from lengthy Porcn truncations or frameshifts with a minority of cases exhibiting single point mutations (Figure 12C).

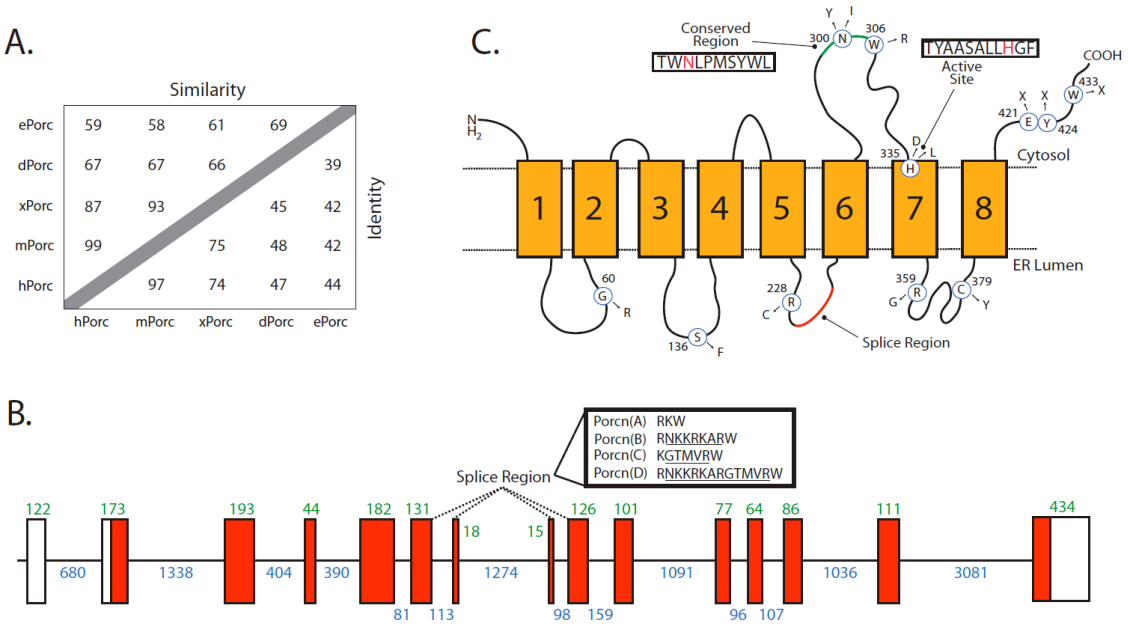


Figure 12: Structure of the membrane bound O-acyltransferase Porcupine. **A)** Porcupine amino acid similarity matrix. Gene symbols are simplified for clarity, h: Human, m: Mouse, x: *Xenopus*, d: *Drosophila*, e: *C. elegans*. Human and worm homologs are occasionally labeled as MG61 and Mom-1 respectively in the literature. Data modified from Tanaka et al. **B)** Human Porcupine mRNA Intron/Exon map. Fifteen exons encompasses a 1386 bp mRNA encoding a 461 aa protein (translated sequences in red). Alternative splicing occurs between exons 6 and 9, potentially truncating the final product by 5, 7, or 11 amino acids. Green and blue numbers are exon and intron lengths respectively. Data modified from Caricasole et al. **C)** Predicted Porcupine membrane topology. The acyltransferase has 8 transmembrane regions with N and C terminal tails protruding into the cytoplasmic space. Based upon homology with other MBOATs, the active site is believed to consist of (in part) a short stretch of residues surrounding a conserved histidine, shown here in the upper portion of the 7th pass. Due to the mixed

hydrophobicity of the protein's later half, the active site may lie closer to the ER lumen than depicted. Boxed insets represent highly conserved sequences, with polar residues shown in red. Numbered residues have been found mutated in diseases, with substitutions as shown.

Little precedent exists to model chemical inhibition of Porcupine. Studies within the diverse palmitoyl acyltransferase (PAT) superfamily, of which Porcn is a member, have previously uncovered several lipid (e.g. 2-bromopalmitate, tunicamycin, cerulenin) and small polycyclic based (e.g. structures I-IV, Ducker et al) chemical inhibitors [133, 134]. Few of these compounds possess clearly defined mechanisms of action and all are known to inhibit multiple acyltransferases. In contrast, IWPs have failed to exhibit activity against related peptide acylating MBOAT members (Hhat & Goat) in both cell and *in vitro* based assays (separate laboratories, data not shown). This suggests the existence of unique structural determinants for meditating Porcupine/IWP interactions, the exploration of which may advance IWP efficacy and our general understanding of MBOAT function.

Statement of Purpose:

Determine the structural basis for IWP/Porcupine interactions using a combination of protein mutagenesis and IWP derivatization. Relevant findings may allow for the creation of second-generation IWP compounds in addition to providing insight into MBOAT chemical tractability.

Results

Several possible IWP inhibitory mechanisms were tentatively dismissed based upon the characteristics of transiently introduced Porcupine. First was the idea that IWPs may lower Porcn levels, perhaps by inducing a misfolded protein degradation response. Western blotting of Porcn-myc suggested that in fact the opposite was occurring; Porcupine protein increased slightly following compound addition (Figure 13A). Quantitation of this phenomenon by fusing the acyltransferase to *gaussia* luciferase demonstrated a high inverse correlation between intracellular Porcn-GL increases and IWPs IC50 (Figure 13B), with similarly tagged MBOATs unable to phenocopy this result (Figure 13C). IWPs therefore appeared to be simultaneously inhibiting Porcupine activity whilst promoting stabilization.

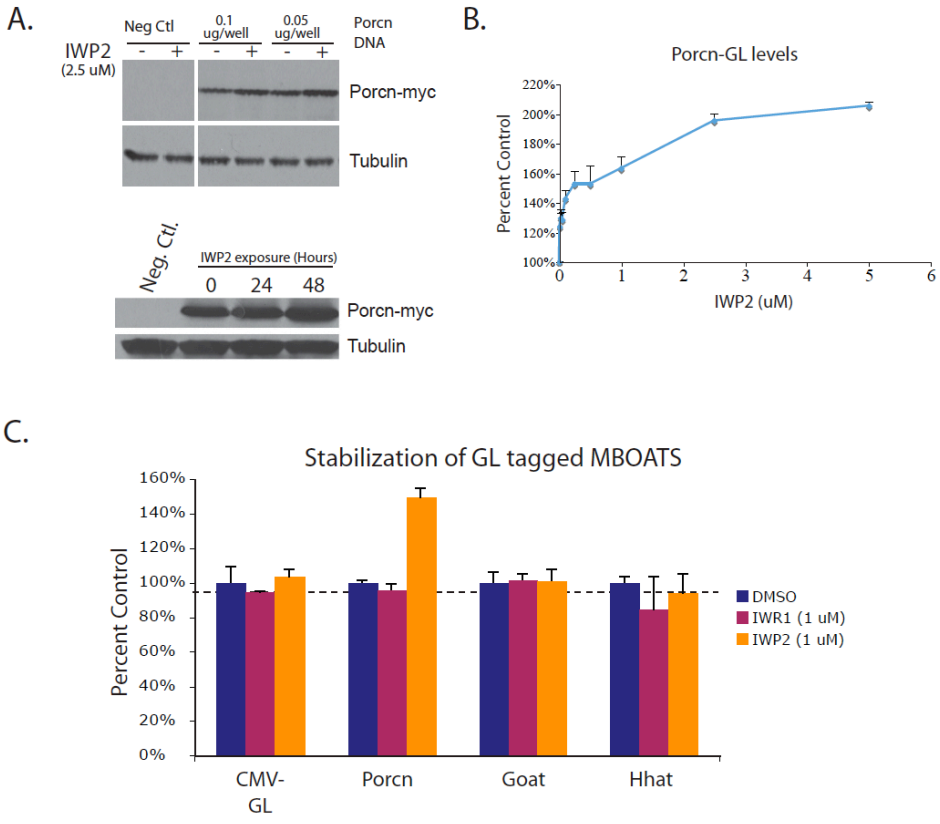


Figure 13: Porcupine protein levels increase in response to IWPs. **A)** Porcupine-myc protein levels increase slightly following IWP treatment. Tagged constructs were overexpressed at varying amounts in Cos1 cells, incubated for 48 hours, and lysates visualized via western blot (upper blot). A time course of these observations (lower blot) shows progressive buildup over the course of several days. (Cells were transfected and harvested at the same time, with compound/DMSO added in the interim). **B)** Compound mediated Porcupine increases are in line with previously observed IC50s (L-Wnt-STF cells). Porcn-GL was transfected into L cells exposed to IWP2 at the noted concentrations for 48 hours. GL counts from whole cell lysates were quantitated. **C)** *Gaussia* luciferase tagged GOAT and Hhat do not increase with IWP exposure. Transfected cells were incubated 48 hours, and processed as in B).

Enzyme mislocalization can effectively prevent functioning though changes in pH, ionic composition, substrate availability, or the presence/absence of regulatory cofactors/proteins. Immunofluorescence against *gaussia* or myc tagged Porcupines revealed an expected ER localization pattern in both instances that failed to change in the presence of IWPs (Figure 14A). Differences in overall protein levels with or without compound were generally unremarkable, with dissimilarities likely due to transfection efficiency variability (Figure 14B). Consequently, changes to Porcn spatial expression did not appear to be influenced by IWP introduction.

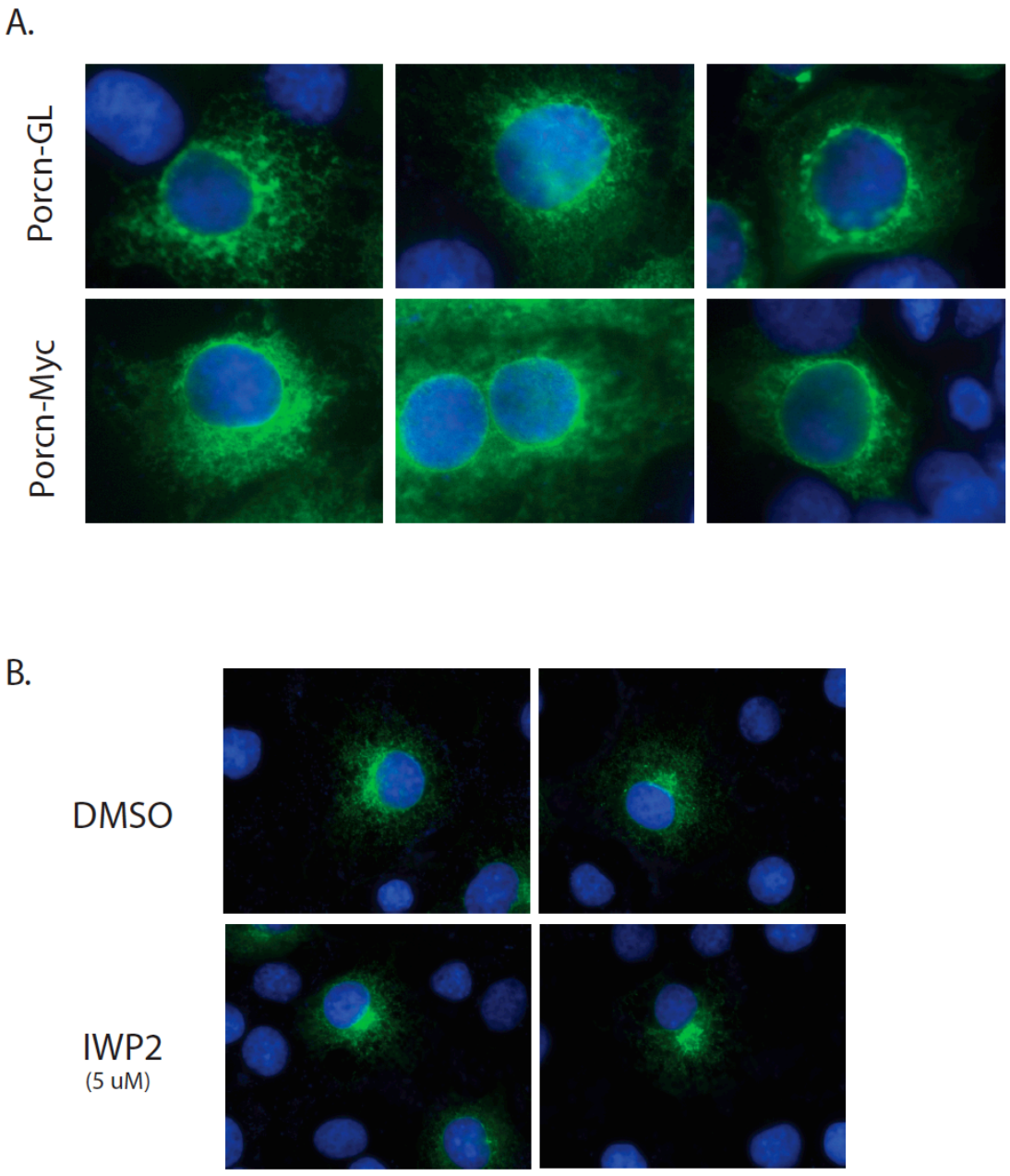


Figure 14: Porcupine intercellular localization is not altered by C-terminal tagging or IWPs. **A)** Porcupine-myc/*gaussia* luciferase fusion proteins exhibit expected ER localization patterns. Consequently, the tagging inherit to all Porcn detection assays does not significantly mislocalization the protein. **B)** IWP2 has no apparent effect on Porcupine intracellular localization. Although the IWPs may still function in part though

changes in Porcupine's station, this does not appear to be their primary inhibitory mechanism. Immunofluorescent experiments conducted with Cos1 cells, fixed 48 hours post transfection.

With initial assays unable to clarify IWPs mechanism, direction was sought from possible compound-Porcupine interaction sites. Guiding this effort was recently uncovered genetics data from patients with Focal Dermal Hypoplasia [128, 131, 132, 135-137]. Individuals harboring single site mutations in Porcupine were of particular interest; functional dependence upon these amino acids may have indicated regions amenable to chemical attack. Unfortunately, such documented residues often concurred *in vivo*, making it difficult to determine *a priori* which were ultimately relevant. Thus, following site directed mutagenesis to reproduce the lesions (Figure 12C), protein function was assayed via IWP rescue experiments (see Figure 8). Constructs were transiently transfected into cells exposed to low levels of compound in an attempt to reverse IWP induced loss of STF and secreted Wnt3a-GL signals (Figure 15A/B). The majority of mutants were unable to match the effectiveness of their wildtype counterpart, suggested by western blotting to be the result of significant protein destabilizations (Figure 15C). However, similar to parental Porcupine, many of these mutants would increase/stabilize in IWPs presence. An exception was the putative active site variant H335Lwhich showed little destabilization (and poor IWP induced stabilization) in both GL and myc tagged forms (Figures 15D & 16A). As many enzyme inhibitors are known to interact with their target's active site, this mutant was selected for further study.

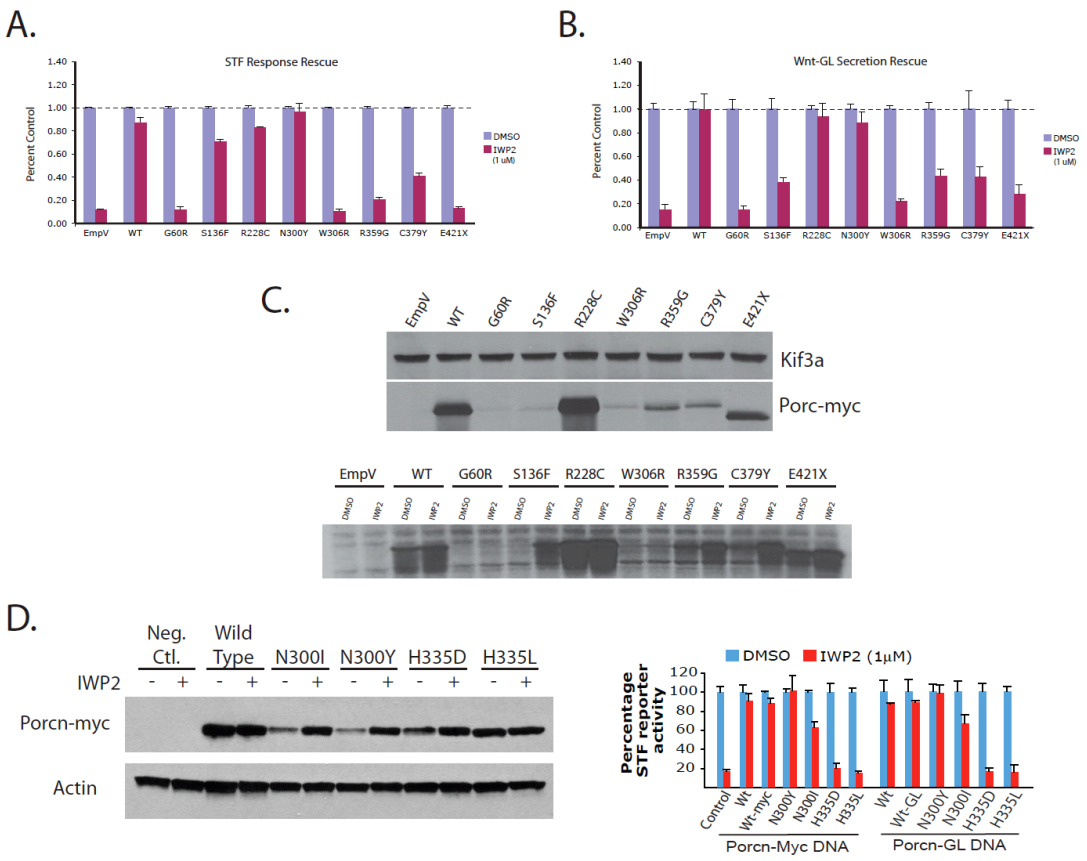


Figure 15: The majority of Porcupine site mutations found in disease are non-functional. **A)** Porcupine point mutations (myc tagged) observed in FDH often compromise acyltransferase function as determined via STF rescue ability. **B)** Porcn mutant (myc tagged) STF rescue capability correlates with preceding Wnt-GL secretion results in A). **C)** Disease associated Porcn mutants have poor expression levels. Similar to wildtype Porcupine, exposure to IWPs results in stabilization. Identical amounts of DNA transfected in all cases. **D)** The putative Porcn active site mutant (H335L) is not unstable, and does not appreciable increase with IWPs. This may indicate a failure to interact with the compound. At right are rescue activities for H335L corresponding to A) and B) parts above. Active site mutants lack activity, although the choice of C-terminal tag (wildtype Porcn) seemingly has no functional impact in these assays.

Porcupine H335L was introduced into the previously described IWP-Cy3 flow cytometry assay (see Figure 10D) to gauge compound interaction potential. Gaussia luciferase variants were substituted for their myc counterparts due to an unusual degree of stability conferred by the tag (Figure 16 A), allowing overexpression of several proteins that may have otherwise produced false negative results due to poor expression. When compared to wildtype Porcupine, H335L transfected cells exhibited significantly decreased IWP-Cy3 positive counts (Figure 16B). Further, this was apparently not due to mislocalization of the protein, either induced by the gaussia tag or single site mutations (Figure 16C).

If IWPs were interacting with the acyltransferase's active site, a similarly functioning molecule should have been capable of competing IWP-Cy3 from wildtype Porcupine. Indeed, this is what was observed in the flow cytometry assay following 2BP addition, a palmitate analogue and promiscuous acyltransferase inhibitor (Figure 16D) [133]. Unfortunately, 2BPs lack of specificity precluded employing the compound at previously reported concentrations without incurring significant cellular toxicity. Noted competition was therefore not as robust as found with unconjugated IWP.

In addition to palmitate, Porcupine function presumably requires conterminous association of Wnts to the active site. In turn, these interactions may be blocked or otherwise modulated by steric changes to the region, including interposition of a small molecule. Consequently, Porcupine affinity for Wnt3a in IWPs presence was determined via Co-IP and found to be moderately reduced (Figure 16E) but not when using a modified negative control (IWP7, see following section). These findings are consistent with active site disruptions.

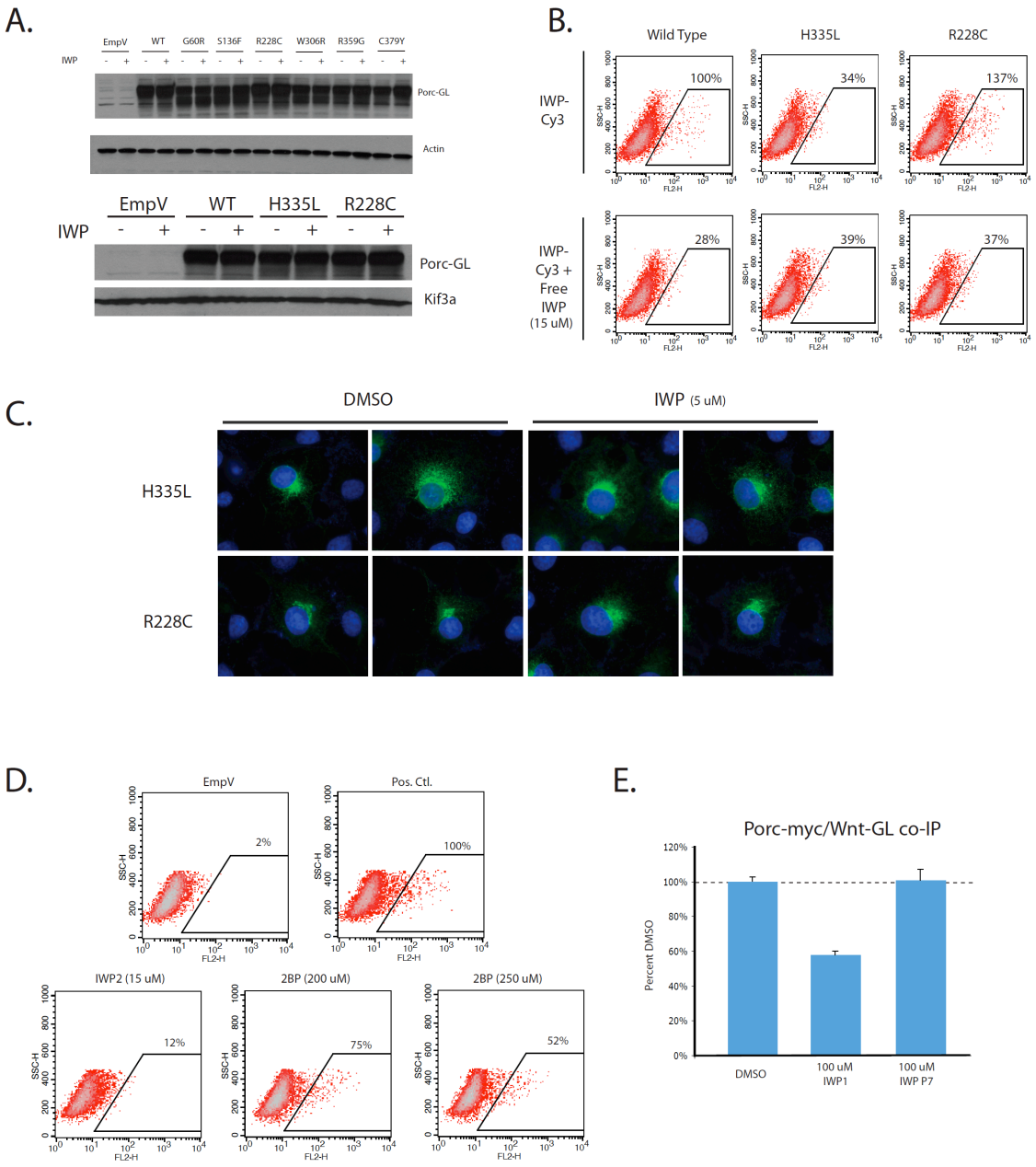


Figure 16: An IWP derivative associates with Porcupine's active site. **A)** *Gaussia* luciferase tagging counteracts mutational instabilities. This allows construct overexpression in other assays (although misfolding or additional problems may still occur). **B)** Cells expressing Porcn-H335L do not associate with IWP-Cy3. An active mutant (R228C) with similar myc tagged expression levels is included as a control. Both

wildtype and R228C Porcupine associate with IWP-Cy3 and respond to free IWP competition. **C)** Intracellular mislocalization of select Porcn mutants does not occur. Similar to concerns with IWP exposure, H335L results may otherwise have been due to loss of ER retention. **D)** 2-BP, a potential active site inhibitor, functions similarly to IWPs at competing off IWP-Cy3 from Porcn transfected cells. Maximum employed concentrations were limited by observed toxicity effects. **E)** Wnt3a-GL is co-immunoprecipitated with Porcn-myc and can be prevented from associating by IWP2 but not a negative control. This assay does not rule out the possibility of another protein creating bridging contacts between Porcn/Wnt, or cleavage of the gaussia tag giving rise to signal. All experiments were conducted in Cos1 cells. Percentages shown for FACS assays reflect gating of respective positive controls.

Subtle structural variations between the four initial IWPs provided little insight into the chemical determinants necessary for compound activity, save for an apparent degree of tolerance for benzyl ring adducts. Generally speaking, limitations inherit to chemical library design necessarily restrict the number of lead structures retrievable from any given screen. Because it is rare for first discoveries to be sufficiently appropriate for defined applications (e.g. disease intervention), further optimizations (e.g. to half life, bioavailability, specificity) are frequently pursued. Concerning IWPs, this involved systematically modifying core scaffolds, either in collaboration with Dr. Chuo Chen's laboratory or by purchasing related structures from existing chemical libraries (Figure 17A). IC50s calculated (L-Wnt-STF cells) for the approximately seventy resultant derivatives were inferior to or, in rare cases, comparable to the lead structures (Figure 17

B). For the most part, additional benzene ring variants maintained activity, particularly a para-ethoxyl group substitution (Figure 17B, compound P10, yellow box) that would later direct selection of IWP-Cy3/IWP-BP's linker attachment point (Figure 10). Subtle differences unique to IWP1 (phthalazin-1(2H)-one group and 2-formamidoacetamide linker) did not affect IC50s when transposed onto an IWP2-4 template (Figure 17B, blue box), thereby expanding possible synthetic options. One of the resultant hybrid molecules, IWPB5, would come to exemplify such findings by combining IWP1s high solubility with the simplicity of IWP2 (i.e. lack of methoxy groups). Using this molecule as a starting point, focus shifted from the benzene ring to modifying the adjacent benzo[d]thiazole group, which was initially believed to be similarly accommodating due to variability at the 6' position (methyl or methoxy) found within the original IWPs. Indeed, substituting in a variety of groups at this position produced little effect (Figure 17B, red box), and it was only by creating a structural isomer (IWPE1, 6' methyl to 4') that activity was lost. Further experimentation revealed that the removal of any adduct from the 6' position, as opposed its relocation, produced this result (Figure 17B, green box), making this area critical for inhibitor function. More drastic truncations of the benzo[d]thiazole and adjoining linker further emphasized IWPs inability to abide changes while suggesting that lead compounds possessed close to optimal activity in cell based reporter assays (Figure 17B, black box).

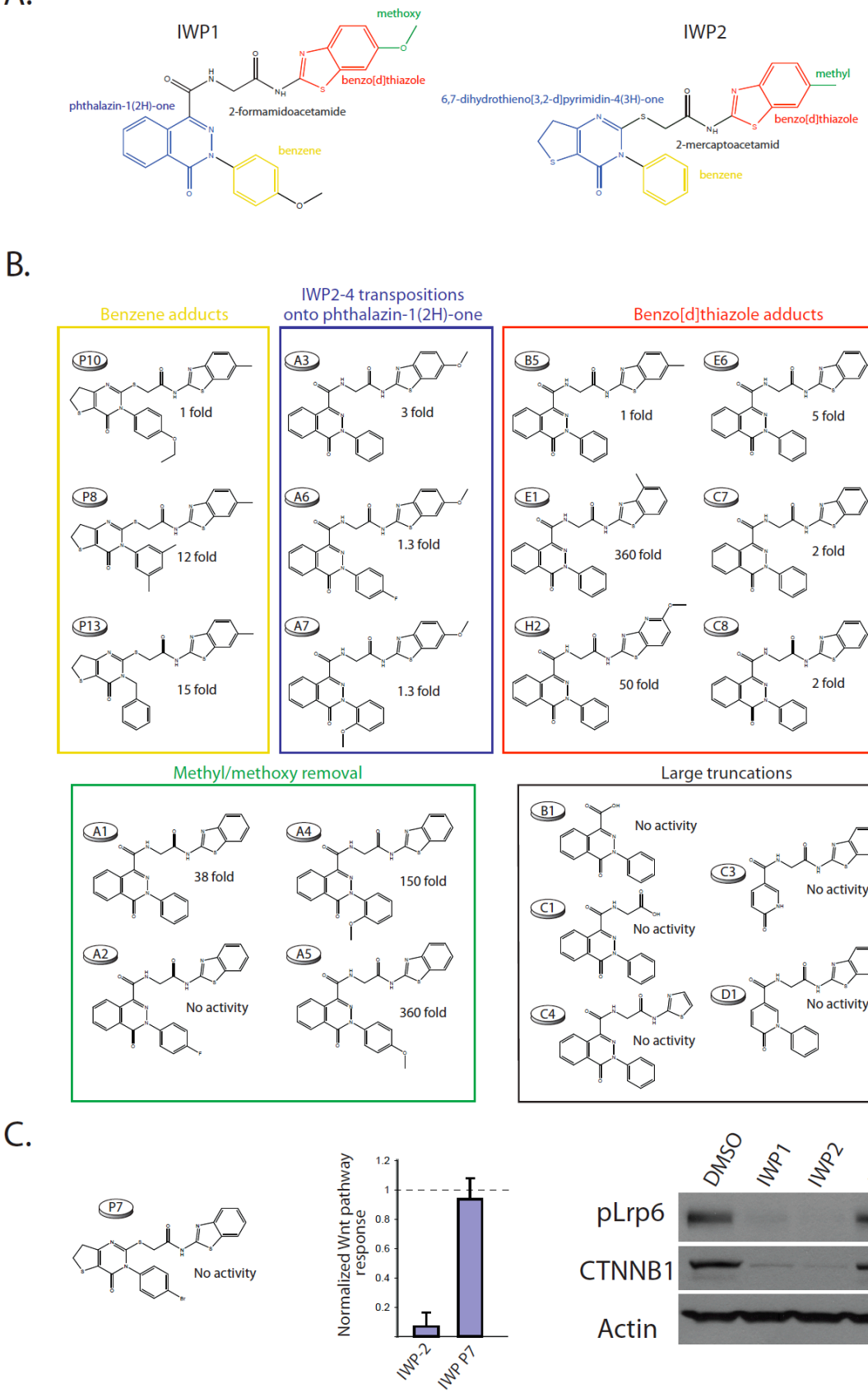
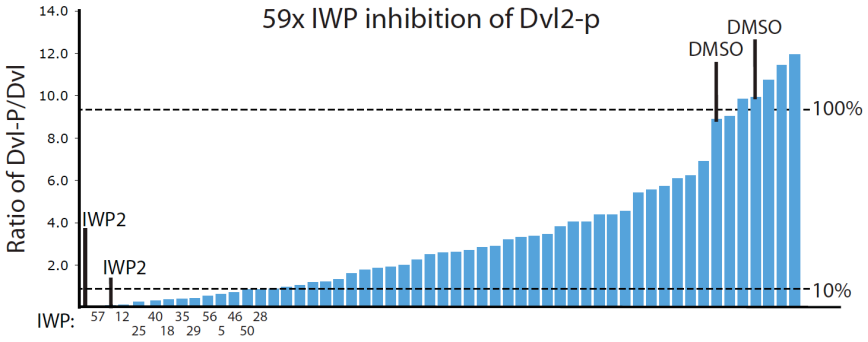

A.
IWP1
methoxy
benzo[d]thiazole
phthalazin-1(2H)-one
2-formamidoacetamide
benzene
IWP2
methyl
6,7-dihydrothieno[3,2-d]pyrimidin-4(3H)-one
benzo[d]thiazole
2-mercaptoacetamid
benzene
B.
Benzene adducts
IWP2-4 transpositions onto phthalazin-1(2H)-one
Benzo[d]thiazole adducts
P10
1 fold
P8
12 fold
P13
15 fold
A3
3 fold
A6
1.3 fold
A7
1.3 fold
B5
1 fold
E6
5 fold
E1
360 fold
C7
2 fold
H2
50 fold
C8
2 fold
Methyl/methoxy removal
Large truncations
A1
38 fold
A4
150 fold
A2
No activity
A5
360 fold
B1
No activity
C3
No activity
C1
No activity
C4
No activity
D1
No activity
C.
P7
No activity
Normalized Wnt pathway response
1.2
1
0.8
0.6
0.4
0.2
IWP-2
IWP P7
DMSO
IWP1
IWP2
P7
pLrp6
CTNNB1
Actin

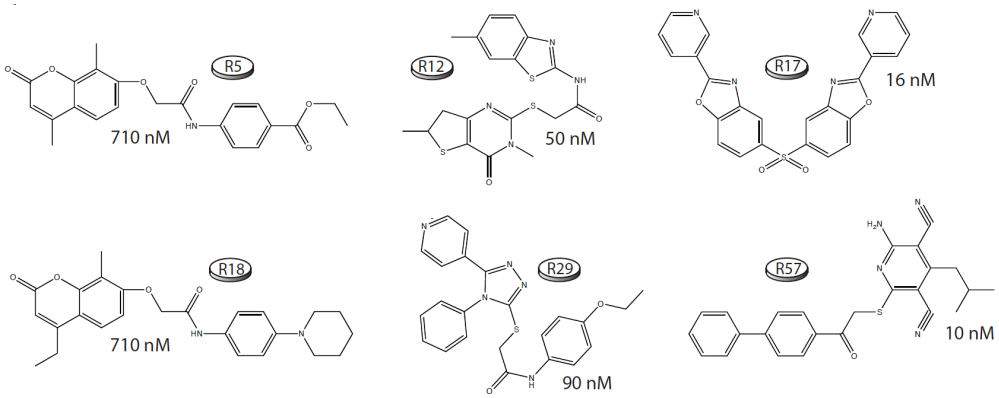
Figure 17: Original IWPs are intolerant to structural changes. **A)** Lead structures for IWPs 1&2. Colorized regions are labeled based upon their protonated form (i.e. lacking bonds to adjoining regions). **B)** Select IWP derivative structures with reciprocal STF IC50 values listed relative to IWP1 (i.e. 1 fold: 100% efficacy, 2 fold: 50% efficacy, 4 fold: 25% efficacy, etc) as determined in L-Wnt-STF cells. 'No activity' applies only to the tested IC50 range; 500 pM to 25 uM, with compounds potentially demonstrating inhibition at higher concentrations. **C)** A slightly modified compound (IWP P7) lacking STF inhibitory activity is unable to block biochemical events downstream of Porcupine. (Compounds tested at 2.5 uM.)

Difficulties encountered while optimizing IWPs impelled the development of an alternative approach, namely reexamination of hits from the 59x compound stage (see Figure 5). Although IWPs1-4 were originally the most potent compounds at inhibiting Wnt3a-GL luciferase secretion (Figure 6), multiple unrelated chemicals similarly faired and may have in fact been superior when accounting for errors in library stock concentration and degradation while in storage. Consequently, the original 59x panel was retested using Dvl2 phosphorylation as a new readout (Figure 18A), with the most potent hits restocked (depending upon availability) for further testing (Figure 18B). Surprisingly, despite their considerable structural divergence, most chemicals phenocopied the original IWP's ability to reduce Wnt hydrophobicity (Figure 18C) and compete away IWP-Cy3 from Porcn transfected cells (Figure 18D). If these alternative compounds were similarly operating at the level of Porcupine, it would imply Porcn node inhibition could be achieved though diverse chemical means.

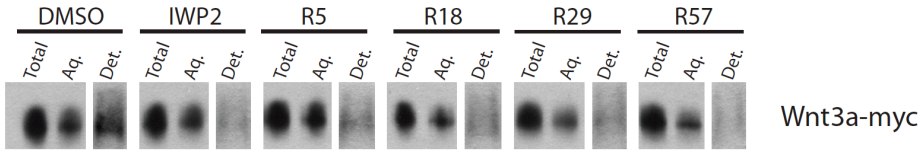
A.


59x IWP inhibition of Dvl2-p
Ratio of Dvl-P/Dvl
14.0
12.0
10.0
8.0
6.0
4.0
2.0
DMSO
DMSO
100%
10%
IWP2
IWP2
IWP:
57 12 40 35 56 46 28
25 18 29 5 50

B.


R5
710 nM
R12
50 nM
R17
16 nM
R18
710 nM
R29
90 nM
R57
10 nM

C.


DMSO
IWP2
R5
R18
R29
R57
Total
Aq.
Det.
Wnt3a-myc

D.

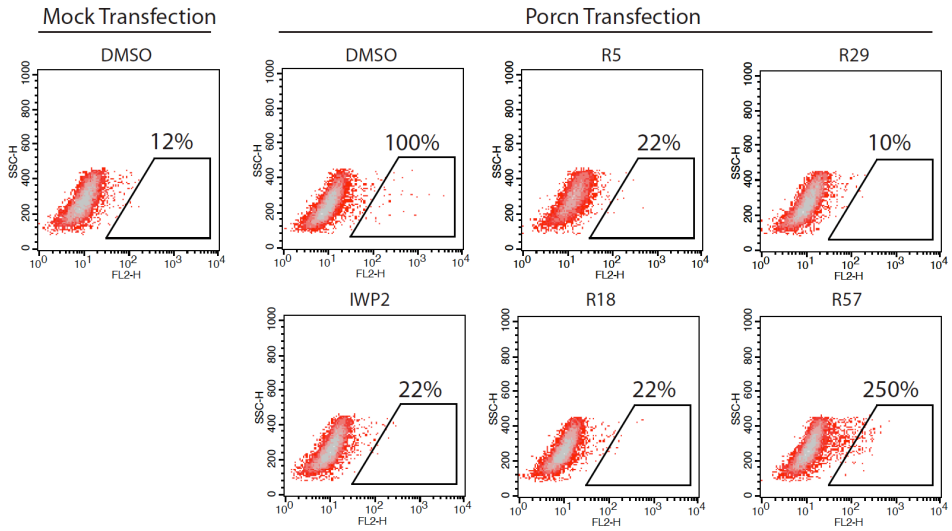

Mock Transfection
Porcn Transfection
DMSO
DMSO
R5
R29
IWP2
R18
R57
12%
100%
22%
10%
22%
22%
250%
SSC-H
FL2-H

Figure 18: Putative second generation IWPs have diverse chemical structures. **A)** Several potential IWPs from the 59x compound screen stage (Chapter 1) inhibit Dvl2 phosphorylation. Compounds were incubated with HeLa cells and resultant Dvl2 western blot bands quantitated with ImageJ. 100% (no net activity) was set as the average of DMSO controls. **B)** Select second generation IWPs. Chemicals were selected/restocked based upon Dvl2 potency (10% cutoff), structural uniqueness and commercial availability. Two compounds (R17, R57) have L-Wnt-STF IC50s several fold superior to original IWPs. **C)** Wnt3a-myc palmitoylation is reduced by select restocked IWPs. TritionX-114 phase separation was used to determine Wnt hydrophobicity, as previously described. For clarity, detergent fractions derive from a second, longer exposure. **D)** New IWPs compete IWP-Cy3 from Porcn expressing cells. Compounds tested at 15 uM, as in Figure 10D.

Discussion

Examining the relationship between Porcupine and IWPs was a logical outgrowth of initial discoveries. Aside from an ability to leverage previously established methodology, further studying this novel inhibitor/enzyme interaction held implications for targeted MBOAT therapies within multiple fields. At the very least, more potent IWPs may have been derived to help facilitate the classes' transition into model organisms.

Little if any known correlation exists between Porcupine's primary sequence and Wnt processing activity. Information derived from earlier MBOAT studies was generally

not applicable to Porcn/IWP interactions due to considerable sequence divergence and functional nuances (e.g. protein vs. oligosaccharide substrates) existing within the family [31]. Thus, in order to narrow down possible Porcn/IWP interface regions it became prudent to simplify the problem by assuming a correlation existed between chemically and genetically tractable residues. While by no means necessarily true, this hypothesis allowed focus to be placed upon known Porcupine mutations as defined by FDH patients. Encouragingly, the majority of corresponding mutant Porcn constructs created were unable to rescue IWPs inhibitory effects on Wnt-GL secretion or STF activity (Figure 15A/B), implying *in vivo* observations were meaningfully related to function. Loss of activity may have been partly due to observed protein destabilization (Figure 15C), although increased stability conferred by a gaussia luciferase tag was alone insufficient to restore signaling (Figure 16A). Instead, these observations may have been symptomatic of an underlying Porcn misfolding anomaly- expected to be largely unaffected by expression level changes (i.e. GL tagging). (Circular dichroism, dual polarization interferometry or a number of additional protein folding techniques could later be employed to elaborate upon this issue, though this digresses somewhat from experimental intent). In rare instances, acyltransferase mislocalization appeared to occur, notably in the case of a C-terminal tail truncation mutant that represented one of the more frequently observed FDH lesions. Remaining point mutations that did not influence Porcn activity or localization were potentially neutral or nearly neutral in nature and may have been coincidentally present in FDH cases.

Loss of function Porcupine-myc mutants were often significantly stabilized in the presence of IWPs. This effect was also observed with GL tagged fusions (mutant and wildtype) but due to their relatively high basal expression levels, the results proved less robust. Nevertheless, quantitation of Porcn-GL stabilization revealed close inverse coupling to previous IWP IC50s. Not only was this consistent with a direct interaction model but it provided a rapid means of testing Porcupine/IWP associations. However, because a mechanistic understanding of this phenomenon was lacking, the assay could at best serve as a starting point for follow up studies (transcriptional regulation was an unlikely explanation due to the use of CMV promoters). Such was the case of the active site Porcn H335L mutant, which exhibited an atypical stabilization pattern in both myc and gaussia tagged forms.

The inability of Porcn-H335L-GL to associate with IWP-Cy3 during flow cytometry (Figure 16) suggested an intact active site contributed to compound interaction(s). Precisely why Porcn-H335L yielded negative results during this assay was not readily discernable, although several possibilities existed. First was exclusion of IWP from the active site due to local electrochemical or steric differences induced by the histidine to leucine substitution; histadine's additional bulk and tendency to take on a positive charge may have previously facilitated compound docking to wildtype Porcupine. This interaction need not have been direct and could have involved coordination of neighboring residues to make the appropriate IWP contacts. Along these lines, the point mutation may have alternatively created more significant instabilities within Porcupine, essentially distorting overall folding and preventing IWP binding at another (possibly remote) region. This was thought less likely due to the comparative

lack of PorcH335L destabilization observed vs. other mutant constructs. However, because it remained possible that Porcn-H335L misfolded in a manner unconnected to expression levels, alternative means were sought to further confirm an IWP/Porcn active site interaction.

As a substrate analogue, 2BP could potentially interact with Porcupine's active site so as to preclude occupation by other small molecules (i.e. IWPs). FACS observations supported this assessment, with a partial loss of IWP-Cy3 positive cells noted in the presence of 2BP (Figure 16). Further competition with 2BP at concentrations sufficient to completely inhibit Porcupine activity was not possible due to significant cellular toxicity, likely the result of broad palmitoyltransferase inhibition. Another explanation for these results whereby 2BP functioned as a detergent could not be ruled out although this was deemed unlikely to have produced favorable IWP completion at low 2BP concentrations since such a non-specific model would be cytotoxic long before significant contributions were made to Porcn inhibition.

Apart from palmitoyl/palmitic acid, Wnt proteins, representing Porcupine's remaining known substrate class, are similarly thought to dock near the active site during acylation [129]. IWP seemingly interfered with this interaction in Porcn-myc/Wnt3a-GL Co-IP assays, consistent with the proposed model. However, as with 2BP competition, IWPs hydrophobic characteristics may have blocked protein-protein interactions though non-specific associations. To counter this possibility, a hydrophobic but non-functional IWP control was employed (IWP P7) and found unable to abrogate Porcn/Wnt3a binding. Of greater concern was the potential for IWPs to specifically bind to an alternative region of Porcupine or Wnt, thereby preventing their association. Given the preceding

experiments however, direct inhibition of Porcn's active site by IWPs was viewed as the most parsimonious explanation.

In contrast to Porcupine mutagenesis, structurally altering IWP compounds presented several novel challenges. Importantly, no comparable SAR information existed to guide early alterations, forcing the undertaking of a more empirical strategy. Such an approach was in turn further limited by synthetic practicalities, reagent costs, manpower, and broad pharmacological considerations (e.g. Lipinski's rule of five). The net result, though not comprehensive, provided important insights into IWPs functional determinants. These include the necessity of a 6' benzo[d]thiazole adduct and phthalazin-1(2H)-one group, contrasting sharply with the seemingly dispensable nature of the lower benzene ring and its adducts (exemplified by IWP R12). It is important to note however, that these findings cannot be easily extrapolated to IWP/Porcupine interactions, as the IC50 assays from which these conclusions derive cannot discern inhibitory mechanism. Nonfunctional compounds may lack activity for reasons other than steric considerations, including changes to cell permeability, transporter efflux, or stability. Although additional experiments may help differentiate between these possibilities, compounds that failed initial cell based reporter assays may not warrant the same level of follow-up as their more functional counterparts.

Like the core IWP compounds, several additional small molecules from the 59x preliminary IWP screen stage (Figure 5) effectively blocked Wnt-GL secretion. At the time, these structures were set aside due to their dissimilarity from both each other and the top four, nearly identical IWP hits. However, upon revisiting, the majority of chemicals within this new group were able to influence Wnt hydrophobicity and IWP-

Cy3/Porcn associations, thereby essentially reproducing earlier IWP1-4 findings. (The only exception was IWP R57 during flow cytometry, the result of autofluorescence). If new IWPs similarly inhibited Porcn function, the resultant susceptible to diverse chemical structures would give considerable flexibility to future compound development.

Methods:

Reagents and Equipment: Immunofluorescent samples visualized with a Zeiss Axioplan 2E equipped with a Hamamatsu monochrome digital camera. Images processed with OpenLabv5.5 (PerkinElmer). IWPs categories A-H were synthesized by Dr. Chuo Chen's lab, UTSouthwestern medical center, and are grouped (A,B,C, etc) based upon the order of synthesis. Category P and R compounds were ordered though Interchim (Paris, France) and AnaLogix (Moscow, Russia). Immunofluorescence blocking buffer consists of PBS, 0.2% Trition X100, and 5% normal goat serum. Anti-gaussia luciferase antibody was purchased from New England Biolabs.

Cloning: PCR based site directed mutagenesis was conducted as noted within the Porcn (C variant) vector, with resulting fragments digested and ligated into pcDNA3.1 myc/GL vectors. All open reading frames were sequenced in their entirety.

Porcn-myc/Wnt-GL Co-Immunoprecipitation: Porcn-myc and Wnt-GL were separately expressed in Cos1 cells for 48 hours. Following 1%NP40/PBS lysis, fractions were combined in a 1:1 ratio, compounds added, and Porcn-myc pulled down with

ProteinA/myc. After washing 4x with lysis/binding buffer, 10 min each, beads were loaded into a 96 opaque well format and luciferin added (Progmega).

59x IWP Dvl2 quantitation: Hela cells were seeded into a 24 well format, 50K cells/well, and compounds added (2 uM). After 48 hours incubation, lysates were visualized via western blotting and phosphorylated to unphosphoylated Dvl2 ratios calculated with ImageJ.

Immunofluorescence: Cos1 cells were seeded onto glass cover slips in 6 well plates, 150k cells/well. Following Fugene6 (Roche) transfection the following day, cells were incubated for an additional 48 hours, cover slip transferred into new 6 well plates, and washed 3x with PBS. Cells were fixed with 3.7% paraformaldahyde for 15 min, washed 3x with PBS, and permeabilized with blocking buffer for 10 min. Primary antibody was then added per the manufactures recommended concentration (typically 1:50-1:500) for 30 min, samples washed 3x times with PBS, and secondary antibody (1:300) added for 30 min. A final 3x PBS washes were performed, and Vectasheild (Vector Labs) used to mount coverslips onto glass slides. All steps and reagents were at room temperature.

Previous Methods: See Chapter 1 for discussion of IWP-Cy3 flow cytometry, STF reporter/rescue experiments, Wnt-3a GL secretion, western blotting, IC50 generation and phase assays.

Chapter 3:

Chemically targeting Wnt dependant carcinogenesis

Introduction

The importance of Wnt signaling during cancer progression gives provenance to establishing relevant treatment modalities. As with most solid tumors, Wnt associated carcinomas have historically been addressed using chemotherapy (adjuvant/neoadjuvant) combined with surgical resection or radiological based approaches [138]. The exact specifics vary on a per patient basis dependant upon cancer subtype, staging, ontogenetic markers, comorbidity, etc. Broadly speaking, the more advanced or metastatic a cancer is, the more chemotherapy is relied upon [139, 140]. This in turn creates a number of unique clinical challenges arising from chemotherapy's inherit limitations. First, what are often subtle differences between normal and cancerous tissue frequently translate into significantly reduced therapeutic windows. This is because most properties considered 'unique' to cancer are more properly viewed as deviations in normal signaling and encompass pathways controlling increased replicative potential, angiogenesis, or tissue invasion and decreased sensitivity to immune, apoptotic, or growth suppressing cues [141, 142]. Many of these characteristics in turn synergize to support rapid cell division, the principle phenotypic target of anti-cancer chemicals (Table 3). While clinical approaches focused upon countering aberrant cell growth can successfully treat underlying disease, care must be taken to maintain a balance between antineoplastic and off-targeting effects, the later commonly manifesting as hair follicle, bone marrow, or

intestinal mucosa damage [143]. Resultant declines in overall health frequently present a significant contraindication to ongoing therapy, effectively impairing patient prognoses.

Generic	Tradename Example	Class	Subclass	Target/mechanism
Busulfan	Myleran	Alkylating agent	Alkyl sulfonate	General crosslinking
Thiotepa	Tepadina	Alkylating agent	Ethylene imine	General crosslinking
Chlorambucil	Leukeran	Alkylating agent	Nitrogen mustard analogue	General crosslinking
Cyclophosphamide	Cytoxan	Alkylating agent	Nitrogen mustard analogue	General crosslinking
Carmustine	BiCNU	Alkylating agent	Nitrosourea	General crosslinking
Lomustine	CeeNU	Alkylating agent	Nitrosourea	General crosslinking
Dactinomycin	Cosmegen	Antibiotic	Actinomycin	DNA Replication
Doxorubicin	Adriamycin	Antibiotic	Anthracycline	DNA Replication
Epirubicin	Ellence	Antibiotic	Anthracycline	DNA Replication
Methotrexate	Trexall	Anti-metabolite	Folic acid analogue	DHFR
Pemetrexed	Alimta	Anti-metabolite	Folic acid analogue	DHFR
Cladribine	Leustatin	Anti-metabolite	Purine analogue	DNA Replication
Clofarabine	Clolar	Anti-metabolite	Purine analogue	DNA Replication
Azacitidine	Vidaza	Anti-metabolite	Pyrimidine analogue	DNA Replication
Fluorouracil	Adrucil	Anti-metabolite	Pyrimidine analogue	DNA Replication
Procarbazine	Matulane	Other antineoplastic	Methylhydrazine	General crosslinking
Cisplatin	Platinol	Other antineoplastic	Platinum	General crosslinking
Oxaliplatin	Eloxatin	Other antineoplastic	Platinum	General crosslinking
Gefitinib	Iressa	Other antineoplastic	Protein kinase inhibitor	EGFR
Imatinib	Gleevec	Other antineoplastic	Protein kinase inhibitor	PDGFR
Temsirolimus	Torisel	Other antineoplastic	Protein kinase inhibitor	mTor
Etoposide	Toposar	Plant alkaloid	Podophyllotoxin	Topoisomerase II
Docetaxel	Taxotere	Plant alkaloid	Taxane	Microtubules
Paclitaxel	Onxol	Plant alkaloid	Taxane	Microtubules
Vinblastine	Velban	Plant alkaloid	Vinca alkaloid	Microtubules
Vinorelbine	Navelbine	Plant alkaloid	Vinca alkaloid	Microtubules

Table 3: Common chemotherapeutics. Examples are from the WHO ATC L01 classification system (antineoplastic agents) and exclude several items, notably monoclonal antibodies and sensitizers (L01XC and L01XD respectively). With the exception of several protein kinase inhibitors, most compounds possess broad mechanisms of action. DHFR: Dihydrofolate reductase. EGFR: Epidermal growth factor receptor. mTor: Mammalian target of rapamycin. PDGFR: Platelet derived growth factor receptor.

Although not as apparent as chemotherapy's systemic effects, difficulties in therapeutically discriminating between cell types also occurs within tumor microenvironments where incomplete, as opposed to widespread toxicity, becomes a principal shortcoming. Paradoxically, attempts to limit small molecule off-targeting contribute to this outcome; increased chemically mediated tumor destruction (e.g. via dosage changes, polytherapy) correlates with increased damage to healthy tissues. Resultant constraints placed upon drug administration account for some but not all observed 'fractional kill' cases, or the ability of some tumor cell subpopulations to survive single rounds of chemotherapy [144]. A second shortcoming is found within the cells themselves; tumor heterogeneity produces differential drug sensitivity stemming from cell cycle variability (e.g. asynchronous division), reduced accessibility to chemotherapeutics (e.g. distance to blood vessels), and changes in protein function or expression (e.g. drug efflux pumps, resistance mutations, drug metabolization). Many of these items were historically attributed to genetic instabilities existing within tumor cells (random or otherwise) coupled with differences inherit to the cells local environment (e.g. oxygen levels, inflammatory factors) [145-147]. Together these considerations led to what would become the 'clonal evolution model', postulating that cancers arose from successive somatic mutations that were selected based upon the survival/replicative advantage conferred to daughter cell populations [148, 149] (Figure 19A). Conceptually elegant from an evolutionary biology perspective, the clonal evolution model was most significantly applied to Wnt signaling by Bert Vogelstein in 1990 when he postulated familial colon cancers progressed from APC, to k-Ras, to finally p53 mutations (among other aberrations) [96]. Still, many issues remained unresolved, including: why did

advanced tumors consist primarily of post-mitotic cells, how could mutations accumulate in populations turning over, and what caused some cancers remerge years after treatment? Answers to many of these questions would present themselves by postulating the existence of a new tumor cell subtype; 'cancer stem cells' (CSCs).

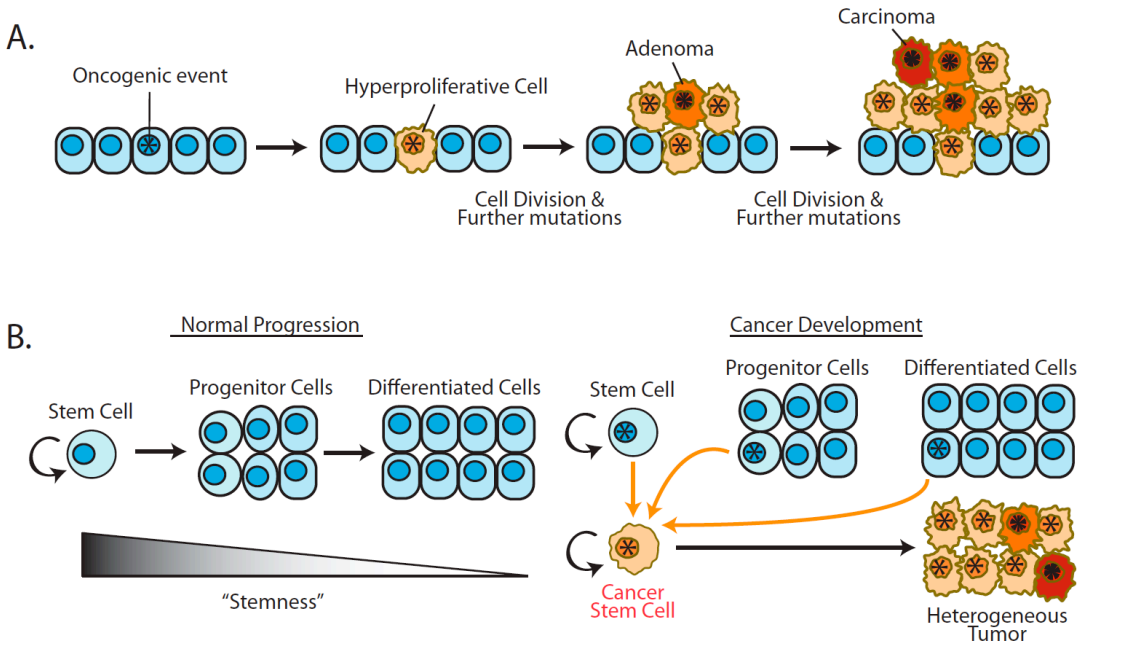


Figure 19: Cancer development hypothesizes for solid tumors. **A)** Clonal evolution model for an adenocarcinoma. An oncogenic mutation occurs within a healthy cell, conferring increased replicative potential. Further mutations within this or daughter cells advance the tumorigenicity of subsequent populations, leading to a selective growth advantage. Although a formal cancer requires changes to occur in a progressive/accumulative manner, cells at any particular stage within a tumor may mutate in a divergent manner to increase overall heterogeneity. Nuclear asterisks denote mutations. **B)** Cancer stem cell model. Depending upon the cell of origin, tissue specific stem cells may incur oncogenic changes, transforming into CSCs. Conversely, differentiated cells already possessing lesions may revert back into a more stem-like

state. In both cases, the cancer stem cells undergo self-renewal while maintaining the bulk of the tumor by producing daughter cells capable of further differentiation. The clonal evolution and cancer stem cell models are not mutually exclusive, with some combination thereof potentially existing in practice.

As their name implies, CSCs are essentially cancer cells possessing stem-like properties [150, 151]. These include exceptionally long lifetimes punctuated by periods of relative dormancy, capacity for self-renewal, and the ability to produce differentiating daughter cells. Molecular signatures characteristic of normal stem cells are also often present (e.g high telomerase activity, expression of ABC transporters, elevated Yamanaka factors) leading some researchers to postulate that CSCs are *bona fide* stem cells that have experienced oncogenic incursions [152-154] (Figure 19B). (Were this the case, CSCs would properly represent the cancer's "cell of origin" or a "tumor initiating cell"- interchangeable terms found in some literature to functionally describe CSCs.) Support for this hypothesis was first observed by conditionally knocking out JunB in hematopoietic stem cells, inducing a myeloproliferative disorder similar to human CML [155, 156]. A more recent (and Wnt pertinent) study has reported analogous adenoma formation in murine Lgr5+ intestinal stem cells following APC truncation [93]. Ultimately however, these findings succeed only in demonstrating stem cell to CSC experimental feasibility, leaving unanswered whether this transition naturally occurs in disease. Several contrary CSC inception models postulate the above process essentially proceeds in reverse, with somatic cells acquiring stem like abilities following oncogenic events (Figure 19B). Indeed, researchers have shown that terminally differentiated cells

are receptive to pluripotent reprogramming by a handful of transcription factors, although again, natural *in vivo* examples are lacking [157-159]. Consequently, the actual mechanism of CSC generation remains largely unresolved.

Experimentally identifying CSCs has proven difficult as many of these cell's hypothesized properties only became apparent following isolation. This in turn has prompted the creation of a widely applied litmus test: CSCs are a subset of tumor cells with shared characteristics capable of engraftment and recapitulation of source tumor heterogeneity within immuno-compromised mice [160](NOD/SCID). 'Shared characteristics' in this instance could potentially refer to any experimentally discriminable markers, although in practice they comprise cell surface molecules detectable via FACS. These antigens were historically stochastically derived with sometimes no readily apparent connection to underlying stem cell biology; their inclusion as CSCs isolation tools instead simply came from an ability to designate increasingly distinct cell populations capable of fulfilling the above CSC xenograft definition. Salient examples include CD44 (a hyaluronic acid receptor) and CD133/Prominin1 (a cholesterol binding protein) for sorting breast and brain cancer CSCs respectively [161, 162]. Both of these markers have been studied to a degree as to provide several general insights into CSC FACS methodology. First, individual markers alone have rarely proved sufficient for isolating potent CSC fractions due to the markers variable specificity. CD44+ for example is also found on some normal breast cells that would represent false FACS positives unless excluded by another measure, such as simultaneous CD24 (a GPI anchored sialoglycoprotein) negativity [163]. This leads into a second point; CSC markers possess limited applicability across tissues. CD133 has perhaps come closest to

serving as a universal marker, having been documented in cancers as diverse as prostate, lung, liver, skin, and pancreas. However, more recent isolations of CSCs from these tissues via alternative means has cast doubt upon initial CD133 enthusiasm, seemingly relegating the maker back to more traditional CNS cancers [164]. Taken together, a thorough, empirical approach is required for each CSC subtype.

The possibility of extant CSC populations following standard chemotherapy has lead to a reevaluation of clinical practices [150, 154, 165-168]. Like normal stem cells, CSCs are believed to possess a number of characteristics (see above) that facilitate survival and eventual disease reemergence. Specifically eliminating CSCs will require exploiting these differences, either by inducing cell death outright or by driving CSC differentiation into more chemically susceptible populations. In both cases, disruption of Wnt signaling represents an attractive therapeutic avenue given the pathways established roles in stem cell regulation and disease progression [105, 165, 169, 170].

Finally, it is worth mentioning disease contributions engendered by atypical Wnt responses. Collectively these events are classified as "non-canonical", all sharing in common ligand signal mediation that bypasses CTNNB1 dependent mechanisms (Figure 20). This includes among others, Wnt induced signal transduction through intracellular calcium, JNK, PKA, NLK, and mTOR pathway associated intermediaries [171, 172]. Wnts functioning in this manner were initially identified based upon an inability to induce differentiation of the mouse mammary epithelial cell line C57MG and include members 2, 4, 5a, 5b, 6, 7b, and 11 [102]. Subsequent research has shown this distinction to be imprecise, as several non-canonical associated Wnts are capable of inducing β-catenin accumulation within certain contexts. CTNNB1 pathway divergence

may instead occur at the level of disheveled regulation [171] following receptor activation, although a mechanistic understanding of these events remains largely unknown. Regardless of the pathway utilized, most non-canonical signaling events ultimately influence gene transcription, with secondary effects on actin and microtubule dynamics associated with cell orientation and migration [173, 174].

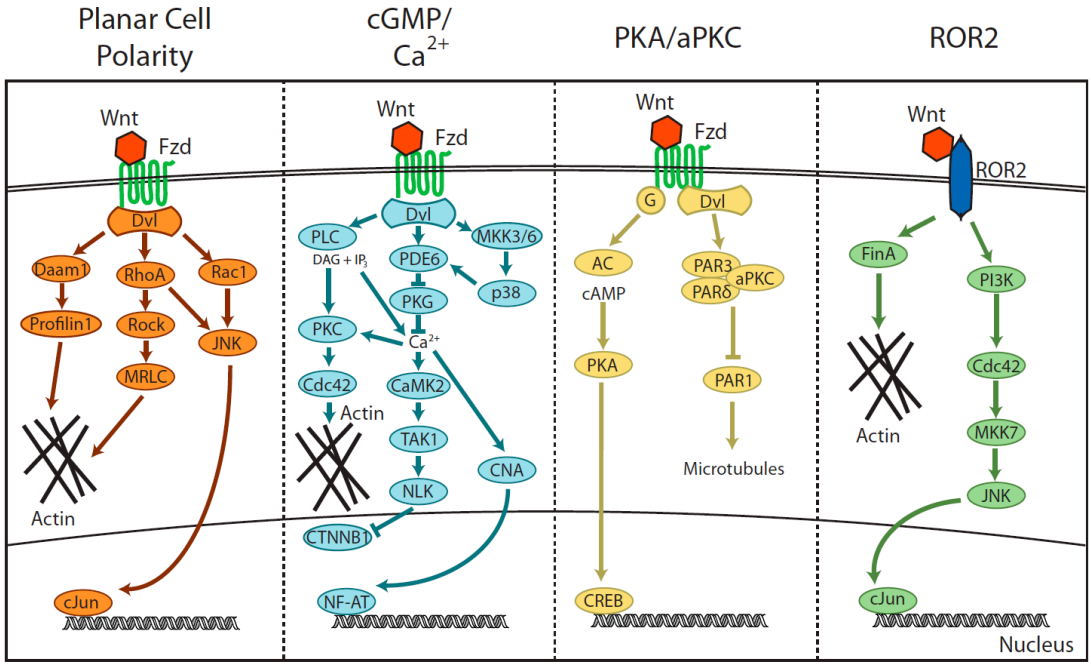


Figure 20: Selected non-canonical Wnt pathways. With a few exceptions, non-canonical pathways transduce initial signals through one or more Frizzled and Disheveled proteins shared with canonical signaling. Factors delimiting CTNNB1 dependent and non-canonical signaling are poorly understood but likely involve specific combinations of components, crosstalk between pathways, and non-Wnt related priming events. Among the pathways shown, only the PCP and Ca2+ pathways have been reasonable well characterized, particularly in *Drosophila*. Unfortunately, researchers have been largely unable to extricate non-canonical Wnt signaling from converging nodes, leaving these pathway's contributions to diseases relatively unknown.

Statement of Purpose:

Characterize potential IWP anti-cancer effects with an emphasis on obtaining mechanistic insight into corresponding ligand dependent contributions. Previous studies have largely overlooked this aspect of Wnt signaling, focusing instead upon oncogenic events at or below the level of CTNNB1.

Results

The chemical tractability of Wnt acylation as revealed by IWPs can potentially be exploited for therapeutic gains. This need not necessarily involve IWPs themselves, as acylation dependent pathway activation encompasses multiple enzymatic activities subject to modulation (Figure 21A). For example, TOFA (5-tetradecyloxy-2-furoic acid), an inhibitor of acetyl-CoA carboxylase (ACC), was able to effectively prevent Wnt3a modification, secretion, and induction of Dvl2 phosphorylation in cell based assays (Figure 21B-D). Targeting ACC or alternative fatty acid anabolic steps may therefore serve as useful Wnt inhibitory tools under some circumstances, albeit with significant implications for divergent acylation events (e.g. Ras trafficking, SNARE protein stabilization, etc). IWPs conversely possess a distinct practical advantage: both they and Porcupine appear specific to Wnt ligand production.

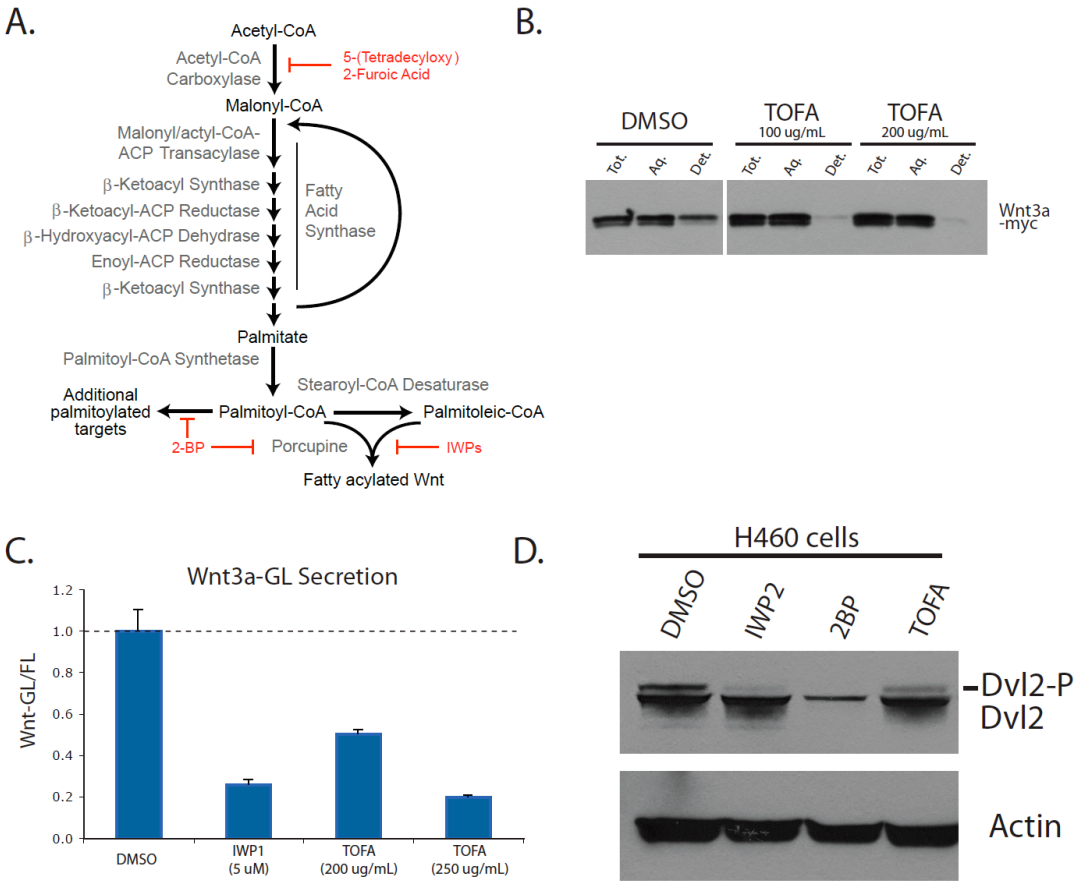


Figure 21: Wnt palmitoylation can be disrupted by IWP independent mechanisms. **A)** Fatty acid synthesis from Acetyl-CoA to Wnt palmitoylation. In mammals, chain elongation is largely confined to fatty acid synthase (Type I), a homodimeric complex capable of shuttling intermediate products though iterative passes. The large number of steps required for eventual Wnt modification may allow significant flexibility in achieving ligand based pathway inhibition, albeit with consequences for other acylation dependent processes. Although not depicted, it is conceivable that IWPs inhibit additional enzymes or that Porcupine modifies further substrates. **B)** Wnt3a-myc acylation is inhibited by an acetyl-CoA carboxylase inhibitor (TOFA). Palmitoylation status was determined using a TX-114 phase assay. **C)** Wnt3a-GL secretion can be

reduced by disrupting various points along Wnt's fatty acylation route. L cells were transfected with Wnt3a-GL, CMV-FL and processed as previously described. **D)** Loss of Wnt secretion translates into a reduction in Disheveled2 phosphorylation, a marker of downstream Wnt pathway activity. The loss of total Dvl2 levels in 2-BP treated samples may be due to toxic or otherwise nonspecific effects.

IWPs successfully inhibited cell growth in a variety of cancer types, resulting in near complete loss of viability by 2 uM (Figure 22A). Several unique observations emerged during these studies, notably an apparent dependence upon cell density for compound effect. Earlier experiments performed at relatively high densities (~70% confluence) had produced negative results, even at IWP concentrations several hundred-fold in excess of established STF IC50 values (Figure 22B); only when cell numbers were reduced to clonal levels (<5% confluence) did growth inhibition/cell death occur. Analogous genetically based assays have previously noted that under stressful conditions (i.e. reduced serum or at clonal density), Wnt pathway activity both increases and facilitates cell survival [175-177]. While possibly a contributing factor to these more recent observations, further experiments suggested IWP could function through alternative means. Foremost was the possibility of off-targeting effects, either through loss of another Porcn dependent process (e.g. lipidation of an unknown factor) or IWP antagonism of separate enzymes. Addressing the later possibility, slightly modified non-functional IWPs were unable to induce cell death (Figure 22C), suggesting difficulty in separating Porcn inhibitory activity from other potential effects (although alternative explanations cannot be discounted such as control reduced solubility, increased efflux,

etc). Second, siRNAs targeting Porcupine phenocopied chemical results (Figure 25B) and exhibited a similar cell density growth dependence (Figure 22B). Finally, as previously noted, IWP compounds failed to disrupt the related MBOATs GOAT and Hhat in both cell based and *in vitro* experiments, perhaps among the more likely candidates to manifest off-targeting.

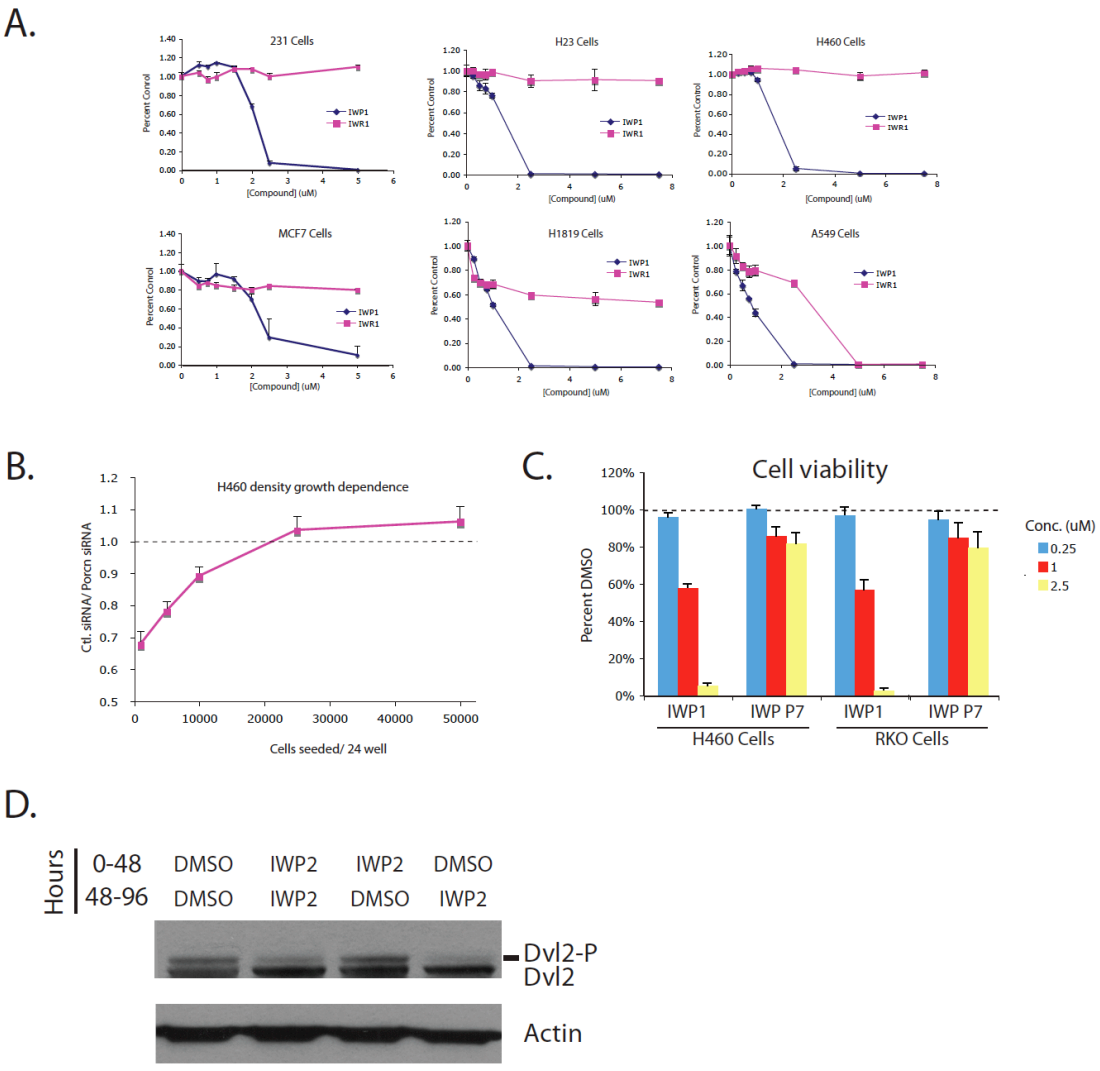


Figure 22: IWPs are cytotoxic/cytostatic to a variety of cell lines. **A)** Select *in vitro* clonal density cell growth curves using IWP1. Two thousand cells were seeded

(triplicate) into a 24 well format and incubated for 6 days. Viability was measured via cell titer glow. **B)** Reductions in cell growth associated with Porcn inhibition are inversely proportional to cell density. H460 cells were transfected with Porcn or control siRNA and seeded at the stated cell numbers. After 48 hours, viability was measured and the ratio between knockdowns plotted. **C)** Structurally similar but non-functional IWPs were unable to induce cell death. **D)** IWP2s effects within signal receiving cells are transitory. Replacement of media containing IWP2 (2.5 uM) to one with DMSO demonstrates Dvl2 phosphorylation recovery within 48 hours.

Among the cancer types affected by IWPs were several cell lines thought to be insensitive to Wnt antagonism based upon established genetics. This included lung cancers, which historically have been associated with p53, K-Ras, Stk11, and Egfr pathway mutations rather than hyperactivations in Wnt signaling [178]. Consistent with such holdings, super-top-flash levels were negligible in a lung cancer panel (Figure 23A), although Desheviled2 phosphorylation, a widely accepted indicator of canonical/non-canonical pathway activation, was oddly prevalent (Figures 23B). The further sensitively of Dvl2 to IWP exposure or Porcn siRNAs argued strongly for Wnt ligand signaling, most likely transmitted through non-canonical means (Figure 20). This was consistent with separate observations of Porcn mRNA upregulation in primary lung cancers [179, 180], suggesting these diseases may benefit from aspects of ligand mediated signaling not induced by typical CTNNB1 responses.

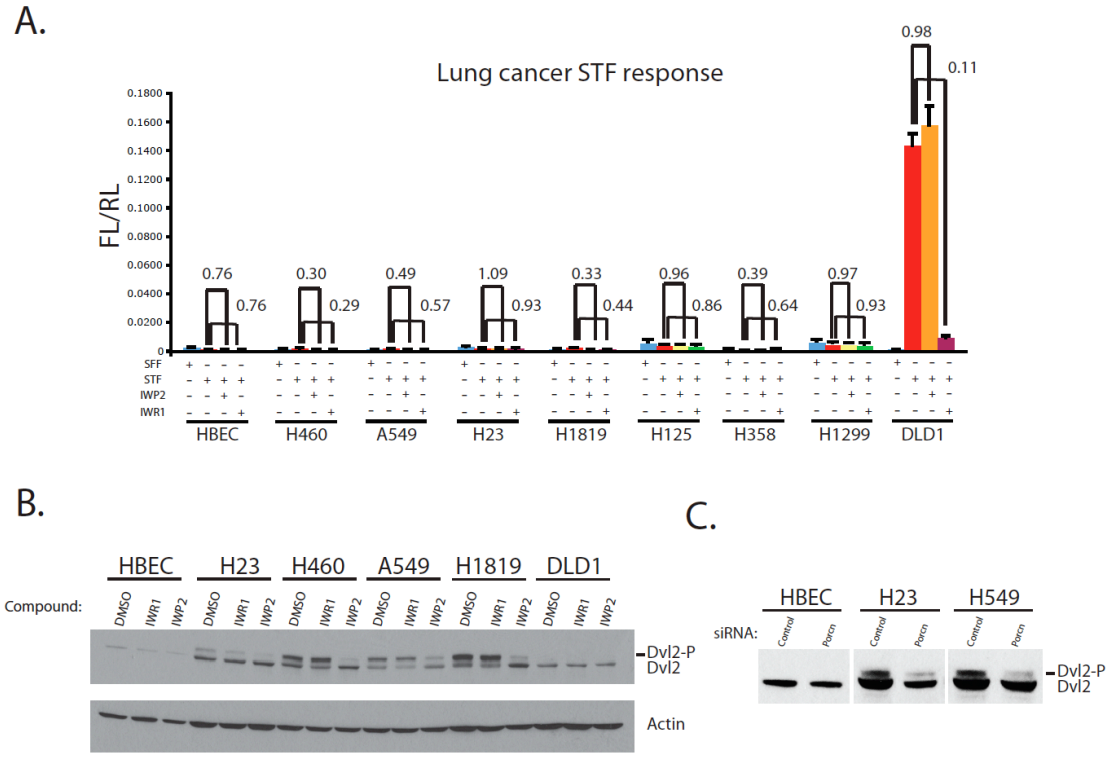


Figure 23: Lung cancers are dependent upon non-canonical Wnt signaling for survival. **A)** Lung cancer cells have relatively low canonical Wnt pathway activation. Pathway status was determined by normalizing transient STF expression levels to CMV-FL. DLD1 cells have high constitutive pathway activation due to an APC truncation, and are included for reference. Numbers represent fold difference between indicated triplicates. **B)** Constitutive Dvl2 phosphorylation is reversed by IWP in several lung cancer lines. This suggests Dvl2 phosphorylation is Wnt/Porcn dependant. **C)** Porcn siRNAs abrogate Dvl2 phosphorylation. Select lines from B) were transfected with Porcn targeting or control siRNA pools. Loss of Dvl2 phosphorylation indicates preceding chemical effects were Porcn dependant.

Non-canonical Wnt signaling pathways are notoriously difficult to parse from converging effectors, often necessitating the utilization of indirect approaches. Before studying non-canonical signaling in any particular case, (i.e. lung cancer) it was paramount to first establish whether IWPs possessed relevant inhibitory capacity. To begin this process, all 19 Wnts were cloned into *gaussia* luciferase expression constructs and expressed in a variety of cell types. Of those constructs that were detectable, all experienced reduced secretion in the presence of IWP (Figure 24A/B), suggesting Wnt palmitoylation was generally widespread, necessary for secretion, and subject to small molecule disruption. As no evidence existed to support retained Wnt ligand signal initiation capacity, it was reasonable to infer a correspondingly reduction Wnt derived non-canonical signaling. Examining one of the more visible aspects of non-canonical signaling validated this initial assumption: inhibitory crosstalk with canonical signaling. This was best observed with the non-canonical Wnt5a, whose presence could prevent CTNNB1 mediated transcription though Ror2 activation (Figure 24C). If in this context, canonical activation was induced by Wnt3a conditioned media, subject to the inhibitory effects of overexpression derived Wnt5a, then IWPs would be expected to increase canonical pathway response (being able to only effect produced Wnts, not conditioned media). This somewhat counterintuitive result was indeed observed, providing the first indirect evidence of non-canonical pathway disruption by IWPs (Figure 24D). Unfortunately, mediators of this process (e.g. PKC, Ror2) were too weak to biochemically confirm, and efforts were redirected to the more frequently studied PCP pathway. Again, IWPs demonstrated inhibitory activity, this time against phosphorylated Jnk and a Jun firefly luciferase reporter (Figure 24E/F).

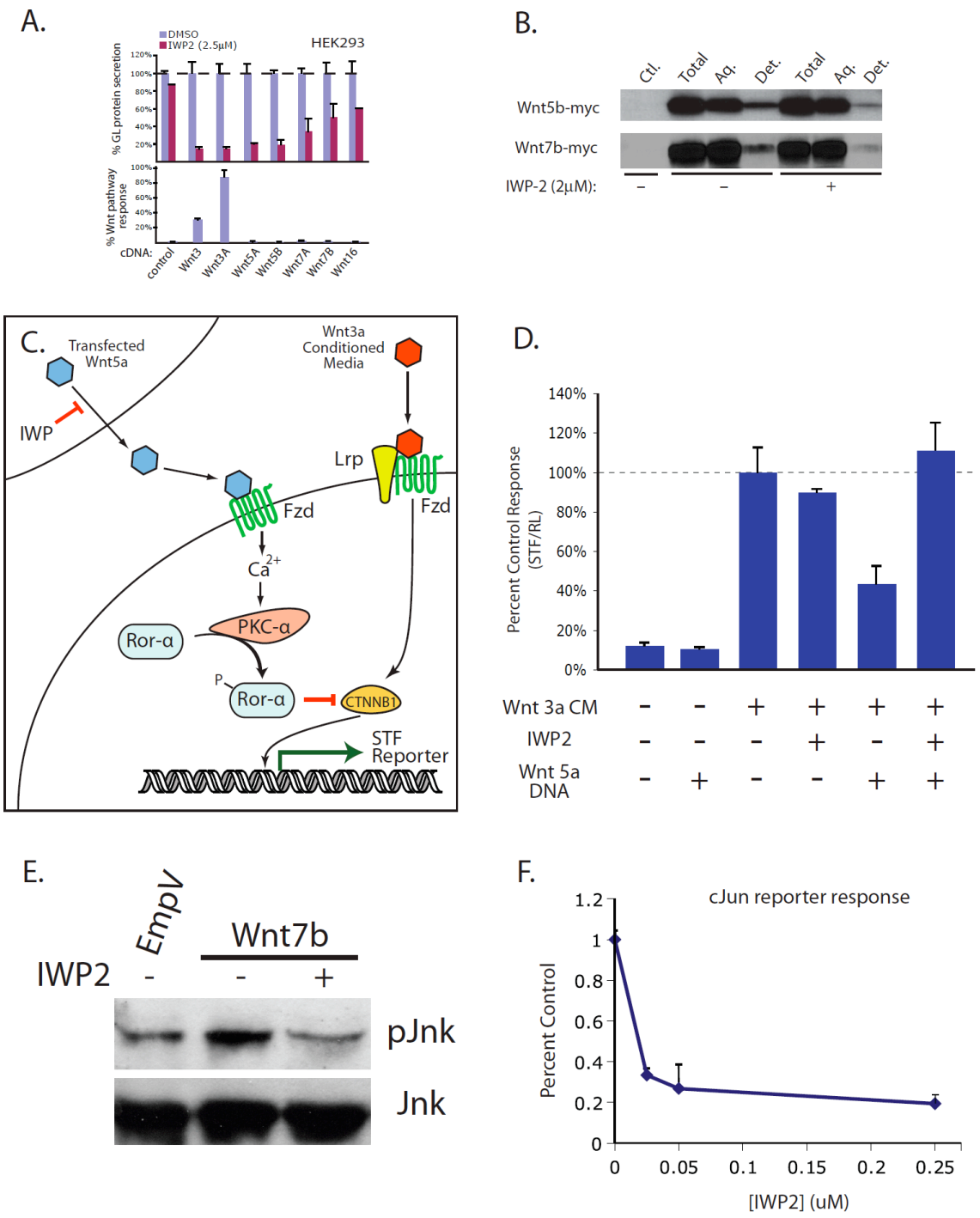


Figure 24: IWPs effectively inhibit non-canonical Wnt pathways. **A)** IWPs inhibit the secretion of multiple Wnt-GL fusion proteins from producing cells. Shown below is the corresponding STF stimulating capacity, a crude means of assigning canonical

functionally. **B)** Two representative "non-canonical" Wnts have reduced hydrophobicity in the presence of IWPs. Palmitoylation status was measured using a TX-114 phase assay. **C)** Setup for Wnt5a non-canonical suppression of Wnt3a/CTNNB1 signaling. Wnt5a exposure can induce cytosolic calcium release, activating PCK-α, which in turn phosphorylates serine 35 of Ror-α, creating a binding interface with CTNNB1 that precludes other transcription factor activities [181]. Wnt3a conditioned media is capable of activating canonical target genes, while at the same time remaining unaffected by IWP introduction. **D)** Transfected Wnt5a inhibits STF reporter activation by Wnt3a conditioned media. IWPs are unable to block signaling by Wnt3a CM, but are functional against Wnt5a, leading to STF de-repression. CM: Conditioned media. All data points are in triplicate. **E)** Wnt dependent phosphorylated Jnk, a common mediator of non-canonical signaling, is decreased by IWPs in L cells. Total kinase levels are unaffected. **F)** A Jun firefly luciferase based reporter is suppressed by IWP2 in H460 cells.

Non-canonical Wnt pathway mediators facilitate lung cancer cell growth under some circumstances [182, 183]. The difficulty ultimately becomes one of origination; is the Wnt pathway their sole activator, a contributor in combination with other signals, or ultimately irrelevant to one or more of these pathways? To shed light on these issues, two broad approaches were adopted. The first strategy employed systematic siRNA knockdown of all Wnts to identify which if any of these ligands were necessary for cell growth. Reductions in multiple Wnts independently affected viability, the specific pattern often changing with reference to the line in question (Figure 25A). The one exception was Wnt7b, which dramatically influenced growth across multiple lung

cancers (Figure 25B). This result was not altogether unexpected, as Wnt7b was already an established (and indispensable) regulator of normal lung development (Table 1). Wnt7b's apparent role within cancerous contexts was however more unusual, and was further complicated by the ligands ability to activate both canonical and non-canonical signaling pathways (in other contexts). Thus, transcriptome wide microarray analysis was further employed to interrogate possible non-canonical mechanisms. H358 cells (a bronchioalveolar non-small cell lung cancer) formed the foundation for these experiments; due to the lines inherit flexibility for studying lung cancer stem cells (see below). Within this context, overexpression of Wnt7b upregulated a diverse subset of genes, broadly categorized as metabolic (HMGCS1, PSAT1, ASNS), transcriptional (ATF3, XBP1, FAM129A), or mitotic (CCND2, TPX2, GDF15) in nature (Figure 25C). Genes implicated in non-canonical Wnt signaling were also present, particularly ATF3 (a CREB pathway transcription factor) and ULBP1 (a ligand that alternatively activates the cGMP/Ca+ pathway), although such upregulation was not an indicator of respective pathway activities. Wnt7b overexpression surprisingly did not influence known target genes, favoring non-canonical mechanisms as possible explanations for the ligand's effect.

In contrast to Wnt7b's presence, IWP and IWR compounds down regulated canonical target genes in H358 cells (Figure 25C), consistent with the low basal canonical pathway activation existing within these cells (Figure 23A). No target genes overlapped between the compound sets, an unexpected result with several possible explanations. First, IWPs inhibition of multiple Wnts may influence crosstalk existing between canonical and non-canonical signaling components, modifying the specific

output of the former. Analogues IWR inhibition of Tankyrase function could also impinge upon non-Wnt related events (e.g. vesicle trafficking, insulin response), perhaps similarly reflected in canonical Wnt output. Finally, feedback elements present in either treatment condition could exacerbate subtle initial differences, such as DKK1 loss in the IWR target set. This target gene functions as a inhibitor of canonical responses (but not non-canonical pathways) through induction of Lrp5/6 co-receptor internalization. Consequently, small changes in DKK1 or related pathway specific modulators could account for some differences between data sets.

While not a known Wnt target gene, ALDH3A1 transcript reduction in IWP treated cells garnered special attention. This gene is a member of the aldehyde dehydrogenase family, a group of enzymes whose expression associates with expected lung cancer stem cell (LCSC) properties, including increased soft agar growth, matrigel evasion, and engraftment into immuno-compromised mice (~1,000 ALDH+ cells injected) [184-187]. Unlike most putative stem cell markers, the presence of ALDH can be discerned indirectly by using a modified fluorescence dye, such as BODIPY-aminoacetaldehyde (BA3, sold commercially as Aldefluor). Aldehyde dehydrogenase enzymes process BA3 as a substrate, oxidizing it into an acetate derivative (BA2) and imparting a negative charge that retains the product within cells in proportion to overall ALDH activity (Figure 25E). Unprocessed BA3 background levels can optionally be accounted for by treating an identical test set with 4-Diethylamino-benzaldehyde (DEAB), a general ALDH inhibitor.

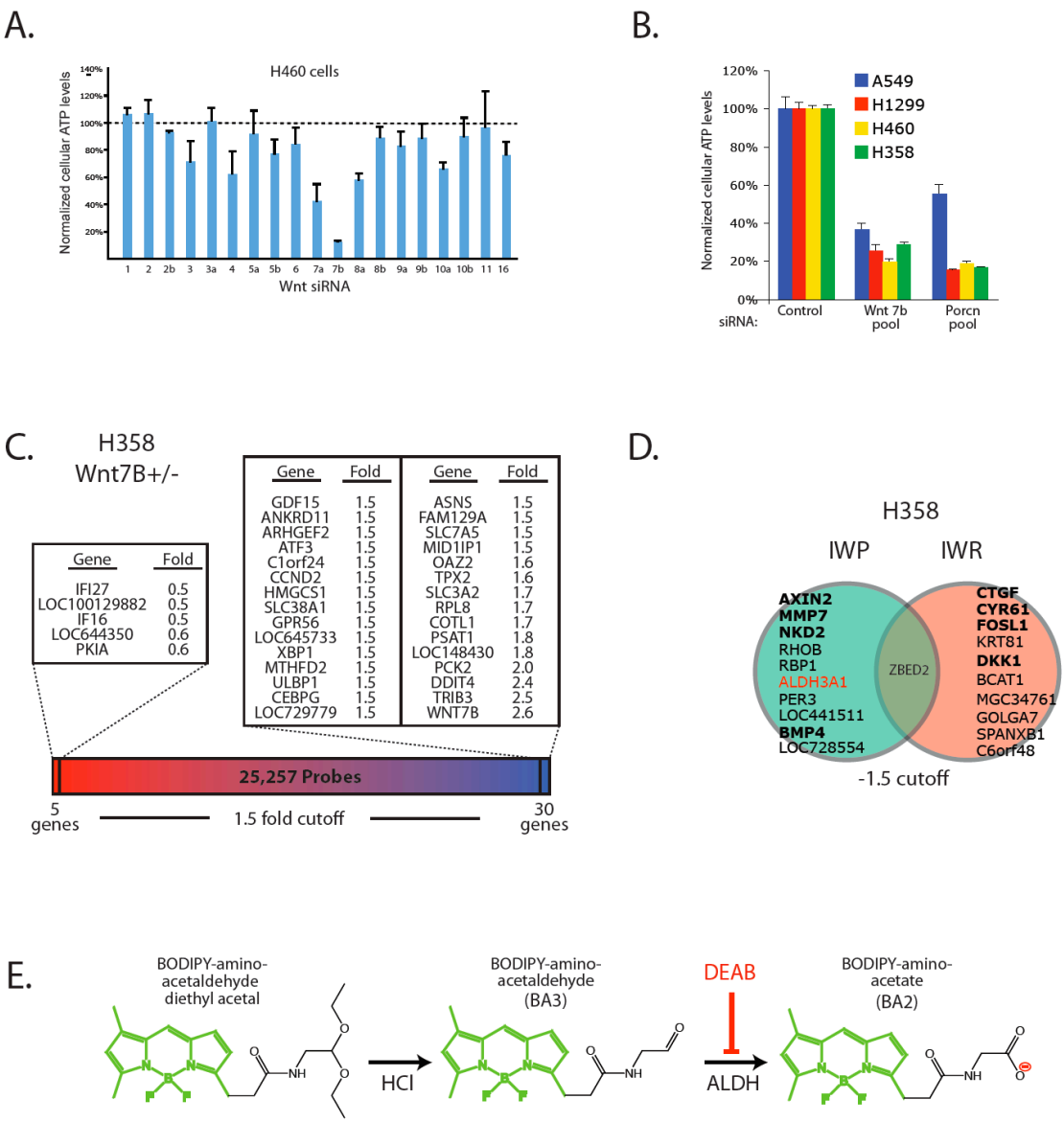


Figure 25: Wnt7b is important for lung cancer cell growth. **A)** Systematic siRNA knockdown of Wnt ligands disrupts lung cancer cell growth (sample H460 data shown). Cells were transfected with 4x siRNA pools and seeded at clonal density for 6 days. Viability was measured with cell titer glo. **B)** Wnt7b loss is detrimental to most lung cancer lines. Cells were treated and grown as in A). **C)** Wnt7b overexpression up-regulates a wide range of genes, as measured via microarray. Because there is no defined

non-canonical gene signature, the exact mechanism of Wnt7b function cannot be inferred from this data alone. **D)** IWP and IWR compounds decrease known Wnt target genes (bold) in microarray samples. ALDH family members (red) have been previously shown to associate with putative lung cancer stem cells. **E)** Activation and retention process for BA3, a measure of ALDH activity. The diethyl acetate form of the regent (left) is converted to BA3 under acidic conditions, neutralized, and incubated with live cells at 37C. ALDH converts BA3 into a final anionic form that can no longer passively diffuse though membranes (BA2). Additional inhibitors within the reaction buffer prevent ABC transporter efflux. The methylated BODIPY core is shown in green, possessing excitation and emission wavelengths of 503/512 nm respectively.

Unlike most stem cell markers (e.g. CD24, CD44), BA3/Aldefluor rarely produces distinct positive populations in cell lines derived from solid tumors. Instead, a global 'shift' occurs with higher ALDH+ cells preferentially migrating towards the population's periphery. IWP1 (as well as several second generation IWPs, data not shown) inhibited this process in two lung cancer lines possessing established ALDH activity, consistent with earlier microarray results (Figure 26A). It is unclear if this result originated from a reduction in stem like cells, or if ALDH is a Wnt target gene (direct or indirect) disconnected from underlying 'stemness.' While xenograft studies are being implemented to more appropriately address this issue, early overexpression and knockdown experiments with Wnt7b support a role for pathway signaling in ALDH positive cell maintenance (Figure 26B).

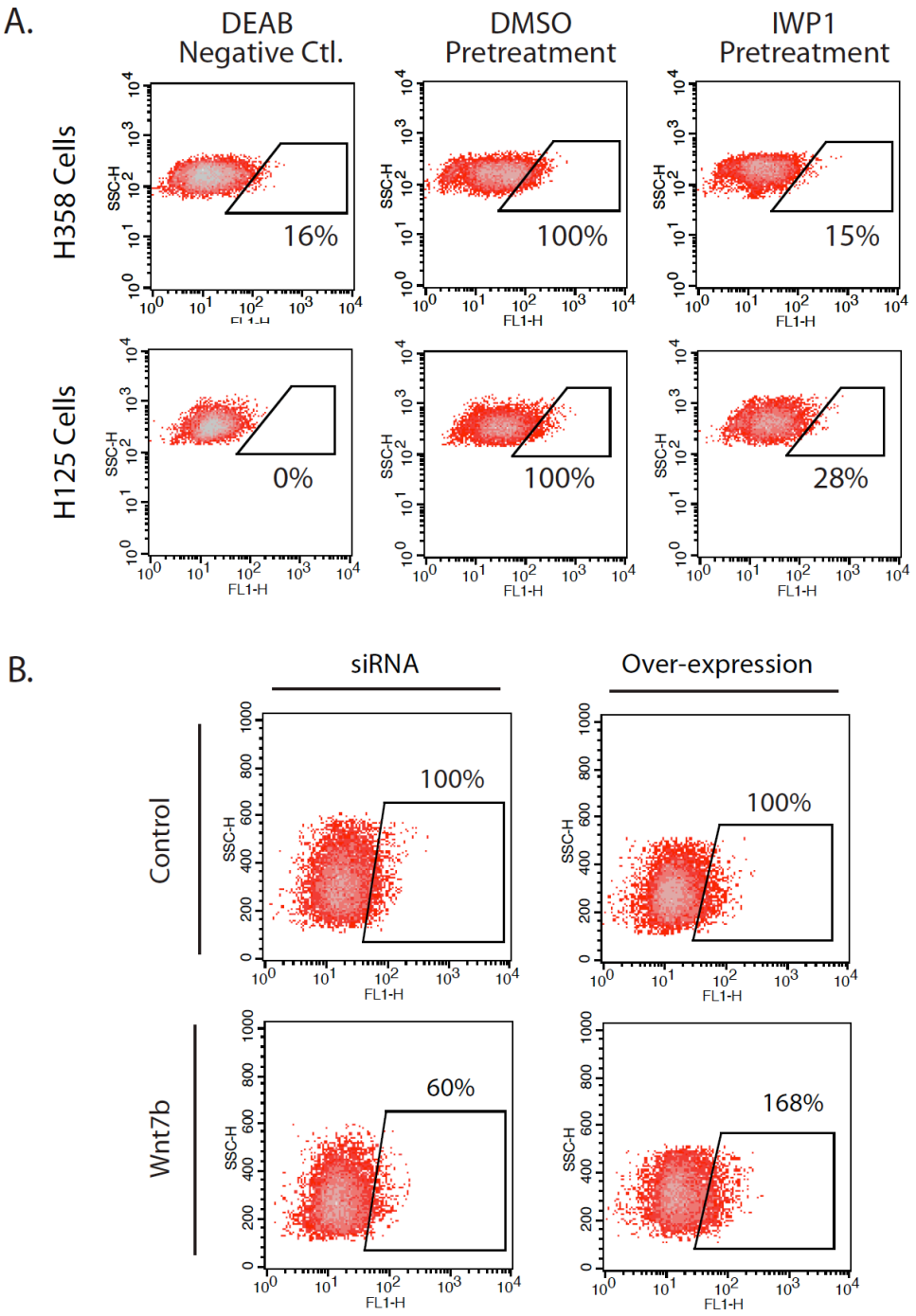


Figure 26: Putative lung cancer stem cells are targeted by IWPs. **A)** H358 and H125 cells, possessing previously characterized ALDH+ populations, are sensitive to IWP1 pretreatment (1 uM, 48 hours). **B)** Wnt 7b overexpression and siRNA mediated

knockdown respectively increases and decreases the H358 ALDH positive population. Percentages shown were normalized to DMSO treated controls. Viability for all cells was assessed by counter-staining for PI negativity and normalizing FCS and SCS parameters.

Discussion

The shortcomings of traditional chemotherapy have fueled the pursuit of alternative strategies. Facilitating this process is an ever-expanding knowledgebase of cancer's epigenetic, genetic, and proteomic underpinnings, allowing researchers to develop target-based therapies. Arguably, the most tangible result has been the deployment of monoclonal antibodies designed to kill cancer cells though varying mechanisms. These include marking cells for immune surveillance (e.g. Rituxan/CD20), blocking specific growth signals (e.g. Erbitux/EGF), or interfering with angiogenesis (e.g. Avastin/VEGF). Side effects, while still present, are generally reduced and partly a reflection of increased specificity. However, although antibodies offer substantial benefits over chemotherapy in certain instances, widespread adoption has been hampered by prohibitive cost, limited targeting potential (i.e. extracellular/intramembrane antigens) and to a minor extent, intravenous administration requirements. All of these challenges are potentially addressable by using small molecules to influence cancer's constituent drivers.

To this day, no clinically viable chemicals exist for disrupting the underlying mechanisms of Wnt dependant diseases. IWP compounds, while still far from fulfilling

this role, demonstrate the feasibility and effectiveness of targeting upstream ligand production. This includes not only the acyltransferase Porcupine, but potentially any component involved in Wnt processing. Factors thought to be pathway specific (e.g. Evi/Wls) would naturally make for ideal starting points, although disruptions to more general processes (e.g. fatty acid metabolism) may also prove useful.

Multiple cancer types were sensitive to IWPs, an effect inversely correlated to confluence levels. This result, while previously associated with Wnt signaling, lacked a definitive explanation, although a switch from autocrine to paracrine signaling may have been a factor. Off-targeting effects could also have been present, particularly given the number of cancer types affected. However, several lines of evidence argued against this interpretation, including the aforementioned dependence upon cell density, phenocopying of Porcn loss (via siRNA), and the compound's inability to inhibit related MBOATs. Another reasonable explanation, loss of Wnt independent Porcupine functions, could not be dismissed, but would require further experimentation beyond the scope of this project to properly vet.

Rationalizing IWP induced cell death in cancer lines with documented Wnt aberrations and/or IWR susceptibilities was relatively straightforward given their apparent canonical dependencies. More difficult to explain was toxicity imparted to lines lacking Wnt hyper activations, particularly numerous lung cancer stocks. Unexpected high levels of Dvl2 phosphorylation, reversible by IWPs, argued for active Wnt ligand production within these cells unable to manifest in canonical responses. By apparently bypassing CTNNB1, this means of signaling fell under the umbrella of "non-canonical" Wnt dependent events, a diverse group of pathways only recently uncovered.

Beyond a requirement for Wnt ligands, little is known about non-canonical components, signal transduction, or downstream effects. Largely this is due identified factors' (i.e. Jnk, calcium, cAMP) involvement in decidedly non-Wnt related functions, making it difficult to discern the contributions made by Wnt ligands. Coupled with the existence of over half a dozen named pathways, non-canonical events likely comprise a dynamic network, the output of which can only be narrowly studied at this time.

By favoring Wnt-GL retention within producing cells, IWP compounds effectively prevented ligands from becoming available for receptor initiation of non-canonical responses. The universality of these observations remained unclear however, as not all Wnts successfully expressed after tagging with gaussia luciferase. It was possible, though unlikely, that one or more family members functioned without modification. To date, only drosophila WntD has shown activity absent palmitoylation, a result attributed to considerable sequence divergence compensating for critical cysteine /serine substitutions [188]. No corresponding vertebrate ortholog of WntD exists, and all known family members retain conservation at Porcn recognized residues. More definitively exploring the extent of Wnt palmitoylation will require alternative approaches, including the systematic use of phase assays, radiolabeling, or secretion based methods.

siRNA loss of function experiments identified Wnt7b as a possible non-canonical mediator in lung cancer. Transcriptome wide microarray results confirmed an absence of CTNNB1 dependent signaling following Wnt7b overexpression, instead demonstrating up-regulation of a diverse set of transcription factors and genes associated with various non-canonical pathways. Unfortunately, no single non-canonical mechanism emerged

from this data, possibly reflecting interconnectivities existing between downstream responses or the contributions of multiple endogenous Wnts. A surrogate microarray assay using DMSO, IWP or IWR treated cells (the more appropriate Wnt7b siRNA microarray having failed for technical reasons) produced contrasting results, with hits reflecting a loss in basal canonical signaling and curiously, an ALDH isozyme. Members of this gene family were of particular interest due to the recent association of their activities with putative lung cancer stem cells. With the caveat that microarray levels do not measure enzyme activity, one interpretation of these results was that IWPs were lowering ALDH levels though loss of a distinct ALDH positive population. Initial Aldefluor based flow cytometry experiments supported this hypothesis by demonstrating a reduction of ALDH+ cells following IWP pretreatment. Future xenograft experiments will ultimately be required to verify that these changes are functionally relevant to lung cancer progression and not simply a disconnect between a marker and phenotype. However, if true, IWPs would represent the first chemical Wnt antagonists of putative cancer stem cells, a population some researchers believe to be at the very heart of cancer [189].

Methods

Reagents and Equipment: TOFA (Cayman Chemicals). Aldefluor (Stem Cell Technologies) Jun reporter. Jnk1-2C6 and phospho-Thr183/Tyr185-Jnk-G9 antibodies were from cell signaling technologies. Microarray data was collected by the UTSouthwestern Microarray core using an Illumina HumanHT-12v4 Expression chip,

with data analysis performed using BeadStudio v3 (Illumina). Lung cancer cell lines were a generously provided by Dr. John Minna (UTSouthwestern).

Wnt5a repression of Wnt3a: HEK293 cells were seeded into a 48 well format, 50K cells/well and transfected with STF/Ren, EmpV, and Wnt5a (40:20:1 ratio) using Fugene6. IWP2 (7.5 uM final) or DMSO was added to select samples, along with Wnt3a or control conditioned media (L cell derived, following standard ATCC protocol). STF activity was read after 72 hours.

IWP/IWR cell death assays: Cells were seeded into 24 well format, 2K cells/well, triplicate plates and compounds added at specified concentrations (0.5% DMSO final). After 6 days incubation, media was discarded, and cell titer glo reagent (Promega) combined with fresh media (1:3 ratio, 200 uL total). After lysing at RT for 15 min, 100 uL of samples were transferred into a 96 format and luminescence measured.

siRNA cell death assays: 10K cells were seeded into a 96 well format, 10K cells/well. Assayed siRNAs were transfected with Effectene, 50 nM final concentrations, triplicate. Following 48 hours incubation, control wells were counted and an equal volume of cells transferred into 24 well format to give 2K cells/well. After 6 additional days, cells were processed identically to compound cell death assays (above).

Aldefluor (ALDH) Flow Cytometry: Cells were processed based upon the manufacturer recommendations. Briefly, H358/H125 cells pretreated with compounds

for 48 hrs were trypsinized, pelleted, and resuspended in sample assay buffer, 1x10^6 cells/mL. Activated Aldefluor reagent was added (5 uL/mL) and half of each test sample was immediately transferred to tubes containing DEAB (10 uL/mL). After incubating for 30 min at 37C, cells were pelleted, resuspended in fresh assay buffer (typically 750 uL), 1 uL of propidium iodide (1 mg/mL stock) added, and samples placed on ice. Cells were gated based upon PI negativity (FL3) and normal FCS/SCS parameters, with final data depicting FL1 BODIPY signal.

RNA extraction: Total RNA was prepared using a Trizol (Invitrogen) extraction, following the manufacturer's instructions. Cell pellets were resuspended in 0.5 mL Trizol by pipetting 30 times with a P1000 pipette. After incubating at room temperature for 5 min, 100 uL of chloroform was added, samples vigorously shaken for 15 seconds, and allowed to partition at room temperature for 2 minutes. Phases were then separated by centrifuging at 15k g for 15 min, 4C. The top (clear) layer from each sample was transferred to new tubes, 250 uL isopropanol added, samples inverted to mix, and incubated at RT for 15 min. After an additional centrifugation (15k g, 10 min), pellets were quickly rinsed with ethanol, and allowed to dry at RT. Resultant RNA was resuspended in water.

Previous Methods: Please see Chapters 1 & 2 for western blotting, luciferase reporters, and phase assay methodology.

Conclusions

Accumulating research over the past decade has underscored the importance of aberrant Wnt signaling during oncogenic progression. The pathway's potent cell fate directing capabilities, naturally intended for embryogenic functions, are frequently co-opted by nascent cancer cells to confer a survival advantage. Consequently, there is growing interest within the medical community in achieving chemically based control over pathway responses.

Preceding Wnt chemical screening efforts have historically focused upon inhibition of signaling at or below the level of ligands/receptor interactions. Alternatively targeting upstream events, such as the production of Wnt ligands, was generally omitted for two practical reasons. First, in contract to signal transducers within receiving cells, few components specific to Wnt production were known. Second, most pathway mutations occurred downstream of pathway receptors, an area thought to be insensitive to ligand regulation. However, following the more recent discoveries of Porcupine, Wntless, and downstream non-canonical signaling events, both of these assumptions appeared increasingly conjectural.

Within this context, our laboratory initiated a 200K compound chemical screen to identify Wnt antagonists. By utilizing cell autonomous Wnt production in the primary screen, potential hits could fall within the majority of the pathway's signaling space. This would later allow for the identification of several molecules lacking activity in the presence of Wnt conditioned media, so called Inhibitors of Wnt Production.

IWP compounds represent the first specific chemical inhibitors of Wnt ligand

production. They function by binding to and inhibiting the active site of Porcupine, an acyltransferase responsible for Wnt palmitoylation. Loss of this modification prevents ligand secretion and downstream receptor activation, with profound implications for cancer cell survival. By further utilizing IWPs as tools to study Porcn dependent processes, several additional observations could be made:

- Reconstitution and inhibition of Porcn activity *in vitro*, providing the first direct evidence that Porcn possesses acyltransferase activity
- Second generation IWPs are represented by a diverse collection of small molecules, possibly indicating that significant flexibility exists for designing future Porcn inhibitors
- Porcn mutations observed in FDH patients are functionally meaningful
- Loss of Porcn activity, either through chemical or genetic means, is deleterious to a number of cancer lines
- Multiple lung cancer cells exhibit non-canonical Wnt signaling effects, further reversible by IWPs

Future efforts will attempt to transition IWPs into *in vivo* cancer and developmental contexts, in the hopes of obtaining therapeutic utility. Already, two compound derivatives (IWPs P12 and G9) have demonstrated potent activity in zebrafish and mouse models respectively. By simultaneously contrasting these new molecules with IWR effects (similarly functional *in vivo*), we may in some cases be able to further differentiate between canonical and non-canonical contributions. Finally, IWPs hold

implications for a number of other MBOAT dependent pathways, including Hedgehog (Hhat) and Ghrelin (GOAT) signaling. Determining the specific residues required for IWP/Porcn interactions (e.g. via crystallography) may facilitate the creation of new drug classes targeting MBOAT related disorders.

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